

# Vitamin C and oxidative damage to DNA

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## 11.1 Introduction

Numerous epidemiological studies (for citations and review, see Ames, 2001; Halliwell 2001), have pointed at a close correlation between vitamin C intake on one side and arteriosclerosis and cancer incidence on the other side. Clearly vitamin C is important for humans: we cannot synthesize it; deficient intake results in scurvy; there are efficient carrier proteins for its uptake (Levine, 1999); it is a necessary cofactor for several enzymes and metabolic roles (Halliwell, 2001); and it is the most important water soluble reductant. It is present in high levels in sperm fluid (see section 11.4) and may also be important as an inhibitor of nitrosamine formation in the stomach (Mirvish *et al.*, 1998).

Among all the functions of vitamin C there has been a considerable focus on its function as an antioxidant in the prevention of various diseases such as arteriosclerosis, cancer, degenerative cerebral diseases and aging. Vitamin C is present in high concentrations in cells and body fluids and it is straightforward to assume that it could prevent oxidation of important macromolecules such as DNA. Surprisingly, in spite of huge, long-lasting and costly trials with antioxidant vitamins, none of these have tested out vitamin C as a single compound, and only very few have included vitamin C in a cocktail together with  $\beta$ -carotene and vitamin E.

In this review we will therefore focus on the scientific data available about the relationship between oxidative DNA damage and vitamin C, including whether vitamin C can act as an antioxidant as well as a prooxidant *in vivo*.

## 11.2 Measurements of DNA oxidation

There is a generally accepted concept that changes in DNA are necessary but not sufficient for cancer development. There also are suggestions that DNA changes are of importance for arteriosclerosis development, however low density

lipoprotein (LDL) oxidation is still considered the prime initial event. The attack of free radical and reactive oxygen species (ROS) formed by cellular metabolism and external factors modifies DNA mainly in the base region and these modifications are mutagenic in simple *in vitro* systems. However only a small portion of the induced modifications are assumed to induce mutations *in vivo* because of efficient repair mechanisms (Ames, 2001; Bohr, 2002; Cooke *et al.*, 2002; Halliwell 2001; Jackson and Loeb, 2001; Poulsen *et al.*, 1998c).

The methods available to measure DNA oxidation include measurement of the levels of oxidized lesions in tissue or cell samples, measurement of the urinary excretion of oxidized bases that most likely is the result of DNA repair, and measurement on single cells using the Comet assay.

Measurement of the level of oxidative DNA modifications in tissues or cells, and the excretion into urine of modified bases or nucleosides, is most often done by either chemical measurement, e.g., HPLC with mass spectrometry or electrochemical detection, details on these methods can be found elsewhere (ESCODD 2000, 2002a, 2002b; Hartmann *et al.*, 2003; Lunec, 1998; Riis and ESCODD, 2002). ELISA methods are also available but are still not sufficiently specific for urinary analysis. Other approaches such as ligase-mediated PCR and mutation analysis are outside the scope of this review, as there are no data relating to vitamin C. The interpretation of these measurements in tissue or cells on the one hand and of the urinary excretion on the other hand is quite different although the methodology of the chemical estimation is very similar (Poulsen *et al.*, 1998a; Poulsen and Loft, 1998). Assuming that the individual under investigation is in steady state, e.g., not acutely but chronically exposed, the urinary excretion e.g., in 24-h urine is interpreted as the rate of damage to DNA by oxidative stress. It is important to stress that although the excretion of the 8-hydroxylated form of guanine (8-oxodG), or other indicators, in contrast to tissue concentration measurement, is based on the DNA repair mechanism; the measure is independent of DNA repair in the sense that a change in repair will not influence the measurement in steady state. Also it should be noted that the urinary excretion is an average of the modification from oxidative stress in the body and cannot be related to a single organ. Measurement of the tissue level, on the other hand, results from a balance between the rate of formation (oxidative stress) and the rate of removal (repair). If one or both of these factors change the level will change, e.g., a decreased repair will give increased levels, so will increased oxidative stress. By measuring tissue levels it is not possible to distinguish whether repair or oxidative stress is behind the change.

We recently assessed the rate of damage related to the presently known major DNA modification 8-oxodG to a minimum of about 500 hits per cell per day in humans (Poulsen *et al.*, 1998b), which would result in a doubling time for this particular modification of about 60–190 days, meaning that after a lifespan of 8 years 1% of DNA would be oxidized, a situation not compatible with life. This situation is avoided by specific repair mechanisms that keep the levels down to about 1 per million bases.

Estimations of the tissue levels of 8-oxodG in normal human tissues and cells are very controversial, but there is agreement that the levels as estimated by different methods are about 1 per million guanines (ESCODD, 2000, 2002a; Lunec, 1998; Riis and ESCODD, 2002). The levels of 8-oxodG in mitochondrial

DNA reported in the literature vary to an extreme degree (Beckman and Ames 1999) and there is no final conclusion about the true mitochondrial level except that it seems to be higher than in genomic DNA.

Although 8-oxodG is the only lesion studied extensively, about 100 different modifications in DNA from oxidation have been demonstrated (Dizdaroglu, 1992; Dizdaroglu *et al.*, 2002) of which maybe 20 are relevant *in vivo*.

Measurement of genomic and mitochondrial DNA modifications from oxidation requires isolation, extraction and digestion of DNA before subsequent analysis. The methods available are HPLC-ECD, GC-MS, LC-MS, LC-MS/MS (ESCODD, 2000, 2002a, 2002b; Lunec, 1998; Riis and ESCODD, 2002). A major problem in the analysis is the possibility of artificial oxidation of guanine/deoxyguanosine during the work-up procedures, in nuclear as well as in mitochondrial DNA. The ratio of nonoxidized guanine/deoxyguanosine to the oxidized ones is 1 : 1 000 000. Slight oxidation, i.e., just 0.1 or 0.01%, during the preparative and the work-up procedures therefore will result in very high measured levels and will obscure most changes from increased or decreased oxidative stress. In the literature there are many reports of very high levels of 8-oxodG in tissue or cells; these data should be interpreted with great care and in this review we have avoided citing papers with such levels. Measurement of 8-oxodG or the corresponding oxidized base in urine is generally accepted as accurate, since the levels of unoxidized guanine in urine are low. Thus, the problem of artefacts due to *post hoc* oxidation is much less severe, if it exists at all.

The Comet Assay in principle detects strand breaks as movement of DNA fragments in agarose gels. In the nucleus the DNA is organized on the nuclear scaffold as loops of supercoiled helices that unwind by introduction of strand breaks (Collins *et al.*, 1997). The procedure for making this visible consists of several steps. First the cells are lysed in solutions containing detergents and high salt concentrations, thus producing a nucleus-like structure that is virtually devoid of proteins (referred to as a nucleoid). A subsequent alkaline electrophoresis forces the DNA to migrate towards the anode. Upon staining with a fluorescent dye the migration can be visualized in a microscope as images that resemble the tail of a comet. In an enzyme-modified version of comet assay, nucleoids are incubated with DNA repair enzymes that excise specific types of oxidatively damaged DNA from the strand. The most frequently used repair enzymes have been formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (ENDOIII) (Collins *et al.*, 1997). The FPG protein recognizes mainly oxidized purines, e.g., 8-oxodG and formamidopyridine derivatives of guanine and adenine, whereas ENDOIII recognizes a diverse array of oxidized pyrimidines, e.g., thymidine glycol, urea residues, deoxyuridineglycol, 5-hydroxydeoxycytosine and 5-hydroxydeoxyuridine.

Quantification of the Comet Assay can be done by visual scoring or by automated image analysis systems. A detailed discussion of the quantification methods is not included here.

### 11.3 Antioxidant and prooxidant properties of vitamin C

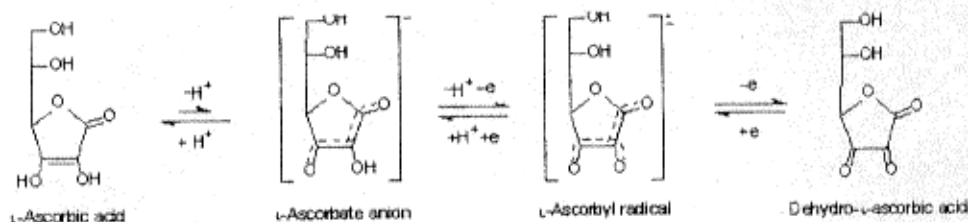
Vitamin C has been called the most important water soluble antioxidant in plasma (Frei *et al.*, 1988) and its antioxidant properties both *in vitro* and *in vivo* have been

documented extensively (Carr and Frei, 1999). However, the compound also has the ability to reduce transition metal ions such as copper and iron and can therefore potentially act as a prooxidant (Halliwell, 1996). While Fenton type systems employing vitamin C as prooxidant have been frequently used *in vitro* (Wills, 1969), it has been subject to much debate whether similar reactions occur *in vivo* and if so what the biological implications are.

Vitamin C, or ascorbic acid (ASC), fulfils the criteria of an effective antioxidant. The oxidation of ASC is outlined in *Figure 11.1*. ASC and even the MDHA have low reduction potentials making it possible for them to serve as electron donors for a variety of potentially damaging radicals and oxidants. The MDHA itself has low reactivity due to its highly resonance-stabilized structure (*Figure 11.1*). Furthermore, two MDHAs have been shown to readily dismutate into one molecule of ASC and one of DHA thereby lowering their energy state even further (Buettner and Jurkiewicz, 1996). DHA – the two electron oxidation product of ASC – is most likely formed via dismutation or further oxidation of the MDHA. DHA rapidly breaks down nonenzymatically to 2,3-diketogulonate as well as oxalate, threonate and other oxidation products with a half-life of only a few minutes at physiological pH (Bode *et al.*, 1990).

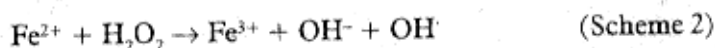
Consequently, efficient ways of regenerating ASC have evolved. ASC can be regenerated from MDHA by means of an NADH-dependent semidehydroascorbate reductase (Wells and Jung, 1997). However as reported by Meister (1992), regeneration of ASC from the MDHA was not sufficient to protect GSH-deficient animals from oxidative damage, indicating that this route is probably not of major importance (Lykkesfeldt, 2002).

DHA is readily reduced back to ASC by cellular antioxidants such as glutathione and lipoic acid (Wells and Jung, 1997; Wills, 1969, see Chapter 8 by May and Asard). The efficient chemical reduction of DHA under physiological conditions (e.g., via glutathione) has previously led to the conclusion that ASC recycling occurs non-enzymatically *in vivo* (Wills, 1969; Winkler, 1992; Winkler *et al.*, 1994). However, more recently ASC regeneration from DHA has been shown to occur intracellularly via enzymatic as well as non-enzymatic pathways. Thus, several enzymes are now known to possess DHA reductase activity including the NADPH-dependent thioredoxin reductase (May *et al.*, 1997) and the glutathione-dependent enzymes glutaredoxin and protein disulfide isomerase (Park and Levine, 1996; Wells *et al.*, 1990). Moreover, enzymatic regeneration of ASC *in vivo* was recently demonstrated in guinea pigs in a vitamin C deficiency model (Lykkesfeldt, 2002).



**Figure 11.1:** Oxidation of ASC by means of two successive one-electron oxidation steps. Direct formation of DHA by two-electron oxidation is also possible. Furthermore, two molecules of MDHA can dismutate into one molecule of ASC and one of DHA.

The classical system of hydroxyl radical formation *in vitro* involves ASC in the role of prooxidant:



In fact, ASC acts as an antioxidant in reducing  $\text{Fe}^{3+}$  while itself getting oxidized (Scheme 1). However, due to the ability of free  $\text{Fe}^{2+}$  to participate in reactions generating hydroxyl radicals (Scheme 2), it is overall regarded as a prooxidant function.

From a chemical point of view, it is clear that depending on the circumstances, vitamin C can act as an antioxidant or as a prooxidant. In the *in vivo* situation it remains a matter of controversy whether Fenton type reactions occur and if so to what extent they play a role in oxidative damage to cellular macromolecules, e.g., DNA. In the organism there are efficient systems to control free transition metals. Prime examples of this are the hemoglobin molecule and the cytochrome P450 enzyme superfamily, where the special affinity between iron ions and oxygen is utilized in oxygen transport and in controlled oxidation reactions of foreign compounds. In healthy humans, iron and copper ions are largely bound in complexes making them unavailable for free radical reactions (Halliwell and Gutteridge 1986, 1990). Presently there is no evidence that demonstrate an *in vivo* uncontrolled prooxidant mechanism of vitamin C, and if existing it may be biologically well controlled and without any importance in the intact organism. More relevant are perhaps disease conditions, in which elevated levels of transition metal ions are found *in vivo* either as a result of traumatic tissue injury, iron overload as seen in hemochromatosis or in severe infectious conditions with massive production of nitric oxide, e.g., septic shock. However, data concerning DNA oxidation in these conditions are scarce and inconclusive.

## 11.4 Vitamin C and oxidative DNA damage in sperm

Oxidative damage to DNA is abundant in sperm. Moreover, the male fertility appears to be closely related to the level of 8-oxodG in sperm DNA, indicating important effects of oxidative stress (Loft *et al.*, 2003; Ni *et al.*, 1997; Sharma and Agarwal, 1996; Shen *et al.*, 1999; Shen and Ong, 2000). Similarly, vitamin C levels are high in sperm plasma and may influence both the extent of oxidative DNA damage and fertility. In a US study the sperm count was significantly lower in samples with low vitamin C concentration than in samples with high vitamin C (Fraga *et al.*, 1991; Shen and Ong, 2000). Moreover, the 8-oxodG level in sperm DNA was highly dependent on the intake and seminal plasma concentration of vitamin C as shown by a significant correlation among 24 men and a depletion and replenishment study in eight men (Fraga *et al.*, 1991; Jacob *et al.*, 1991). In a small study in infertile men, administration of a combination of vitamin C,  $\alpha$ -tocopherol and glutathione for 2 months decreased the levels of 8-oxodG in sperm DNA to the levels of fertile men (Kodama *et al.*, 1997). Furthermore, supplementation with vitamin C, 200 or 1000 mg per day, increased sperm

concentrations and viability in heavy smokers (Dawson *et al.*, 1992). *In vitro* addition of ascorbate (ASC) to sperm samples protects from induction of DNA damage by hydrogen peroxide or irradiation, whereas addition of ASC together with tocopherol and/or glutathione may enhance damage (Donnelly *et al.*, 1999a, 1999b; Hughes *et al.*, 1998).

Smokers have decreased plasma levels of vitamin C (Lykkesfeldt *et al.*, 1997) due to both increased expenditure and lower intake as compared to nonsmokers (Lykkesfeldt *et al.*, 1996; Zondervan *et al.*, 1996). In a study on 8-oxodG in sperm from US men, the intake of vitamin C assessed by questionnaire in a subset was 26% lower in smokers than in nonsmokers although the level of vitamin C in seminal plasma was not significantly different between the groups (Fraga *et al.*, 1996). Nevertheless, the level of  $\alpha$ -tocopherol was significantly lower in seminal plasma from smoking as compared to nonsmoking US men (Fraga *et al.*, 1996). It would thus be expected that smokers would have higher levels of 8-oxodG in sperm. This was in fact found in studies of men from Argentina, California and China (Fraga *et al.*, 1996; Shen *et al.*, 1997). Incubation of sperm cells from nonsmokers in sperm plasma from smokers leads to decreased viability of the cells (Zavos *et al.*, 1998). However, in a large Danish study (Loft *et al.*, 2003; Ni *et al.*, 1997; Sharma and Agarwal, 1996; Shen *et al.*, 1999; Shen and Ong, 2000) there was no difference between smokers and nonsmokers with respect to 8-oxodG in sperm DNA. Another study (Sergerie *et al.*, 2000) found no association between smoking and DNA fragmentation in sperm. It is possible that the apparent discrepancies could be related to differences in genetic background, sperm counts and diet, including vitamin C intake between the populations.

### 11.5 Vitamin C intervention trials relating to oxidative DNA damage and related disease

Searching the PubMed for ASC or DHA gives 23 072 hits, restriction to 'human' gives 11 474 hits, restricting to controlled trials gives 164 hits (search performed February 2003). A more refined search may give more hits but will not be able to change the pattern of many chemical and biological (e.g., cell) studies and very few controlled intervention trials with vitamin C. When restricted to cancer trials with hard end-points or to surrogate markers for cancer development, the number of trials falls dramatically to those listed in *Tables 11.1 and 11.2*. Three controlled trials, two with a large number of subjects, have been conducted (Harris *et al.*, 1989; Heart Protection Study Collaborative Group, 2002) with diseases as endpoints, however, none of them used vitamin C as a single substance and all of them included beta-carotene or vitamin E as well as other antioxidant vitamins or substances. All three provided negative results in the sense that vitamin C did not show any positive or negative effects (*Table 11.1*). *Table 11.1* also includes the studies we have been able to identify that use a global biomarker for oxidative stress, all these results are also negative (*Table 11.1*).

Negative studies also dominate *Table 11.2*, which lists the 15 studies where all except one have used lymphocytes as a surrogate tissue on a relatively small number of subjects, range 8–86. The positive studies indicate that the level of

Table 11.1: Clinical trials with mortality/morbidity or global biomarker measurement.

Subjects (n)	Vitamin C dose	Plasma vitamin C	Marker/tissue	Effect	Other vitamins	Duration	Reference
20 536	250 mg·day <sup>-1</sup>	33% up	Mortality, morbidity, hospitalization	None	600 mg vitamin E, 20 mg β carotene	5 years	Harris <i>et al.</i> , 1989; Heart Protection Study Collaborative Group 2002
18 000 (approx)	120 mg·day <sup>-1</sup>	Increased by 240% (relative to placebo)	Death by cancer and cerebrovascular disease	None	Factorial with retinol, zinc, riboflavin, niacin, molybdenum, β-carotene, selenium, vitamin E		Blot <i>et al.</i> , 1993
205	1000 mg·day <sup>-1</sup>	n.d.	Colorectal adenoma	None	Factorial with vitamin E 400 mg	4 years	Greenberg <i>et al.</i> , 1994
184	500 mg·day <sup>-1</sup>	Increased by 30%	8-oxodG in urine (ELISA)	None	Factorial with vitamin 400 mg	60 days	Huang <i>et al.</i> , 2000
142	500 mg·day <sup>-1</sup>	Increased by 70%	8-oxodG in urine	None	Factorial with vitamin E 182 mg	60 days	Prieme <i>et al.</i> , 1997
48	500 mg·day <sup>-1</sup>	30%	8-oxodG in urine	None	Alone or with vitamin E 182 mg	36 months	Porkkalia-Sarataho <i>et al.</i> , 2000
23	1000 mg·day <sup>-1</sup>	n.d.	8-oxodG in urine	Decreased in HIV-positive after zidovudine treatment	600 mg vitamin E	1 month	Asuncion <i>et al.</i> , 1996
11	1000 mg·day <sup>-1</sup>	Increased by 30%	8-oxodG in urine	None	553 mg vitamin E, 10 mg β carotene	1 month	Witt <i>et al.</i> , 1992

ELISA, Enzyme linked immunosorbent assay; n.d., not determined.

Table 11.2: Clinical trials with vitamin C using biomarkers on surrogate tissue.

Subjects (n)	Vitamin C dose	Plasma vitamin C	Marker/tissue	Effect	Other vitamins	Duration	Reference
80	100 mg·day <sup>-1</sup>		Comet/lymphocytes	Decrease ENDOIII sites and sensitivity to H <sub>2</sub> O <sub>2</sub>	Vitamin E 280 IU, β carotene 25 mg	20 weeks	Duthie <i>et al.</i> , 1996
72	1000 mg·day <sup>-1</sup>	Increased by 66%	Comet/esophagus biopsy cells	None	Vitamin E 200 mg	12 weeks	White <i>et al.</i> , 2002
63	500 mg·day <sup>-1</sup>	Increased by 40%	8-oxodG/lymphocytes	None	400 IU vitamin E, 12 mg β carotene	6 month	Jacobson <i>et al.</i> , 2000
48	60–6000 mg·day <sup>-1</sup>	Increased by 23–80%	Comet/lymphocytes	None	None	2 weeks	Anderson <i>et al.</i> , 1997
38	60–260 mg·day <sup>-1</sup>	Unchanged, n = 20 Increased by 47%, n = 12	8-oxodG/lymphocytes 12 other DNA modifications	Decrease in n = 12 with low initial plasma vitamin C	14 mg Fe	12 weeks	Rehman <i>et al.</i> , 1998
24	1000 mg·day <sup>-1</sup>	Increased by 15–110%	Micronuclei/lymphocytes	Reduction, particular in smokers	None	7 days	Schneider <i>et al.</i> , 2001
21	350 mg·day <sup>-1</sup>		8-oxodG/lymphocytes	None	250 IU vitamin E, 60 mg β carotene, 80 μg Se	4 weeks	Weich <i>et al.</i> , 1999
20	500 mg·day <sup>-1</sup>	Increased by 50%	Comet/lymphocytes	None	200 IU vitamin E, 9 mg β carotene, 1.8 g red ginseng	4 weeks	Lee <i>et al.</i> , 1998
20	260 mg·day <sup>-1</sup>	Increased by 15%	8-oxodG/lymphocytes 10 other DNA modifications	None	14 mg Fe	6 weeks	Proteggente <i>et al.</i> , 2000



20	500–5000 mg·day <sup>-1</sup>	Increased by 50%	8-oxodG, NK cell activity, cell cycle progression, apoptosis	None	None	2 weeks	Vojdani 2001
19	60 mg·day <sup>-1</sup>	Increased by 26%	8-oxodG/lymphocytes	Decreased after supplementing	3 mg vitamin B, 30 IU vitamin E, 40 mg Zn, 2 mg Cu	60 days	Howard <i>et al.</i> , 1998
15	1000 mg·day <sup>-1</sup>	n.d.*	Micronuclei/lymphocytes	Reduced micronuclei	None	1 month	Perminova <i>et al.</i> , 2001
14	1000 mg·day <sup>-1</sup>	Increased by 50%	Comet/lymphocytes	No effect to H <sub>2</sub> O <sub>2</sub>	None	42 days	Brennan <i>et al.</i> , 2000
12	1000 mg·day <sup>-1</sup>	n.d.	Comet/lymphocytes	Sensitivity to H <sub>2</sub> O <sub>2</sub> down	None	Single dose	Panayiotidis and Collins, 1997)
6	35 mg·day <sup>-1</sup>	n.d.	Comet/lymphocytes	Sensitivity to ionizing radiation <i>ex vivo</i> down	None	Single dose	Green <i>et al.</i> , 1994

n.d., Not determined; IU, international units — 100 IU vitamin E corresponds to 91 mg vitamin E; NK, natural killer.

oxidative DNA modification is not changed by vitamin C, however, some protection may be provided against increased oxidative stress.

By means of the Comet assay it has been possible to study effects of vitamin C supplementation on DNA damage in human subjects. These studies are listed in *Table 11.2* together with studies using other endpoints, mainly measurement of 8-oxodG and micronuclei. In general a preventive effect is apparent within a few hours after intake, whereas continued administration has failed to yield significant effects. In studies with administration of a single high dose of vitamin C, the findings include decreased levels of strand breaks and alkali labile sites, and lower sensitivity to *ex vivo* challenge with H<sub>2</sub>O<sub>2</sub> and ionizing radiation in leukocytes (Green *et al.*, 1994; Panayiotidis and Collins, 1997). The effect of vitamin C appeared to peak early, within 2–8 h after consumption (Panayiotidis and Collins, 1997). In contrast, in three studies the steady state level of strand breaks and alkali labile sites were unaffected by vitamin C supplementation (Anderson *et al.*, 1997; Brennan *et al.*, 2000; Welch *et al.*, 1999). Since strand breaks are unaltered in most antioxidant intervention studies, the unaltered strand break levels in long-term vitamin C studies may also reflect the inability of this particular endpoint to reveal antioxidant effects (Moller and Loft, 2002). In *ex vivo* lymphocyte challenge studies, two investigations found no effect of either X-ray or hydrogen peroxide (Anderson *et al.*, 1997; Welch *et al.*, 1999), whereas one study shows decreased sensitivity to hydrogen peroxide after 42 days of 1000 mg·day<sup>-1</sup> vitamin C consumption (Brennan *et al.*, 2000). So far, no study has examined the effect of prolonged administration of vitamin C alone on oxidation of DNA bases assessed by the comet assay. In a study with administration of cocktail of vitamin C (100 mg·day<sup>-1</sup>), vitamin E (280 mg·day<sup>-1</sup>), and  $\beta$ -carotene (25 mg·day<sup>-1</sup>) for 20 weeks, the level of ENDOIII sensitive sites were decreased in smokers (Duthie *et al.*, 1996).

## 11.6 Conclusions

There is a plethora of evidence for antioxidant functions of vitamin C *in vitro* and in cell systems and a large number of epidemiological studies consistently showing relations between vitamin C intake and cancer.

Although vitamin C can act as a prooxidant in chemical systems, there is no substantial evidence that such reactions occur in humans *in vivo*. Large scale clinical intervention trials are scarce and the three trials with hard end-points, two with thousands of volunteers, have not been able to identify a cancer preventive effect of vitamin C. Studies using surrogate measures for global oxidative stress to DNA or using surrogate tissue (lymphocytes) for measuring the number of oxidative modifications of DNA (8-hydroxylation of guanine) generally have not been able to identify a long lasting protective effect of vitamin C. Vitamin C seems to have a particular function in protecting sperm DNA, however there are negative studies as well.

We conclude that there are surprisingly few intervention trials with vitamin C with regard to prevention of cancer or its surrogate markers. Consequently, and in view of the abundant *in vitro* and epidemiological evidence, there still is a real need for well designed clinical intervention trials using both hard end-points and surrogate end-points on surrogate tissue. We also conclude that the methodology

for using surrogate endpoints and surrogate tissue is still in its infancy and that much development is needed.

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