Antioxidant Intervention Studies Related to DNA Damage, DNA Repair and Gene Expression

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In human cells, oxidatively modified nucleobases can be measured in the DNA and strand breaks can be detected by Comet assay, optionally with the use of repair enzymes inactivating breaks at oxidized bases. Oxidized bases and nucleobases from DNA repair, the nucleotide pool and cell turnover can be measured in urine. The excretion rate represents the average rate of damage in the body, whereas the level of oxidized bases in DNA is a concentration measurement in the specific cell. The expression of genes relevant for the defense against oxidative DNA damage, antioxidant and DNA repair enzymes can be assessed at the mRNA, protein and activity level. Functional assays can involve susceptibility to, and disappearance of, damage induced in vitro in lymphocytes.

Vitamins C and E, coenzyme Q as well as various vegetables, fruit and carotenoid rich products, have been assessed with biomarkers primarily including damage in lymphocytes DNA by Comet and chemical assays and urinary excretion of oxidized bases and nucleobases. The basal levels as well as the reported effects of the interventions have been rather variable, possibly reflecting differences in the populations, regimens, functional covariates of the biomarkers as well as between laboratoresses and assays. However, the data suggest that a depleted state due to nutritional deficiency and/or an increased oxidative stress might facilitate demonstration of protective effects of antioxidants with respect to DNA damage. The effect of antioxidants on gene expression has been little studied in humans and only at the activity and protein level.

New RT-PCR based techniques may improve this in the future.

The proper application of biomarkers of oxidative DNA damage in human studies may provide proof of causal relationships with cancer and aging and improve prevention.

Keywords: Biomarkers, oxidative DNA damage, gene expression, cancer, aging, antioxidants, intervention, human

Abbreviations: ROS, reactive oxygen species; h-norBG, 7-hydroxy-8-amino-7,8-dihydro-qtetine; 8-oxoG, 7,8-dihydro-8-oxoguanine; 8-oxoGua, 7,8-dihydro-8-oxoguanine; 8-oxoGuo, 8-oxoguanosine; FapyGua, 2′-deoxynucleoside-6-hydroxy-5-fluoroorumidine (very open guanine); 8-oxoAdo, 8-oxoadenine; UG/UTG, thymine/thymidine glycol; 5-FluoroU, 5-fluoro-2′-deoxyuridine; Fpy, FapyGua glycosylase; EndoIII, endonuclease III; HPRT-OC, high performance liquid chromatography with electrochemical detection; GC/MS, gas chromatography-mass spectrometry; ITS, environmental tobacco smoke; IOD, superside discriminant GL; glutathione reductase; GST, glutathione-S-transferase; GPX, glutathione peroxidase; ELISA, enzyme linked immunosorbent assay

Oxidative modifications of DNA are abundant, mutagenic and thought to be important in carcinogenesis and aging as supported by experimental studies in animals and in vitro. Indeed cells...
are constantly exposed to oxidants from both physiological processes, such as mitochondrial respiration, and pathophysiological conditions such as inflammation, ischemia/reperfusion, foreign compound metabolism and radiation. The bodily defences against oxidants include an extensive system of antioxidant enzymes and radical scavengers and chain breakers, of which many are nutritionally dependent. Nevertheless, damage to DNA is continuously ongoing but is also repaired with high efficiency in the cells in the body.

Descriptive epidemiological studies support important protective properties of antioxidants by a highly reproducible close relationship between a high dietary intake of vegetable and fruits rich in antioxidants and a low risk of epithelial cancers, particularly in the airways and upper gastrointestinal tract. So far, however, the large scale intervention studies of the effects of single and combinations of antioxidants, vitamin E and β-carotene have been negative and even indicated a cancer promoting effect of the latter in smokers. Due to the very large study populations and enormous costs required for such studies only a very limited number of supplements has been tried in these studies focusing on high risk groups, such as middle aged heavy smokers who may very well be beyond prevention. Accordingly, mechanistically based biomarkers of oxidative DNA damage and the involved defence systems may serve as intermediate endpoints in human intervention studies, which may target the optimum strategies for new large scale intervention. Biomarker approaches may also provide support for a causal relationship between oxidative damage and cancer and aging. In parallel, the biomarker approaches are applicable in mechanistic animal experiments and cancer bioassays as well as in vitro. The present review focus on the use of biomarkers of oxidative DNA damage and repair and expression of the involved defence genes in relation to intervention studies involving antioxidants.

**BIOMARKERS IN ANTIOXIDANT INTERVENTION STUDIES**

**Biomarkers of Oxidative Damage in Cellular DNA**

In DNA more than 100 different oxidative modifications have been observed. However, so far only a few of the base modifications have been used as biomarkers and of these the oxidative C-8 adduct of guanine is by far the most studied as either the nucleoside or base. In principle, the level in nuclear or mitochondrial DNA from target or surrogate tissues or cells or the excretion of repair products into the urine can be measured.

Oxidatively modified nucleobases in DNA can be measured by various chromatographic techniques, including high performance liquid chromatography with electrochemical detection (HPLC-EC) (or MS or UV), gas chromatography-mass spectrometry (GC/MS), thin layer chromatography with 32P-postlabelling, and some immune assays. Recently, an ultra-sensitive capillary electrophoresis assay with immuno-extraction and laser based detection for Tg in DNA was published. The required isolation of DNA and hydrolysis by enzymes or acid at high temperature may result in oxidation of the abundant unmodified nucleobases causing artificially high values. Particularly, the derivatization with silyl groups for the GC/MS is prone to give rise to oxidation unless the unmodified base are removed or possibly with control of temperature and other conditions. New, approaches involving release of 7-hydro-8-oxo-guanine (8-oxoGua) and other modified bases from DNA by means of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (ring open guanine) (FapyGua) glycosylase (Fpg), endonuclease III (EndoIII) and other enzymes before HPLC or GC/MS may solve that problem. Provided the reaction is complete, 32P-phosphate used for post-labelling can oxidize guanine and thus explain some reported rather high
values \(^{27,28}\). Even with the HPLC-EC method the reported values for 7-hydroxy-8-oxo-2-deoxyguanosine (8-oxodG) in leukocytes DNA vary from 0.1 to 13 pg \(10^{6} \text{cells}^{-1}\) \(^{29,30}\). Indeed, DNA extraction procedures may cause oxidation, particularly the use of impure phenol and drying of the DNA after ethanol precipitation. \(^{31,32}\) The lowest values have been obtained with anaerobic DNA extraction and/or the use of sodium iodide and desferrioxamine or other antioxidants.\(^{30,31}\) Work is in progress regarding interlaboratory validation of the measurement of 8-oxodG in DNA as reported elsewhere\(^{32}\) and at the present conference. So far, the variation between different methods and laboratories is surprisingly large.

Oxidative damage to nucleobases can be assessed indirectly as strand breaks, DNA unwinding or relaxation, or supercoiling induced by treatment of DNA by the relevant repair enzymes, i.e. Fpg and EndoIII, for purines, including 8-oxodG, and pyrimidines lesions, respectively.\(^{33,34}\) This approach is used in alkaline elution or unwinding of DNA as well as in alkaline single-cell gel electrophoresis.\(^{35,36}\) The 8-oxodG values estimated by those assays are around 0.3 pg \(10^{6} \text{cells}^{-1}\) in lymphocytes.\(^{37}\) I.e. close to the lowest values obtained by HPLC-EC. So far, it is unknown whether the enzyme-based assays are missing 8-oxodG in some of the DNA as suggested by a completeness of only 50% of Fpg repair of damaged DNA in vivo.\(^{38}\) A strong correlation \((r = 0.89, \ p < 0.01)\) has been shown between 8-oxodG concentration and the Comet assay value after 2-methylpropanone induced DNA damage in rat bone marrow cells in vivo.\(^{39}\) Recently a strong correlation between 8-oxodG in DNA and Fpg-sensitive sites measured by the Comet assay was shown in monocytes exposed to different levels of gamma irradiation.\(^{40}\) We have shown a fair correlation between 8-oxodG levels, strand breaks \((r = 0.50)\) and Fpg-sensitive sites \((r = 0.11)\) in lymphocytes from 29 healthy subjects (unpublished data); whereas others have found poor correlations.\(^{41}\) Interestingly, the Comet assay shows considerable seasonal variation, which is important to take into account in antioxidant intervention studies.\(^{42}\) Recently, a highly sensitive spot blot assay was introduced for determination of apurinic/apyrimidinic sites.\(^{43}\) However, there are as yet no reports on systematic interlaboratory validation of these enzyme based assays. Moreover, the application of the elegant ligation-mediated PCR reaction for determination of hot spots for base oxidation at specific nucleotide positions is relevant given the biomarker purposes requires further development.\(^{44}\)

### Urinary Excretion of Oxidation Products as Biomarkers of DNA Damage

Oxidized bases and nucleotides, are poor substrates for the enzymes involved in nucleotide synthesis, fairly water soluble, and generally excreted into the urine without further metabolism.\(^{45,46}\) In animal experiments injected 8-oxodG is readily excreted, unchanged into the urine without contamination from 8-oxodG in the diet or oxidation of dG.\(^{47,48}\) Among the possible oxidation products from DNA 8-oxodG, 8-oxo-8-deazaG, 8-oxo-8-deazaguanine (8-oxoAde), thymine glycol (Tg), thymidine glycol (dTg), 5-hydroxyuracil (5-HU) and 5-hydroxy-5,6-dihydro-2H-pyrimidin-4-one (5-OHHC-M) have so far been identified in urine (Figure 1). Refs. \(2,4,20,51–56\). Of these 8-oxodG and the thymine derivatives are the most intensively studied ones. Although interlaboratory validation of these urinary biomarkers has yet to be reported the levels of concentration and excretion of the oxidized bases and nucleotides obtained in different laboratories are generally in the same range.\(^{49}\)

The assays for the urinary DNA repair products include HPLC with deoxycytosine for 8-oxodG and 8-oxoGua and by UV absorbance for dTg and Tg, whereas all the repair products can potentially be measured by GC/MS.\(^{52,53,57,58}\) The major problem with all these assays involves separation of the very small amounts of analyte from urine that is a
very complicated matrix requiring extensive clean-up procedures. Thus, quantification of recovery may be difficult although this problem can be solved by the use of stable isotopes for GC/MS and LC/MS-MS assays. With HPLC/MS-MS sample preparation required is minimum, providing high capacity, and true identity of the compound excreted is verified. This technique yield similar values as HPLC-EC and may be the optimum assay at present. Enzyme linked immuno sorbent assay (ELISA) based on monoclonal antibodies for estimation of 8-oxoG in urine samples generally yield 3-8 times higher values in human and rat urine than the chromatographic assays and poor correlation with an HPLC assay has been reported in smokers.

Collection of urine for 24 h may present some practical problems and spot urine samples collected for creatinine would be simpler. However, the excretion of 8-oxoG per kg body weight in 24 h correlated rather poorly (r=0.50) with the ratio of 8-oxoG to creatinine in a spot urine sample from a subsequent voiding. Thus, this approach is probably more valid for longitudinal studies where subjects are their own controls.

An important aspect of the urine assay is the great stability of 8-oxoG, i.e. at least 7 years at -20°C whereas DNA in cells and tissues oxidizes quickly even at very low temperatures. Thus, for tested case control studies on biobank material only urine assays appear to be feasible. Little is known regarding the stability of the other urinary DNA damage products.

Interpretation of Nuclear Levels and Urinary Excretion of Oxidized DNA Products

In DNA from tissues or cells the level of oxidized bases is in a steady state determined by the balance between damage and repair rates as outlined in Figure 2. The major contributor is
oxidation of bases within the DNA whereas incorporation of oxidized nucleotides is probably of minor quantitative importance although highly mutagenic and thus qualitatively important. For the repair of the modified bases a number of enzymes are active. The human 8-oxoGua glycosylase (OGG1) has been cloned and repairs 8-oxoGua opposite pyrimidines, whereas another enzyme, OGG2, repairs 8-oxoGua incorporated opposite purines. Interestingly, overexpression of OGG1 in mammalian cell lines increases the repair rate of 8-oxoGua residues induced by potassium bromate or a photomodifier with light without affecting the mutation rate. Nucleotide excision repair has been shown also to contribute to the repair of 8-oxoGua in DNA. 8-oxoGua will also come from the highly specific 8-oxoGua phosphodiesterase (MutT) and 8-oxoGMP nucleotidase enzymes sanitizing the nucleotide pool. This human MUTT enzyme was recently shown to sanitize 2-hydroxy-dATP as well. Digestion of DNA from apoptotic cells and turnover of mitochondria could also be a source of 8-oxoGua. A few of the 8-oxoGua lesions in DNA will after replication without repair or misrepair lead to mutations. 8-oxoGua formed within DNA can lead to T to G transversions whereas incorporation of 8-oxoGua into DNA results in both G to T and G to A transversions.

In theory an increase in the rate of damage to DNA due to oxidative stress should increase the nuclear level until an increased repair rate matches the damage rate and a new steady state is achieved. The increased repair rate can result just from the increased substrate availability as well as from increased enzyme activity as shown for OGG1 in lymphocytes from smokers. The concept of steady state in DNA damage is supported by the limited accumulation with age shown in various human and animal cells and tissues investigated to far. Moreover, in cultured human cells induced levels of oxidized nucleotides return to initial values within some hours. In human and experimental animals exposed to radiation the increase in oxidized bases in DNA from leukocytes or liver as well as urinary excretion of repair products is temporary. Indeed, the repair efficiency of 8-oxoGua in DNA has been calculated to 99.4% and without repair the level of oxidized bases would double in DNA in about 20–90 days. The concept of a steady state indicates that repair flux must equal influx, i.e. that the rate of damage can be estimated from the rate of excretion of the repair product, e.g. 8-oxoGua (Figure 2). The unknown in that equation are the contributions of the glycosylase pathway to the repair and the contribution from cellular and mitochondrial turnover to 8-oxoGua formation.

In a recent study the excess 8-oxoGua excreted into urine accounted for most of the excess 8-oxoGua induced in target organs by the liver carcinogen 2-nitropropane. It should be noted that the urinary excretion represents the cumulative body burden and that it usually cannot be determined if the organism originated from an impact to all body cells or from a much higher insult to one
or several organs. In contrast, tissue or cellular levels represent the measure of a concentration, reflecting the balance between rate of oxidation and the rate of repair. Moreover, in human studies the cellular levels are frequently measured in surrogate cells, such as lymphocytes, rather than in true target tissues. Accordingly, the levels of modified bases in DNA from cells or tissues and the urinary excretion of the corresponding bases or deoxynucleosides represent supplementary kinetic biomarkers. However, much more knowledge regarding repair pathways and kinetics are warranted for the optimum interpretation of these biomarkers.

Biomarkers of DNA Repair and Gene Expression

Mechanistic consideration and knowledge on cell biology indicate that antioxidants and oxidative stress can trigger transcription of a battery of genes via the antioxidant-responsive element (ARE). Among the antioxidant-inducible genes are those encoding antioxidant enzyme activities such as glutathione S-transferases (GSTs) and NADPH:quinone oxidoreductase (NQO1) active in detoxification of reactive substances such as oxygen radicals and redox cycling (semi)quinones (Figure 2). Enhanced expression of the antioxidant enzymes such as GSTs, γ-glutamylcysteine synthase and the heme oxygenase as a response to oxidative stress has also been reported in experimental systems. Recently, the importance of SOD in carcinogenesis was suggested by an increased risk of breast cancer found in women with a distinct MnSOD genotype and a low consumption of fruits and vegetables. So far, however, few biomarkers of DNA repair capacity and gene expression have been employed in published studies on antioxidant interventions in humans. Moreover, interlaboratory validations have yet to be reported.

A few assays are available as biomarkers of repair of oxidative DNA damage. The direct activity of OGG1 can be assessed in extracts from lymphocytes although the effects of antioxidants have yet to be studied in humans. The DNA repair capacity can also be estimated by means of the Comet assay in lymphocytes, e.g. by following the levels during incubation or by applying cell extracts to gels of cells with defined oxidative damage. By modifying the assay conditions the repair of, e.g. strand breaks, purines and pyrimidines may be studied. Assays for the expression of the OGG1 protein and corresponding mRNA in human biomarker material have not yet been published although RT-PCR has been reported. Simple, often kit based, spectrophotometric assays are available for assessment of activity of antioxidant enzymes, including GSTs, superoxide dismutase (SOD), glutathione peroxidase (Gpx), glutathione reductase (GR), NQO1 and catalase in plasma, lymphocytes, red cells and or available tissues. As discussed below such assays have been used in a number of antioxidant intervention studies activity but systematic interlaboratory validation efforts do not appear to have been published. At the level of enzyme protein some ELISA kits have also been used in antioxidant intervention studies. RT-PCR based techniques should allow the study of the mRNA of the enzymes in e.g. lymphocytes.

ANTIOXIDANT INTERVENTIONS AND BIOMARKERS OF DNA DAMAGE, REPAIR AND GENE EXPRESSION

The apparent cancer-protective effect of fruits and vegetables shown in epidemiological studies may partly be related to a number of agents with antioxidant capacity such as carotenoids, tocopherols, vitamin C, selenium and flavonoids. Glucosinolates and their many potent breakdown products found in cruciferous vegetables may have special protective effects. A number of intervention studies with such compounds have been carried out in humans employing
biomarkers of oxidative damage to DNA and the enzymes involved in repair and antioxidant defense as end points, i.e., markers of a putative cancer risk (Table 1). The results have not been completely consistent, possibly due to the differences in the employed biomarkers, their functional correlates and assays as well as in the populations and regimens.

Studies on DNA Damage

With HPLC-EC based assays depletion of or supplementation with up to 300 mg vitamin C had no effect on the level of 8-oxoG in lymphocyte DNA, whereas sperm cell DNA was highly susceptible to depletion. In an uncontrolled sequential study, a cocktail of vitamins C, E, and β-carotene, selenium, zinc, and copper brought increased levels of 8-oxoG in DNA extracted from whole blood found in subjects exposed to environmental tobacco smoke down to levels found in unexposed subjects. Similarly, after administration of vitamin E or red ginseng the 8-oxoG levels decreased in leucocytes in a small number of smokers. However, effects of lycopene administered as tomato products on the 8-oxoG level in lymphocytes measured by HPLC-EC failed to reach significance. Interestingly, a recent study reported a north–south gradient in the levels of 8-oxoG in lymphocyte DNA from men from 5 European countries corresponding to the heart disease mortality whereas there was no such gradient among women.

With GC/MS based assays complex patterns were reported after vitamin C interventions. In one study 8-oxoGua decreased while 8-oxoAde increased in lymphocytes. In another study combining ascorbic acid with an iron supplement the sum of the levels of 13 different oxidative damaged bases (DNA extracted from whole blood increased in subjects with initially high ascorbate levels in plasma whereas the levels decreased in subjects with low plasma levels. However, even in the subjects with high initial vitamin C value the level of some oxidized bases, e.g., 8-oxoAde and 8-oxoguanine (8-oxoG) actually decreased whereas some others, such as FAPyguanine, showed large increases. It cannot be excluded that the iron supplement and its interaction with vitamin C were partly responsible for oxidant effects. It should also be noted that in both GC/MS based studies the reported levels of base damage, at least of 8-oxoGua, were 100 times higher than the levels reported by HPLC-EC recently.

With the Comet assay on lymphocytes no effect of daily supplementations of up to 60 mg vitamin C was shown on strand breaks and H2O2 resistance or γH2AX whereas increased OxNDR resistance to H2O2 and lonzurin radiation was reported 2–4 h after a single dose of 1000–2000 mg vitamin C. However, a daily cocktail of vitamins C and E and β-carotene decreased levels of oxidized pyrimidines assessed as EndoIII sensitive sites in the Comet assay and increased OxNDR resistance to H2O2. Administration of individual carotenoids had no effect on strand breaks or Fpg or EndoIII sensitive sites in lymphocytes, although there initially were individual correlations between the levels of oxidized pyrimidines and plasma carotenoids and single doses increased H2O2 resistance by 1.24. As compared to a period with carotenoid depletion, carotenoid rich vegetable products decreased strand breaks in the Comet assay and carrot juice also decreased EndoIII sensitive sites.

The urinary excretions of 8-oxoGua or 8-oxoG are a DNA oxidation product, have not been affected by antioxidants, including vitamins C and E, β-carotene and coenzyme Q10 in large well-controlled studies of smokers, non-smokers, and in relation to exercise. In contrast, increased levels were normalized by antioxidant combinations in AZT treated HIV patients. Interestingly, after smoking cessation the decrease in urinary 8-oxoG excretion measured in increase in plasma ascorbate and the relationships of the two with smoking intensity
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<th>Biomarker</th>
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<td>Vitamin C depletion (5-20 mg/day) and supplement (60-250 mg/day) (n = 6)</td>
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<td>HPLC-EC</td>
<td>Increased damage with depletion, reversed after supplementation in sperm, no effect in lymphocytes or on 24 h urinary excretion of 8-ohdG</td>
<td>[104,102]</td>
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<td>Strand breaks and chromosome aberrations in lymphocytes</td>
<td>Comet + H2O2 + bleomycin challenge</td>
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<td>[110]</td>
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<td>8-OhdG in urine, 8-oxoGuai in lymphocyte DNA, 8-oxoGuai + 8-oxoGua and 8-oxoGua in DNA from blood</td>
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<td>Ratio of oxidative increased in urine, decrease in 8-oxoGuai, decrease in 8-oxoGuai and increase in 8-oxoGua</td>
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<td>[109]</td>
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<td>Vitamin C 1000 mg or α-tocopherol 1 g of β-carotene 45 mg in a single dose (n = 12)</td>
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<td>Comet + H2O2</td>
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<td>[111]</td>
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<tr>
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<td>HPLC-EC</td>
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<td>Vitamin C 1000 mg + α-tocopherol 600 mg + α/β-carotene 30 mg/day for 1 month (n = 11)</td>
<td>8-OhdG in urine</td>
<td>HPLC-EC</td>
<td>No effect on 24 h excretion in non-smokers</td>
<td>[118]</td>
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<tr>
<td>Vitamin C 500 mg, α-tocopherol 200 mg, coenzyme Q 10 mg/day or placebo for 2 months (n = 140)</td>
<td>8-OhdG in urine</td>
<td>HPLC-EC</td>
<td>No effect on 24 h excretion in smokers</td>
<td>[117]</td>
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<tr>
<td>Vitamin C 300 mg + α-tocopherol 600 mg + β-carotene 25 mg/day for 21 weeks (n = 130)</td>
<td>Strand breaks and oxidized pyrimidines in lymphocytes</td>
<td>Comet + H2O2 + EndoIII</td>
<td>No effect on strand breaks, decreased oxidized pyrimidines and increased resistance to H2O2 induced strand breaks or site</td>
<td>[113]</td>
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<tr>
<td>Vitamin C 100 mg and α-tocopherol 600 mg/kg of AZT treated HIV patients (n = 7)</td>
<td>8-OhdG in urine</td>
<td>HPLC-EC</td>
<td>70% lower values than in other AZT treated patients (n = 8) who had increased levels compared to healthy controls (n = 7)</td>
<td>[119]</td>
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<td>Vitamin C 40 mg, beta-carotene 5 mg, vitamin E 30 IU, Zn 40 μg, Se 40 μg and Cu 2 mg/day for 60 days in ETS exposed (n = 80; no control)</td>
<td>β-Carotene 20 mg/day or placebo for 1 n = 20; no smokers (n = 132)</td>
<td>15 mg/day or n = 32; no sun, lutein, lycopene or placebo for 12 weeks (n = 40)</td>
<td>β-Carotene 30 mg/day for 1 month or placebo before single exercise bout (n = 14)</td>
<td>Diet low in carotenoids and tomato juice, carrot juice, or spinach powder for 2 weeks, each in sequence (n = 23)</td>
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<tr>
<td>Decreased 8-oxodG (by 62%) from initial levels. SOD increased by 18%. Effects on GSH, GR and catalase not significant</td>
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<td>No effect on 8-oxodG excitation</td>
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<td>No effect of interventions, negative correlation between initial plasma levels of carotenoids and oxidized peroximides (Endolfoddative site)</td>
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<td>Decreased strand breaks, base oxidation, DNA repair proteins, GSTp1, total protein, 8-oxodG in lymphocytes DNA</td>
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<tr>
<td>Increased strand breaks, base oxidation, DNA repair proteins, GSTp1, total protein, 8-oxodG in lymphocytes DNA</td>
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<td>No significant correlation</td>
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were also tumor growth. However, there were no significant correlations in this study or in other large studies combined between 8-oxoG excretion and plasma levels of vitamin C and E, carotenoids, or ascorbic acid. Similarly, there was no significant difference with respect to 8-oxoG excretion between subjects with habitually high and low consumption of fruits and vegetables. Nevertheless, intervention with a diet rich in Brussels sprouts compared to non-cruciferous vegetables reduced the urinary excretion of 8-oxoG by 30% in men and in rats where an extract also prevented oxidative DNA damage induced by 2-nitropropane.

Studies on DNA Repair and Gene Expression

A few studies have addressed the effect of antioxidants on the DNA repair capacity. In a study involving a single 1g dose of vitamin C DNA repair assessed as the disappearance of strand breaks and Endo III sensitive sites in the Comet assay was enhanced in two subsets out of eight with the highest response in plasma vitamin C. Comparing carotenoids in terms of carrot juice with a carotenoid depleted diet the DNA repair capacity of a protein extract from lymphocytes assessed by the Comet assay on damaged indicator cells was enhanced in 9 out of 21 subjects although this effect was not statistically significant. In another study involving administration of vitamin C 500mg/day the concentrations of 8-oxoG estimated by an ELISA assay increased in plasma and urine whereas the levels decreased in lymphocytes DNA interpreted by the authors as indicating an increased repair rate. However, in that study the reported basal plasma concentrations of 8-oxoG were around 1 nM, i.e. 3–4 orders of magnitude higher than measured by HPLC/ECC. Moreover, it is not likely that plasma alone should contain 100 times more 8-oxoG than the total 24 h urinary excretion which is usually less than 30 nM. Similarly in the vitamin C intervention study the urinary 8-oxoG to creatinine ratios were 6–8 times higher than reported from studies with HPLC/ECC based assays.

Antioxidant enzyme activities and other indicators of expression have only been studied in a few antioxidant interventions although at least the former can be measured by relatively simple spectrophotometric assays. After a cocktail of antioxidants, each in modest doses, SOU activity decreased along with 8-oxoG levels in DNA from whole blood whereas other antioxidant enzymes were unchanged. GST activities and/or enzyme protein levels have been induced by different vegetable products. Thus, carrot juice administration increased GSTP1 level as measured by ELISA in lymphocytes from 7 out of 23 subjects when comparing with a carotenoid depleted period whereas the total protein level increased in all subjects. Comparing a diet with 300 g of Brussels sprouts with a diet with a similar amount of non-cruciferous vegetables the activity and protein level of GST-α increased in plasma and the protein level of GST-α and GST-π increased in tumor whereas the levels were unchanged in duodenal and lymphocytes. Supplementation of black currant and apple juice rich in flavonoids and vitamin C increased GSH activity in erythrocytes as well as protein oxidation in plasma whereas the activity of catalase, SOD and glutathione reductase were unchanged.

Comments on Antioxidant Intervention Studies

The data could indicate that a depleted state due to nutritional deficiency and/or an increased oxidative stress might be required to show protective effects of antioxidants. Thus, effects of vitamin C depletion and supplementation in depleted subjects could be shown in sperm cells by HPLC/ECC and in whole blood DNA by GC/MS. Similarly the effects of carotenoids
on the Comet assay were only shown in comparison with a depleted diet. Positive effects of antioxidants and cocktails could be shown or were more pronounced on 8-oxodG in leukocyte DNA or EndoIII sensitive sites in ETS exposed subjects and smokers and in urine from ATZ treated HIV patients. However, no effect of antioxidants has been shown on the urinary biomarkers in smokers or in relation to heavy exercise. Similarly, there are no correlations between plasma levels of antioxidants and the excretion of 8-oxodG. Nevertheless, the urinary excretion of DNA oxidation products has consistently been elevated in subjects exposed to oxidative stress, e.g. in smokers, after radiation and chemotherapy and in relation oxygen consumption and occupational exposures. With the Comet assay and the level of 8-oxodG in lymphocytes there have been inconsistent effects of smoking whereas some occupational exposures and radiation have in general shown the expected effects. Thus, the urine based assays may be at least as sensitive as the lymphocyte based assays in detecting oxidative stress/damage and they are supplementary in their functional correlation in terms of damage rate and damage/repair balance, respectively.

The complex patterns of oxidized base alteration shown in the GC/MB based studies of vitamin C interventions indicate the need for measurements of other DNA oxidation products than 8-oxodG. However, the problems of possible artefactual base oxidation during DNA extraction, hydrolysis and in particular during derivatization need to be solved. One possible solution to important parts of this problem involves the use of DNA repair enzymes to release the oxidized bases.

The rate resistance to H2O2 appears to be more sensitive to antioxidant interventions than the basal level in the Comet assay. This suggests that the presence of antioxidants in samples may be important for possible artefactual oxidation of DNA after collection. In this context it remains to be investigated whether differences measured by the Comet assay or as oxidized bases in DNA apparently induced by, e.g. antioxidant manipulations actually reflects resistance to handling of samples or not, e.g. oxidation during sample work-up or with respect to frozen storage. Moreover, it cannot be determined whether any change in the actual level of DNA damage in tissue or cell is a result of an altered damage rate, e.g. due to improved antioxidant defence or an altered DNA repair capacity.

In the interpretation of data related to these antioxidant and DNA repair enzymes it can be debated whether increased levels are good or bad, i.e. whether they are a signal of a response to oxidative stress or show an improved defence (Figure 2). Indeed, tobacco smoking appears to induce OGG1 in lymphocytes, which could explain that the level of 8-oxodG is not consistently elevated in leukocyte DNA from smokers. Similarly, antioxidant enzymes and markers of oxidative damage to DNA, protein and lipids are increased in bus drivers exposed to urban air pollution. Consequently, SOL and 8-oxodG levels decreased concurrently in ETS exposed subjects after antioxidant intervention. In contrast, increases in GST protein and activities and decreases in 8-oxodG excretion resulted from intervention with Brussels sprouts. A possible explanation could be that Brussels sprouts constitutes turn on the ARE regulated genes without significant oxidative stress whereas the antioxidant cocktail can act as direct free radical scavengers. However, long term prospective or possibly nested case-control studies on bus/health and are required to study and possibly link these biomarkers and mechanisms to risk of disease.

The differences between groups and effects of interventions seen in human studies of oxidized DNA bases and gene expression are usually rather small, less than two-fold and for urinary excretion less than 50%. Moreover, for the nuclear steady state levels, effects are obscured by the huge variation between and even within the various methods and laboratories. Thus, the
CONCLUSIONS

A number of different oxidatively modified nucleobases in cellular and tissue DNA and repair products excreted in urine can be measured with a variety of methods. However, problems remain with respect to the true values and intermethod and interlaboratory variation, in particular regarding the levels in nuclear DNA. Expression and activity of DNA repair and antioxidant enzymes can also be studied by a number of methods although rather few have been employed in intervention studies so far. In that area interlaboratory validation is even more scarce and interpretation more complex.

It should be emphasized that the levels of oxidized nucleobases in tissue/cells and the excretion of the repair products represent two fundamentally different estimates that are supplementary. The urine measurement represents the number of repaired bases removed from all organisms and cells during a given time period, i.e. the rate of damage. The tissue measurement is a concentration measurement in the specific tissue/cells in the moment of sampling dependent on the balance between the rates of damage and repair. It has yet to be determined whether increased expression of antioxidant and DNA repair enzymes are good or bad, i.e. whether they are a signal of a response to oxidative stress or show an improved defense.

In human intervention studies, vitamins C and E, β-carotene, coenzyme Q as well as various vegetable and fruit regimens and products, particularly those rich in carotenoids, have been assessed with these biomarkers. The data could indicate that a depleted state due to nutritional deficiency and/or an increased oxidative stress might facilitate demonstration of protective effects of antioxidants with respect to DNA damage. The Comet assay involving ex vivo challenge with oxidative may be particularly sensitive to antioxidant intervention although the interpretation of the result of this assay is difficult. So far, no particular biomarker appears superior and the markers presented have different functional correlations and thus supplement each other. Generally, the effects of antioxidants are small, in the order of 50% or much less, and statistical power is crucial. In the future, the use of the biomarkers may provide further proof that endogenous and exogenous oxidative stress is involved in the pathogenesis of cancer and degenerative diseases of aging, as well as target preventive measures involving antioxidants. However, much more knowledge regarding the true sources, levels, pathways and kinetics of repair of the oxidized nucleobases and nucleosides as well as the interpretation of the expression of the corresponding repair and antioxidant genes is warranted.

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ANTIOXIDANTS AND DNA DAMAGE


