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ANTIOXIDANTS AND THE COMET ASSAY

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ABSTRACT

It is widely accepted that antioxidants, either endogenous or from the diet, play a key role in preserving health. They are able to quench radical species generated in situations of oxidative stress, either triggered by pathologies or xenobiotics, and they protect the integrity of DNA from genotoxicants. Nevertheless, there are still many compounds with unclear or unidentified prooxidant/antioxidant activities. This is of concern since there is an increase in the number of compounds synthesized or extracted from vegetables to which humans might be exposed. Despite the well-established protective effects of fruit and vegetables, the antioxidant(s) responsible have not all been clearly identified. There might also be alternative mechanisms contributing to the protective effects for which a comprehensive description is lacking.

In the last two decades, the Comet assay has been extensively used for the investigation of the effects of antioxidants and many reports can be found in the literature. The Comet assay, a relatively fast, simple, and sensitive technique for the analysis of DNA damage in all cell types, has been applied for the screening of chemicals, biomonitoring and intervention studies. In the present review, several of the most well-known antioxidants are considered. These include: catalase, superoxide dismutase, glutathione peroxidase, selenium, iron chelators, melatonin, melanin, vitamins (A, B, C and E), carotenes, flavonoids, isoflavones, tea polyphenols, wine polyphenols and synthetic antioxidants. Investigations showing beneficial as well as non-beneficial properties of the antioxidants selected, either at the in vitro, ex vivo or in vivo level are discussed.
INTRODUCTION

The current scientific consensus holds that significant amounts of oxygen (O$_2$) first appeared in the Earth's atmosphere some 2.4 billion years ago due to the photosynthetic activity of blue-green algae [1]. Oxygen, besides being indispensable for the production of metabolic energy in most eukaryotes, is also a life-threatening agent. DiGuiseppe and Fridovich put forward a hypothesis which attempted to explain the toxicity of O$_2$ in a chronological order [2]. Initially, O$_2$ toxicity was thought to be due to the inactivation of enzymes, mainly the thiol group of cysteine residues. Later, toxicity was also attributed to the effects of hydrogen peroxide (H$_2$O$_2$). Ultimately, molecular biology techniques established that the toxic effects of O$_2$ are directly linked to its reactive forms, the radical oxygen species (ROS), acting on cellular components. The link between O$_2$ toxicity and many pathologies, e.g. pulmonary diseases, is very well-established [3], and its effect on swelling of the blood-gas barrier [4], retinal defects [5], bowel disease [6], neurodegeneration [7], cancer [8] and ageing [9]. However, the progressive increase of the O$_2$ content in the atmosphere and the evolution of species on Earth implied that organisms dependent on aerobic metabolism required biochemical defences in the form of antioxidants for their survival. These protectants against O$_2$ have also evolved to prevent potential excessive oxidation of cellular constituents [10,11] and contribute to the complex and integrated biological antioxidant defence system.

The Comet assay or single cell gel electrophoresis (SCGE) is a widely used technique for measuring and analysing DNA breakage in individual cells which can be applied to in vitro, ex vivo and in vivo systems. The methodology developed in the mid-eighties was originally introduced by Östling and Johanson [12], and later, Singh [13]
modified it by including unwinding under alkaline conditions. Small numbers of cells that have been exposed to a physical or chemical agent are embedded in a thin agarose gel on a microscope slide. The cells are lysed and the DNA subsequently allowed to unwind under different pH conditions. By choosing different pH conditions for electrophoresis and the preceding incubation, different levels of damage and sensitivity can be assessed. The degree of DNA migration can be correlated to the extent of DNA damage occurring in each single cell. In vitro investigations can be carried out virtually with any cell type; however, the cell-type-of-choice in biomonitoring is the lymphocyte because blood is easily collected and lymphocytes have proved to be good surrogate cells. For instance, lymphocytes exhibited genotoxicity caused by anticancer agents targeting several different organs [14].

The Comet assay is a useful tool for examining issues related to oxidative stress in human lymphocytes [15]. With regard to its ability to detect oxidative stress in vivo, it has been used as a biomarker of pathologies [16], diet [17], occupational exposure [18] and environmental pollution [19]. The use of particular antioxidants has allowed the elucidation of the mechanism of DNA damage exerted by a broad variety of agents [19-22]. Further, the Comet assay has revealed the prooxidant/antioxidant effects of various endogenous and exogenous compounds [23].

The Comet assay has successfully assessed interactions of antioxidants with genotoxicants [24] and it has also proved a valid technique to evaluate whether antioxidant/micronutrients are able to protect the integrity of the genetic material [25-27]. This review will focus on selected examples of extensively investigated compounds by the Comet assay with antioxidant or potential antioxidants properties.
THE COMET ASSAY ON ENDOGENOUS ANTIOXIDANTS

Catalase

Catalase is present in all major body organs, but is particularly concentrated in the liver. Catalase activity within the cell is largely located in peroxisomes [28]. It catalyses the reduction of H$_2$O$_2$ to O$_2$ and H$_2$O. At high concentrations of H$_2$O$_2$, catalase displays the capability of reducing it. In fact, it is very difficult to saturate the enzyme. By contrast, it decreases its efficiency at low concentrations of H$_2$O$_2$. The reason for this is that catalase requires the reaction of two H$_2$O$_2$ molecules to carry out its reduction and this is more unlikely to occur as the concentration of the substrate falls. In the Comet assay, catalase has been used together with a large variety of compounds to determine whether the mechanism underlying DNA damage is mediated by H$_2$O$_2$. For instance, the presence of catalase prevented DNA damage produced by ozone in vitro [29]. For metals such as chromium (Cr), catalase decreased the extent of DNA damage induced by Cr(VI) but not that induced by Cr(III) in vitro [30]. The addition of catalase in vitro revealed that DNA damage exerted by oestrogenic compounds was mediated by H$_2$O$_2$ in lymphocytes from healthy donors [20,21]. The brain, heart, skeletal muscle and spermatozoa contain lower levels of catalase [31]. Taking this into account, it was interesting to find out whether the addition of catalase supplied in vitro to low catalase cell types could prevent DNA damage induced by ROS. It was observed that catalase exerted a protective effect on sperm incubated with oestrogenic compounds [22]. The detrimental effect of oxidative stress is found in Alzheimer’s and Parkinson’s disease [32]. Mimicking these conditions in vitro, fresh isolated mouse brain cells and cultured astrocytes were exposed to H$_2$O$_2$ and xanthine combined with xanthine
oxidase. No DNA damage was observed when catalase was present [33]. Catalase was co-incubated with disinfection by-products of drinking water such as the highly genotoxic iodoacetic acid achieving a 42% reduction in the DNA damage in vitro [34]. Blood from vitiligo patients who had already undergone treatment was collected. The lymphocytes were isolated and co-incubated with catalase in vitro. A reduction in their basal levels of DNA damage was observed. In the presence of H₂O₂, catalase prevented genetic damage to healthy volunteers and vitiligo treated patients [35]. For the previous investigations, catalase was used at concentrations ranging from 100 to 500 IU/ml which consistently showed, in our laboratory, reduction or abolition of DNA damage levels in the presence of H₂O₂. Since 100 to 500 IU/ml has been a range of concentrations repeatedly used in our research, we also investigated catalase on its own in preliminary experiments. Catalase on its own did not generate DNA damage up to a concentration of 500 IU/ml, which excluded any prooxidant activity.

**Superoxide dismutase**

There are different types of SOD, named in accordance with the ions they contain. Copper-zinc-SODs are stable enzymes present in the cytosol, more particularly in lysosomes and the nucleus. Manganese-SODs are more present in mitochondrias of yeast and animals whereas iron-SODs have not been found in animal tissues. The discovery of SOD enzymes provided much of the basis of the knowledge of antioxidant defence systems, since it led to the superoxide theory of oxygen toxicity [36]. This proposed that O₂⁻ (superoxide radical) is a major factor in O₂ toxicity and that the role of SOD as a scavenger of O₂⁻ by coupling and converting it to H₂O₂ is of utmost importance. The superoxide theory of O₂ toxicity can be proved by the following investigations. *Escherichia coli* with functional SOD genes replaced by
defective ones and *Pseudomonas aeruginosa* lacking SOD genes did not succeed in growing in an aerobic environment [37,38]. Transgenic mice incorporating human copper-zinc-SOD, in addition to mice copper-zinc-SOD, showed increased resistance to O$_2$ toxicity [39]. By contrast, knockout mice with an absence of SOD genes presented a variety of pathologies from which they died [40]. We investigated whether SOD could quench the DNA damage generated by oestrogenic compounds *in vitro* on human lymphocytes [20]. The SOD concentrations, 50 and 150 IU/ml, were selected since they have been proved to reduce the levels of DNA damage in some systems in our laboratory. However, the efficiency and consistency in reducing the DNA damage levels can not be compared to catalase. SOD did not reduce the response generated by the compounds diethylstilboestrol (DES) (100 µM), 17β-oestradiol (50 µM), nonylphenol (50 µM) and H$_2$O$_2$ (80 µM). Conversely, it decreased to some extent the DNA damage in at least one out of two independent experiments when co-incubated with phytoestrogens like equol (250 µM), genistein (250 µM) or daidzein (250 µM). An explanation for this might be the fact that higher concentrations were used for phytoestrogens with regard to DES, 17β-oestradiol, nonylphenol and H$_2$O$_2$, being more suitable for the kinetics of disproportionation of the two O$_2$− carried out by SOD [20]. In other words, the rate of production of O$_2$− for the non-phytoestrogenic compounds is slower than their conversion to further H$_2$O$_2$.

The fact that SOD accelerates H$_2$O$_2$ generation in the cellular environment obligates this enzyme to work in conjunction with enzymes that eliminate H$_2$O$_2$ e.g. catalase or glutathione peroxidase. A contradictory scenario was observed with the active metabolite of the vasodilatator drug molsidomine (SIN-1 or 3-morpholinosydnonimine) which mediates DNA damage via formation of peroxynitrite. It was found that catalase abolished its toxic
effect [41]. However, manganese-SOD and copper-zinc-SOD were cytotoxic. The latter displayed a biphasic dose response being more pronounced at concentrations ranging from 10-100 IU/ml. Moreover, SOD did not prevent DNA damage generated by SIN-1 and two other nitric oxide donors S-nitroso glutathione (GSNO) and Roussin’s black salt (RBS) in the Comet assay where catalase abolished DNA damage [42]. Catalase combined with SOD showed no effect on responses greater than catalase on its own. More recently, an increase in survival has been reported when SIN-1 was incubated with SOD plus catalase in TK6 cells [43]. SOD and catalase, individually, were ineffective in reducing lethality. Thus, it seems that the functionality of SOD is highly dependent on the sensitivity to the agent causing genotoxicity, which could be linked to the fact that the different types of SOD differ in their metal binding ability and their distribution in cell compartments [44]. Clear protective outcomes by SOD have been found against the antinfective drugs (metronidazole and dimetridazole) [45], the carcinogenic aromatic amine benzidine [46] and hyperbaric oxygen (HBO) [47].

Overall, the importance of SOD in the body is undisputable. For instance, the SOD malfunction in the body is a common denominator in a variety of degenerative processes, diseases and syndromes namely atherosclerosis, myocardial infarction, Parkinson’s disease, Alzheimer’s dementia amongst others [48].

**Glutathione peroxidase and selenium**

Glutathione peroxidase (GPx) is widely distributed in animal and human tissues. GPx levels are high in the kidney, liver and whole blood, moderate in lens and erythrocytes and low in alveolar lining plasma and blood plasma [31]. Its electron donor, the reduced form of glutathione (GSH), is found at intracellular concentrations that are
often in the millimolar range [28]. GPx is considered the major peroxide removing enzyme found in human tissue, being highly specific for GSH but low for H₂O₂ [49]. GPx reacts with H₂O₂ as well as peroxides. Selenium, found to be a component of GPx [50], maintains GPx activity. Thus, selenium-GPx plays an important metabolic regulatory role. The deficiency of selenium intake is clearly demonstrated by its low levels in plasma which may be associated with certain forms of heart disease [51,52]. In a recent meta-analysis, selenium supplementation was observed to be associated with reduced cancer incidence in men but not in women [53]. The literature agrees on the protective effect of selenium evaluated with the Comet assay towards a variety of chemical or physical toxic agents. *In vitro* investigations found that selenium impeded DNA damage from H₂O₂ in murine lymphoma cells [54], sodium selenate prevented UVA-mediated DNA damage in human skin fibroblasts [55], sodium selenite and selenomethionine protected keratinocytes from UV-induced oxidative damage [56] and sodium selenate quenched potassium dichromate in human lymphocytes [57]. Such preventive effects have also been corroborated in supplementation studies with selenium compounds in the Comet assay. Beagle dogs with prostatic cancer supplemented with selenomethionine or high-selenium yeast lowered the basal DNA damage when compared to a non-supplemented group [58]. Mice supplemented with κ-selenocarrageenan (0.25 µg/ml) showed protective effects against lead acetate in mice [59]. Selenium has also been suggested to be a DNA repair promoter [60,61]. Seo and collaborators confirmed by means of the Comet assay that selenomethionine induces DNA repair in normal human fibroblasts *in vitro* after a challenge with UV-radiation [62]. Nevertheless, it remains inconclusive which is/are the most suitable selenium compound/s to prevent DNA damage.
Prooxidant responses of selenium compounds have also been reported. We observed DNA damage induced by sodium selenate, sodium selenite and selenous acid on their own on human lymphocytes [57]. Results obtained with the Comet assay allowed the conclusion that selenite induced oxidative stress and apoptosis and these effects were significantly attenuated by SOD, catalase and deferoxamine [63].

**Iron chelators**

The regulation of iron and copper in the body plays a key role as a control in the generation of ROS. During evolution, humans have developed a complex system of transport and storage proteins to ensure that these metals are rarely allowed to roam free, hence being harmful [64]. Iron promotes the formation of the highly reactive HO’ leading to free radical DNA damage. In order to avoid this, the iron entering the circulation in the intestine is bound to transferrin, which also accepts iron released in hemolysis in the spleen, with an iron turn over of about 10 times/day [31]. Most intracellular iron is stored complexed with the ubiquitous protein ferritin which acts as a natural iron chelator. There must be a regulation of the cellular iron balance which involves iron-regulatory proteins being triggered by special base sequences in the mRNAs of ferritin and transferring receptor proteins [65]. The importance of such a mechanism of defence against ROS has been observed by exposing lymphocytes to HBO which induces DNA damage [66]. However, it also leads to an adaptive response. HBO-exposed lymphocytes showed a small but reproducible increase in cellular ferritin levels, which might indicate that the underlying protective mechanism is based on an induction of ferritin, which may act in an antioxidant manner [67]. Blood transfusions are required in thalassaemia patients but if carried out without the presence of iron chelators they might develop into iron overload pathology. The
effectiveness of two exogenous chelating agents (deferoxamine and deferiprone) was evaluated in the Comet assay in healthy and thalaessemia patients whose blood was treated \textit{in vitro} with several different iron compounds, thus, simulating a post-transfusional iron overload. The iron compounds (ferric chloride, ferrous chloride and ferrous sulphate) generated DNA damage in healthy subjects and patients but not hemosiderine. Deferoxamine and deferiprone showed a reduction of DNA damage depending on the iron compound used. However, for a thalassaemia patient both chelating agents prevented DNA damage induced by all iron compounds [68,69].

A tool not to be neglected when investigating the effects of antioxidants is the intervention study. It has to be borne in mind that normal human plasma contains numerous high and low molecular mass active redox components that are able to react rapidly with organic and inorganic oxygen radicals, which has led to their classification as important biological antioxidants [70]. Such molecules are caeruloplasmin, albumin (the protein itself and possibly also albumin-bound bilirubin), transferrin, haptoglobin, and hemopexin [71]. The physiological parameters obtained for these plasma proteins might be necessary in order to interpret the results.

**Melatonin**

Melatonin is a hormone which varies in the diurnal cycle. It is characterised as a powerful antioxidant which, unlike others, does not undergo redox cycling [72]. A single melatonin molecule is reported to scavenge up to 10 ROS/RNS being documented that the free radical scavenging capacity of melatonin extends to its secondary, tertiary and quaternary metabolites [73] and it is likely to be carried out by the donation of a hydrogen of its $–\text{NH}$ group [31]. Furthermore, melatonin has been shown to regulate on the one hand antioxidant enzyme activity and on the other cellular mRNA levels for glutathione peroxidase, superoxide dismutases and catalase.
both under physiological and under conditions of elevated oxidative stress [74]. Its precursor, serotonin, is reported to be a lipid peroxidation inhibitor [75].

In the Comet assay, it has demonstrated clear antioxidant activity against ROS generators like the anticancer drugs adryamicin in rats [76], iadurubicin in lymphocytes and cultured cancer cells [77] or the penicillin derivative amoxicillin [78]. Melatonin has also reduced the effects of γ-radiation in human blood exposed in vitro [79]. The in vivo Comet assay exhibited the protective effects of melatonin on rat brain cells against ionizing radiation [80] and magnetic fields (50 Hz) on rat lymphocytes [81]. Basal DNA damage in patients diagnosed with Graves’ disease diminished when lymphocytes were exposed to melatonin (100 µM, 4h) in vitro [82]. Conversely, melatonin on its own (0.1-1 mM) has also been shown to generate a slight increase in DNA damage in mammalian cells in vitro, a slight reduction when co-incubated with H₂O₂ and no effect with bleomycin [83]. Clastogenic effects have been corroborated on its own at a concentration of 100 µM [84].

Melanin

Melanin is a pigment mainly found in skin and hair. Epidemiological data suggests that the incidence of solar radiation-related skin cancer is higher in individuals with genetically determined poor ability of the skin to tan and low pigmentation [85]. In cultured melanocytes, melanin offered protection against induction of major DNA lesions by UVB [86]. It has been postulated that the ability of melanin to quench excited states of photosensitising dye molecules and singlet O₂, and scavenge reactive radicals is an important factor in the protective action of melanin against oxidative damage and this could take place by the interaction of melanin with oxidising and reducing radicals by the hydroquinone and quinine nature of its subunits, which can
act as efficient electron donors and acceptors, respectively [87]. The same authors also state that whether or not a relatively low influx of ROS expected in melanotic systems under typical irradiation conditions will have significant biological impact depends predominantly on the melanotic system and more particularly its antioxidant capacity and susceptibility to oxidative damage [87].

It has been investigated in the Comet assay in order to evaluate the extent of its protective effects against UV. Recent investigations conclude that melanin protects against UVA-DNA damage [88] and acts as a radioprotectant when cysteine and GSH are depleted [89]. Furthermore, depending on the concentration of carotenoid mixtures, protection against melanogenic intermediates and/or exogenous DNA damage can be achieved in vitro [90]. By contrast, earlier studies showed that melanin [91] and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [92], a melanin precursor, increased DNA damage in the presence of UVA.

THE COMET ASSAY ON DIETARY ANTIOXIDANTS

Vitamins

The antioxidant status of blood plasma is directly linked to diet and is defined by components such as vitamins and flavonoids. It is postulated a role of ascorbic acid in the prevention of cancer, heart disease and the augmentation of immune function such as in the prevention of cold [93-95]. In vivo, vitamin C is able to quench ROS efficiently; thus, reducing DNA damage to protoncogenes and tumour suppressor genes which might explain its anticancer properties [96]. Within the cell, vitamin C is also utilised as an electron donor as part of the interaction between iron and ferritin [97]. Outside the cells, vitamin C acts in conjunction with vitamin E, present in lipid
membranes, to quench free radicals and prevent lipid peroxidation [98]. In this manner, vitamin C may assist in preventing the oxidation of low density lipoprotein, which is thought to be a major initiating event in atherosclerosis [99]. In the Comet assay, vitamin C, 2-4 h after intake, has been observed to provide significant protection to the DNA of isolated lymphocytes when challenged with H$_2$O$_2$ [100]. Further evidence of protection was seen against the effects of H$_2$O$_2$ when vitamin C was present at low concentrations (up to 1 mM); by contrast, there was exacerbation at higher doses (5 mM) [15,23,101]. Results were inconclusive when oestrogenic compounds were co-incubated with vitamin C (0.5 and 1 mM) in isolated lymphocytes showing no common pattern in the responses [20]. There was no evidence of a protective effect of vitamin C against the damage caused by bleomycin [23]. In sperm, for instance, addition of vitamin C to preparations did not affect baseline DNA integrity but did provide sperm with complete protection against H$_2$O$_2$-induced DNA damage [102]. In intervention studies, supplementation of 100 mg/day to 50-59 year-old men led to a decrease in oxidative base damage to non-smokers and enhanced resistance against oxidative damage [17]. The antioxidant effect of vitamin C in a long-term study has been observed, measuring oxidative DNA damage and DNA repair in blood cells with the Comet assay. Male smokers were given vitamin C (2 x 250 mg) daily in the form of plain or slow release tablets combined with plain release vitamin E (2 x 91 mg), or placebo for 4 weeks. The outcome of this study suggested that long-term vitamin C supplementation at a high dose, i.e. 500 mg, together with vitamin E in moderate dose, i.e. 182 mg, decreased the steady-state level of oxidative DNA damage in lymphocytes of smokers [103].

Therefore, the fat-soluble vitamin E (α-tocopherol) also plays a role in preventing free radical damage by disrupting the chain reaction of lipid peroxidation [104]. Vitamin E
works in conjunction with vitamin C since the latter is able to regenerate α-tocopherol from the tocopherol radical formed by the reaction with ROS. Its ability to trap peroxyl radicals and singlet O₂ has also been stated [105,106]. In recent years, vitamin E has been utilised in vitro to protect from genotoxicants and this has been evaluated with the Comet assay. For instance, it conferred protective effects against complex mixtures of organic compounds adsorbed onto ambient air particles [107], bleomycin [108], benzopyrene [24], acrylamide [109], antibiotics like ciproflaxin [110] or streptozotocin [111] and anaesthetic gases [112]. Moreover, Trolox (a vitamin E analogue) displayed a protective effect against H₂O₂ in a modified Comet assay with buccal cells [113].

Vitamin A as well as retinol, its equivalent in animals, are important antioxidants. β-carotene, can either be converted in the intestinal mucosa to two identical molecules of retinal, or vitamin A, depending on the presence of the enzyme β-carotene 15,15’-dioxygenase [114]. However, most carotenoids are not able to generate vitamin A. The full range of antioxidant activities of vitamin A remains incomplete. Moreover, both, harmful and quenching effects have been observed in the Comet assay. Vitamin A is capable of reducing the genotoxic effects of N-nitrosomorpholine in human hepatoma cells HepG2 [115]. It decreased the extent of DNA damage evoked by the anticarcinogenic compound imanitib [116] and, when administered at high doses (50000 IU/ml), prevented DNA damage in rats treated with the hepatocarcinogen p-dimethylaminoazobenzene [117]. Conversely, retinol supplementation (7 µM, 24 h) induced DNA breaks in V79 cells in vitro [118].

Vitamin B belongs to a family of chemically distinct vitamins that play important roles in cell metabolism. Vitamin B₁ (thiamine) possesses antioxidant properties that protect against the damaging effects of alcohol, smoking and ageing [119]. One
investigation showed that after an intervention of low and middle-dose vitamin C and thiamine in mice added concomitantly with lead acetate, DNA damage measured in the Comet assay was lower than that of groups exposed only to lead acetate [120]. Elevated vitamin B₂ (riboflavin) levels have been reported to provide protection against damage caused by oxidative injury [121]. One study evaluated the antioxidant capability of riboflavin in the Comet assay, which was demonstrated to exert a protective effect against argemone oil/sanguinarine in mice [122]. In contrast, vitamin B₃ (niacin) does not protect DNA integrity from genotoxicants. In fact, it inhibits DNA repair and this has been confirmed in the Comet assay [123-125]. Although some vitamins can not be classified as antioxidants, their roles as dietary factors must be highlighted. For instance, Vitamin B₉ (folic acid), vitamin B₁₂ (cobalamin) and zinc play key roles in DNA metabolism and repair [126]. Folic acid is essential in the synthesis of purine nucleotides and pyrimidine nucleoside thymidine [127]. Thus, its presence at precise levels is indispensable to maintain DNA stability when ROS or other genotoxic compounds reach the DNA.

Coenzyme Q₁₀ (ubiquinone, ubidecarenone, or CoQ₁₀) is a benzoquinone and it is involved in the production of energy in the mitochondria. Coenzyme Q₁₀ is the only lipid soluble antioxidant synthesised endogenously and inhibits, overall, lipid but also protein and DNA oxidation when it is in its reduced form (ubiquinol) [128]. Its antioxidant features have been confirmed in vitro where its supplementation enhances DNA resistance towards H₂O₂-induced oxidation but does not inhibit DNA strand breaks formation as observed in the Comet assay [129]. The formation of 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative stress, has been analysed by the Comet assay after coenzyme Q₁₀ (3 mg/kg/day) supplementation to human probands [130]. During supplementation, delayed generation of 8-OHdG in
lymphocyte DNA was observed. This effect was long-lasting and could be observed even 12 weeks after supplementation stopped.

**Carotenes**

Carotene is a terpene which can be found in two primary forms: α-carotene and β-carotene. β-Carotene is the most common form and can be found in yellow, orange and green leafy fruit and vegetables. Carotenoids are the most potent biological quenchers of singlet O$_2$ [131]. Carotenoids interact with singlet O$_2$ either via a physical quenching mechanism, in which the excited energy from singlet O$_2$ is transferred to the carotenoid and then dissipated to the surroundings as heat, or chemical quenching, in which the carotenoid is destroyed in the process by addition of O$_2$ to its double bond system [132]. The action of carotenoids as chain-breaking antioxidants has also been investigated. The reaction of β-carotene with a lipid radical results in the formation of a carbon-centred-β-carotene radical intermediate. This intermediate structure has two possible fates; it can act as a prooxidant by reacting with O$_2$ or it can react with another lipid radical to form stable products [133]. Despite being listed as an antioxidant [134], its outcomes are often confounding when investigated. In cancer prevention, the usefulness of β-carotene can not be confirmed, as harmful effects have been observed in clinical trials [135,136]. Some, but not all, *in vitro* investigations display its ability to inhibit DNA damage generated by ROS when evaluated by the Comet assay. For instance, carotenes protected DNA integrity against ROS generated through catechol-oestrogens [137]. β-Carotene also quenched O$_2^\cdot$ generated by xanthine plus xanthine oxidase when its concentrations ranged from 1-3 µM but not at higher concentrations [138]. β-Carotene (0.5-1 µM) prevented DNA damage against H$_2$O$_2$ at low concentrations but ambiguous results were found at
higher concentrations [139]. Conflicting conclusions can be found in the literature in ex vivo studies. Lymphocytes challenged with H₂O₂ after supplementation with β-carotene exacerbated DNA damage [139]. By contrast, men aged 50-59 years old supplemented with vitamin C, E and β-carotene increased resistance to DNA damage when lymphocytes were incubated ex vivo with H₂O₂ [17]. When the Comet assay was utilised to evaluate DNA repair capability, promotion of repair by vitamin C, E and β-carotene after radiation-induced DNA damage in mouse leukocytes in vitro was observed [140]. Likewise, rejoining of breaks in the first few hours appeared substantially fast in lymphocytes following supplementation with β-carotene although the authors concluded that while certain carotenoids appear to enhance recovery from oxidative damage, this was most likely a protective antioxidant effect against additional damage induced by atmospheric O₂, rather than a stimulation of DNA repair [141].

Lycopene, a bright red carotenoid pigment, is found in red fruits. Because of the unsaturated nature of lycopene it is considered to be a potent antioxidant and a O₂⁻ quencher [142]. Among all carotenoids, it is considered the most efficient at quenching singlet O₂ [143]. The daily intake of a beverage prototype (Lyc-o-Mato(R)) containing a natural tomato extract was able to modify plasma and lymphocyte carotenoid concentrations. It also significantly reduced DNA damage in lymphocytes subjected to oxidative stress [144].

Lutein, zeaxanthin and astaxanthin are also antioxidants and may act as a filter to protect the macula from potentially damaging forms of ultraviolet light. Observational data suggest that high dietary intake of macular xanthophylls (lutein and zeaxanthin) are associated with a lower risk of advanced age-related macular degeneration [145]. Hence, the antioxidant capacity of the macula carotene-like components has been
investigated in the Comet assay on human neuroblastoma and rat trachea epithelial cells as models. Data obtained after challenge with UVA and radical nitric species (RNS) generators show that the ability of zeaxanthin, lutein and astaxanthin to reduce the DNA damage depends on the type of RNS donor, the carotenoid concentration and the cell model used [146,147].

**Flavonoids**

*In vitro* and animal studies have shown that flavonoids possess anti-inflammatory, antioxidant, antiallergenic, hepato-protective, antithrombotic, antiviral and anticarcinogenic activities [148]. In addition, flavonoids have been identified as fulfilling most of the criteria to be considered as antioxidants: the flavonoids inhibit the enzymes responsible for $\text{O}_2^{-*}$ production [149,150]; the low redox potentials of flavonoids thermodynamically allow them to reduce highly oxidising free radicals such as $\text{O}_2^{-*}$, RO’ and HO’ [151]; and a number of flavonoids chelate trace metals [152]. Besides scavenging, flavonoids may stabilise free radicals by complexing with them [153]. There is clear evidence that radical scavenging activity depends on the structure of the flavonoids and the substituents of the heterocyclic rings [154]. This might explain why ROS-quenching efficiency differs amongst different flavonoids. Quercetin, the most abundant flavonoid in the human diet [155], was recently investigated against the formation of oxidative DNA damage both *in vitro* and *ex vivo* in the Comet assay. A significant dose-dependent protection by quercetin against the formation of oxidative DNA damage generated by $\text{H}_2\text{O}_2$ was observed *in vitro*. *In vivo*, four weeks of quercetin-rich fruit juice intervention led to a significant increase in the total antioxidant capacity of plasma. After intervention, the level of oxidative damage upon *ex vivo* exposure to $\text{H}_2\text{O}_2$ decreased, although not significantly [156]. Human
lymphocytes were pre-treated with a variety of flavonoids on their own, then challenged with \( \text{H}_2\text{O}_2 \) and, finally, measured with the Comet assay [157]. All flavonoids protected against oxidative DNA damage, moreover, it was concluded that free flavonoids (quercetin, luteolin, myricitin and kaempferol) displayed greater protection than conjugated (quercetin-3-glucoside, quercitrin and rutin). Protection against \( \text{H}_2\text{O}_2 \) was confirmed for myricetin, quercetin and rutin in Caco-2 and HepG2 cells [158] and for quercetin and luteolin in murine and human leukaemia cell lines [159,160]. With regard to other genotoxicants, quercetin and rutin displayed antigenotoxic effects on DNA damage induced by mitomycin C, in a concentration-dependent manner [161]. Additionally, rutin, quercetin and naringin protected against the genotoxicity of UVA on mouse fibroblasts [162]. DNA damage in human lymphocytes and sperm after treatment with four oestrogen-like compounds (\( \beta \)-oestradiol, DES, daidzein, and genistein) and its modulation by flavonoids (quercetin and kaempferol) was examined using the Comet assay. Quercetin and kaempferol reduced the DNA damage produced in sperm and lymphocytes by the four oestrogenic compounds and by \( \text{H}_2\text{O}_2 \) (positive control) [163]. Flavonoids (silymarin, mirycetin, quercetin, kaempferol, rutin and kaempferol-3-rutinoside) were selected in order to counteract the effects of food mutagens in lymphocytes and sperm measured with the Comet assay. There were slightly different profiles in lymphocytes and sperm, but antigenotoxic effects were observed over a similar dose range (50-550 \( \mu \text{M} \)) [164,165]. The previous investigations also showed that flavonoids can themselves be genotoxic and can act in a prooxidant/antioxidant way over different dose ranges. Prooxidant effects were observed at around 100 \( \mu \text{M} \) whereas antioxidant effects occurred at 500 \( \mu \text{M} \). Nevertheless, it has to be taken into account that flavonoids preventing DNA damage in particular experimental designs might not be efficient in
other investigations. Hence, reports can be found in the literature where flavonoids failed to protect against oxidative stress [155,159,160].

**Isoflavones**

Isoflavones are found in soy products and they are closely related to flavonoids. Isoflavones are known to interact with animal and human oestrogen receptors causing effects in the body similar to those induced by the hormone oestrogen [166]. Therapeutic effects in cancer [167,168] and cardiovascular diseases [169] have been described. The most studied isoflavones are daidzein, equol and genistein. The mechanism of action of isoflavones is complex and includes several cellular pathways. For instance, genistein has been reported to inhibit steroidogenesis, block several protein tyrosine kinases, arrest the cell cycle, induce apoptosis and has antiangiogenic and antimetastatic properties [170,171]. Their antioxidant properties have been confirmed in the Comet assay. Twenty-four hours supplementation with daidzein and genistein in Jurkat T-cells (2.5 to 20 µM) and in peripheral blood lymphocytes of healthy subjects (0.01 to 2.5 µM) displayed a significantly increased DNA protection from H₂O₂ in both cell types in the Comet assay. Both, daidzein and genistein were equally protective. In addition, since the protective effect was found at concentrations attainable in plasma after soy consumption (less than 2 µM), it can be assumed that the antioxidant activity of isoflavones contributes to the healthy properties of soy [172]. The role for isoflavones in the prevention of male infertility was explored *in vitro* in the Comet assay [173]. Pre-treatment with genistein or equol (a non-steroidal oestrogen metabolised from daidzein) at doses of 0.01-100 µM significantly protected sperm DNA integrity after H₂O₂-mediated damage. Addition of genistein and equol in combination was more protective than administered on their own. Therefore, the
previously described investigations open the possibility of combined effects of flavonoids, which should be considered when designing studies and interpreting data. In an ample review on the genotoxicity of phytoestrogens, Stopper and colleagues report that genistein (10-400 µM) and daidzein (100-400 µM) were found to exert genotoxic effects in vitro when assessed with the Comet assay [174]. By contrast, it is also stated that most published in vitro studies exhibited only weak or no effects for daidzein.

**Tea polyphenols**

Green tea has attained a high reputation as a health-promoting dietary component ascribed to the antioxidant activity of epigallocatechin gallate (EGCG) [175]. EGCG has been demonstrated to act protectively in human cells against bleomycin [176], UV light [177], H₂O₂ and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [178]. The protective effects of EGCG are likely to be due to a combination of several different mechanisms, including modulation of expression of antioxidative systems, direct scavenge of free radicals and promotion of DNA repair [176]. In addition to EGCG, other tea polyphenols such as epicatechin (EC); epicatechin gallate (ECG); epigallocatechin (EGC); and theaflavins (THFs) were studied in the Comet assay on benzopyrene-induced DNA damage in Chang liver cells [179]. EC and ECG impeded DNA damage at concentrations ranging from 10 to 100 µM, whereas EGC, EGCG and theaflavines inhibited DNA damage at concentrations ranging from 10 to 50 µM. Current evidence is growing that tea constituents can be cell damaging and prooxidant themselves as proved by the enhanced genotoxicity of EGCG [175]. This was further corroborated for EGCG and, additionally, for EGC and theaflavins [179].
Wine polyphenols

Resveratrol is found in the skin of red grapes and as a constituent of red wine. The scope of biological interactions by resveratrol is extensive and has been listed in a recent review [180]. Resveratrol has been shown to bind to numerous cell-signaling molecules such as multidrug resistance protein, topoisomerase II, aromatase, DNA polymerase, oestrogen receptors, tubulin and F1-ATPase. It activates various transcription factors, suppresses the expression of antiapoptotic gene products, inhibits protein kinases, induces antioxidant enzymes, suppresses the expression of inflammatory biomarkers, inhibits the expression of angiogenic and metastatic gene products, and modulates cell cycle regulatory genes. Moreover, numerous studies have demonstrated that this polyphenol holds promise against cancer [181]. There is growing evidence that resveratrol can prevent or delay the onset of heart diseases, ischemic and chemically induced injuries, pathological inflammation and viral infections [182]. It has been suggested to be a chemopreventive by virtue of its ability to protect DNA as well as to induce DNA repair [183]. It has also been proposed to have beneficial effects in brain pathologies mediated by oxidative stress [184]. For instance, it has the capability to quench the effects of H$_2$O$_2$ to a certain extent in glioma cells although resveratrol per se induced a slight time and dose-dependent DNA damage. Using H$_2$O$_2$ in peripheral blood lymphocytes as a model of oxidative stress, DNA damage was not observed in the presence of resveratrol [185,186] as well as in the presence of coumarin, curcumin and vanillin [185]. Resveratrol was also efficient at quenching the chemotherapy drugs cisplatin and selenium-cisplatin [187] and the alkylating carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [183]. By means of the Comet assay it has been shown that the anticancer mechanism of
plant polyphenols, such as resveratrol, might be due to the prooxidant action induced by the mobilisation of endogenous copper [188,189].

**Synthetic antioxidants**

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are chain-breaking antioxidant food additives used for the prevention of food spoilage. BHA and BHT block lipid peroxidation which can eventually lead to DNA damage. BHA and BHT have markedly contradictory effects which might be due to their phenolic nature. Precisely, its structure has been addressed as the reason why they exert anticarcinogenic effects by its ability to intercept free radicals [190]. BHA was utilised to counteract the detrimental effect of iodoacetic acid, a highly genotoxic disinfection by-product of drinking water, and showed a reduction in DNA damage measured with the Comet assay [34]. By contrast, it showed no reduction when co-incubated with MNNG [191] but significantly reduced the level of formamidopyrimidine-DNA-glycosylase plus endonucleaseIII-sensitive sites, which at least partially are caused by oxidative DNA lesions [192]. The genotoxic compound benzidine was examined for DNA damage in human lymphocytes using the alkaline Comet assay [46]. Its toxicity was highly decreased when co-treated with BHT. Other synthetic antioxidants used in animal food like salts of ethoxyquin have been screened in the Comet assay and genotoxic effects have been observed for the salts although lower than that of ethoxyquin [193]. On the other hand, ethoxyquin salts, similarly to ethoxyquin, effectively protected the cells from the oxidative effect of H$_2$O$_2$. Complexes of the antioxidant ethoxyquin with rutin or quercetin were studied in human lymphocytes [194]. Such complexes decreased the level of DNA damage induced by H$_2$O$_2$ on its own.
WHAT MAKES THE COMET ASSAY SUITABLE FOR RESEARCH ON ANTIOXIDANTS?

Tice and colleagues suggested several advantages of the in vitro Comet assay compared to other genotoxicity assays [195]: it has demonstrated sensitivity for detecting low levels of DNA damage; there is a requirement for small number of cells per sample; it has flexibility; there is a low cost and ease of application; studies can be conducted using relatively small amounts of a test substance; and a relatively short time is needed to complete an experiment. The Comet assay is commonly used for the investigation of the effects of antioxidants in intervention studies. The Comet assay can be virtually applied on any cell type, as long as a single cell suspension is obtained. While human lymphocytes remain the most popular cell type for monitoring purposes, sperm, buccal, nasal, epithelial and placental cells are also used [196]. The authors consider that it is mainly two lines of development of the Comet assay that will consolidate this assay as a very valuable tool in the research on antioxidants in vitro, in vivo and ex vivo. 1) Much effort is currently addressed to establish the Comet assay as a relatively high throughput technique [197]. A large number of antioxidant compounds e.g. carotenoids (over 600) [31], flavonoids or antioxidants from natural or synthetic origin already exist and the number of reports identifying new potential antioxidant compounds grows rapidly. Thus, there is the need to evaluate them quickly and reliably. 2) An avenue of investigation focuses on the link between antioxidants (and micronutrients) and DNA repair, this being an indirect mechanism to confront oxidative stress. The Comet assay can easily measure DNA repair capacity in human volunteers and, in turn, this can be used as a biomarker to evaluate the effects of dietary supplements [198].
CONSIDERATIONS OF THE USE OF COMET ASSAY FOR RESEARCH ON ANTIOXIDANTS

The Comet assay has a range of detection limited by the structural organisation of the DNA; it is saturated when all the DNA loops are relaxed [199]. Despite its precision not being as great as chromatographic methods, it seems to be more accurate at estimating low levels of damage, this being due to the adventitious oxidation occurring during sample preparation in such methods [199,200]. Two considerations have to be taken into account before interpreting results obtained in vitro [23]. Firstly, the reactions involved in oxidation and reduction are exquisitely sensitive to the chemical milieu in which they occur. It is not surprising, therefore, that contradictory results with a single putative antioxidant can be obtained using in vitro systems based on the use of widely different media. Secondly, the choice of the agent with which to induce oxidative damage and against which the efficacy of an antioxidant can be measured, could display very diverse outcomes.

For interpretation of responses in vivo, the issue of confounding factors must be thoroughly addressed. There are endogenous confounding factors, such as age, gender, and genetic make-up as well as exogenous factors, including lifestyle habits (smoking, drinking, diet, vitamin supplementation, medication, type and duration of employment, stress, etc.). The correlation between biomarkers and the various factors which affect them is complex. Sometimes the variables are not completely independent of each other [196]. It also has to be borne in mind that dietary antioxidant levels in the blood are dependent on the intake of fruit and vegetables and therefore might be expected to show seasonal variation which varies for each fruit and vegetable [201]. Some authors recommend future studies to be carried out with a placebo-controlled, parallel design.
rather than a crossover design, based on the fact that participation in an antioxidant intervention study may cause changes in dietary habits [202].

A glance into Comet assay literature renders many parameters by which data are published, namely, % DNA in Tail, % DNA in Head, Olive tail moment, Tail extent moment, arbitrary units…. In addition, there is a variety of Comet software available, each one with its own specialities. Moreover, the staining method used in each laboratory has to be considered. These aspects eventually lead to the issue of how comparable are results. It is suggested that judicious selection of different parameters, staining methods along with inter-laboratory validation and harmonisation of methodologies will further help in making this assay more robust and widely acceptable for scientific as well as regulatory studies [203].

OTHER TECHNIQUES AVAILABLE FOR RESEARCH ON ANTIOXIDANTS WITH WHICH TO COMPARE THE COMET ASSAY

Oxidative stress is a disturbance in the prooxidant/antioxidant balance in favour of the former [204] as a result from diminished levels of antioxidants and/or increased production of reactive species. Direct measurement of reactive species provides information on the status of the antioxidant system. Most reactive species only exist for a short time in vivo and direct measurement can be extremely difficult. Another approach might focus on the measurement of the end products of the interactions of reactive species with biomolecules. Alternative end products which might be combined with the ones measurable with the Comet assay might provide more compelling information on the efficiency of the antioxidant defence as well as the mechanisms of the genotoxicant or pathology investigated. There is a set of
techniques commonly used on the detection of free radicals despite it is claimed that
one major obstacle for research on free radicals and antioxidants is the lack of specific
and sensitive methods to quantify oxidative stress in vivo and in vitro [205]. The only
technique that can detect free radicals directly is the spectroscopic technique of
electron spin resonance (ESR) or also called electron paramagnetic resonance (EPR)
[31]. It only detects fairly unreactive radicals since reactive species do not accumulate
enough to be measured; and thus, a solution is to add traps or probes [206]. Trapping
permits a molecule to react with a trap molecule to give a measurable stable product.
The method of spin trapping generates a product detectable by ESR. However, there
are a large number of other probes and they are listed in Halliwell and Whiteman’s
review [206]. Berliner and colleagues state that there is no ideal probe that meets the
following criteria: sensitivity of the measuring device, stability of the reaction
products, specificity of free radical or oxidant reactions, localisation, toxicity and
invasiveness [207].

“Fingerprint” or “footprinting” addresses the measurement of end products of
oxidative damage. The main condition is that they must be specific markers and the
advantage is that there is a broad variety of targets. Lipid peroxidation can be
evaluated by measurement of their end products such as malondialdehyde [208], 4-
hydroxynonenal [209] and acrolein [210] among many others. Detection of
isoprostanes appears to be the most comprehensive measurement of lipid peroxidation,
particularly if obtained from urine and plasma. The protein carbonyl assay (PCC),
which measures the amount of protein carbonyl groups, is the most frequently used
biomarker of protein damage [211]. According to Chevion and co-workers, there are
several methodologies for the quantisation of PCC; in all of them 2,4-dinitrophenyl
hydrazine is allowed to react with the protein carbonyls to form hydrazone, which can
be analysed optically by radioactive counting or immunohistochemically; moreover, using PCC as a marker, it could be demonstrated that oxidative damage to proteins correlates well with ageing and the severity of some diseases [212]. There is not yet a “gold standard” with regard to measurement of oxidative DNA damage [206]. Notwithstanding, DNA products of free radicals reactions can be measured by a plethora of techniques namely, high performance liquid chromatography (HPLC) [213], gas chromatography-mass spectroscopy (GC-MS) [214] and liquid chromatography-mass spectroscopy (LC-MS) [215]. Measurement of 8-OHdG is a very common method of assessing DNA damage. However, it has downsides: 1) it is intrinsically unreliable to measure any single reaction product, including 8-OHdG, as an index of oxidative DNA damage since their relative amounts are highly dependent on reaction conditions [206,216] and 2) chromatographic techniques have proved to be prone to generation of artefacts [217]. Consequently, there is no consensus as to what the true levels of oxidised damage are in human DNA [218] and; what is more, it might be that 8-OHdG is only a minor product of oxidative DNA damage.

The use of specific enzymes in the Comet assay has allowed this assay to detect oxidised DNA bases. Endonuclease III excises oxidised pyrimidines. Formamidopyrimidine DNA glycosilase excises 8-OHdG and ring-opened purines resulting from oxidation. If a digestion step is included with the mentioned repair enzymes, then the increase in strand breaks will correspond to the recognition of oxidised bases. In Collin’s view, the Comet assay combined with formamidopyrimidine is the most convenient and reliable method for monitoring levels of 8-OHdG and for assessing oxidative stress in general, although of course it should not be used without proper controls and calibration [219]. Halliwell and Whiteman question whether the reliability of the Comet assay is due to a lower
baseline since artefacts have been minimised or the Comet assay simply just generates a different kind of artefact which leads to an underestimation of DNA damage. In any case, it seems very unlikely that all the oxidised bases in compact DNA can be recognised by exogenously applied enzymes [206]. Further, the Comet assay only indicates that DNA strand breaks have occurred. Breaks can arise from numerous sources – direct damage to DNA, but also from DNA repair intermediates, alkali-labile sites, overt toxicity or even apoptosis.

A very different approach is the total antioxidant capacity (TAC). TAC considers the cumulative action of all the antioxidants present in plasma and body fluids, thus providing an integrated parameter of measurable antioxidants [220]. TAC measured in vitro bears no similarity to in vivo measurements and may not have direct implication in vivo [221]. In most intervention trials carried out, TAC failed to demonstrate an effect of the supplementation of antioxidants and this might be explained by the effect of endogenous antioxidants in addition to those from dietary origin [219]. Moreover, assays for TAC measurement in plasma differ in the type of oxidation source, target and measurement used to detect the oxidised product; thus, providing a wide variety of results [221].

At present it would appear that there is not a single reliable biomarker of antioxidant effects on oxidative stress. Hence, complementary end points and techniques are encouraged when researching the effects of antioxidants with particular emphasis on intervention studies. Even so, the interpretation of data must be done with caution.

CONCLUSION
Despite aerobic organisms generating ROS as a natural by-product of O₂ metabolism, metazoa have evolved mechanisms of defence that cope with this reactive chemical species. However, oxidative stress occurs in situations of imbalance. This is when ROS levels increase and protective compounds, namely antioxidants, are overwhelmed. As a result, significant damage to cell structures like lipids, proteins and DNA is exerted. Thus, O₂ is considered a potential mutagen, elastogen and teratogen that may be responsible for, or at least part of, the background genetic instability [222]. The protective effect of antioxidants is universally accepted, either inherent in the body or ingested. However, certain aspects such as the mechanisms of action, undefined properties of compounds or compounds with dual behaviour (prooxidant and antioxidant) remain unclear and are subjected to investigation. For instance, the elucidation of how antioxidant properties operate in vitro can provide a better understanding of the, sometimes confounding, in vivo situation. It is precisely in the human situation where the Comet assay might assist to define supplements with specific antioxidants which modulate the DNA damage baseline and reveal to what extent antioxidant levels in plasma are involved.

Medicine might benefit from current investigations scrutinising the properties of a vast number of antioxidants as well as delving into the effects of diets. Future medical treatments could rely on co-treatments with antioxidants to minimise diseases involving oxidative stress. Thus, the Comet assay per se provides a reliable and flexible technique to address and approach these type of investigations.
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Table 1: Descriptive listing of publications quoted in the present manuscript.

<table>
<thead>
<tr>
<th>Reference number</th>
<th>Antioxidant (concentration)</th>
<th>Genotoxicant and/or disease</th>
<th>Target cells (Species)</th>
<th>Type of experiment</th>
<th>Antioxidant effect observed</th>
<th>Publication, year.</th>
</tr>
</thead>
<tbody>
<tr>
<td>[16]</td>
<td>Vitamin C (0.04 – 5 mM)</td>
<td>$H_2O_2$</td>
<td>Lymphocytes (human)</td>
<td>In vitro</td>
<td>Generated DNA damage on its own, slight reduction DNA damage at low concentrations, increase at higher concentrations.</td>
<td>Anderson et al., 1994.</td>
</tr>
<tr>
<td>[17]</td>
<td>Vitamin C (100 mg/day) + vitamin E (280 mg/day) + β-carotene (25 mg/day)</td>
<td>Non/Smokers +/- $H_2O_2$</td>
<td>Lymphocytes (non/smokers 50-59 years old men)</td>
<td>In vivo and ex vivo</td>
<td>Reduction DNA damage baseline, increased resistance to DNA damage ($H_2O_2$)</td>
<td>Duthie et al., 1996.</td>
</tr>
<tr>
<td>[20]</td>
<td>Vitamin C (0.5-1 mM)</td>
<td>Diethyldibenzofluor[17β-oestradiol/ non/phenol/ equol/ genistein/ daidzein/ $H_2O_2$</td>
<td>Lymphocytes/ sperm (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage or no effect (no apparent pattern)</td>
<td>Anderson et al., 2003</td>
</tr>
<tr>
<td>[22]</td>
<td>Catalase (100-500 U/ml)</td>
<td>Triiodothyronine/ L-Thyroxine sodium salt/ noradrenaline</td>
<td>Sperm (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
<td>Dobrzynska et al., 2004.</td>
</tr>
<tr>
<td>[23]</td>
<td>Vitamin C (0.04-5 mM)</td>
<td>$H_2O_2$</td>
<td>Lymphocytes (human)</td>
<td>In vitro</td>
<td>Slight reduction DNA damage (up to 1 mM). Increase DNA damage (5 mM)</td>
<td>Anderson and Phillips, 1999.</td>
</tr>
<tr>
<td>[24]</td>
<td>Vitamin C (40 or 100 µM) +/- vitamin E (30 or 100 µM)</td>
<td>BaP</td>
<td>Lymphocytes (human female)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
<td>Gajek et al., 1999.</td>
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<tr>
<td>[33]</td>
<td>Catalase (250 U/ml)</td>
<td>$H_2O_2$/ xanthine + xanthine oxidase</td>
<td>Cortical cells (adult mice)/ cultured astrocytes (rat)</td>
<td>In vitro</td>
<td>Abolition DNA damage</td>
<td>Cemeli et al., 2003.</td>
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<tr>
<td>[34]</td>
<td>Catalase (500 U/ml)</td>
<td>Iodacetic acid</td>
<td>CHO (hamster)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
<td>Cemeli et al., 2006.</td>
</tr>
<tr>
<td>[34]</td>
<td>BHA (10-100 µM)</td>
<td>Iodacetic acid</td>
<td>CHO (hamster)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
<td>Cemeli et al., 2006.</td>
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<tr>
<td>[35]</td>
<td>Catalase (250 U/ml)</td>
<td>Vitiligo +/- $H_2O_2$/ vitiligo +/- 17β-oestradiol</td>
<td>Lymphocytes (human)</td>
<td>In vitro/ ex vivo</td>
<td>Reduction DNA damage</td>
<td>Schaller et al., 2006.</td>
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<tr>
<td>[38]</td>
<td>SOD (200 U/ml)</td>
<td>SIN-1, GSNO or RBS</td>
<td>HIT-T15 (hamster)</td>
<td>In vitro</td>
<td>No effect DNA damage</td>
<td>Delaney et al., 1997.</td>
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<td>[42]</td>
<td>SOD (100 U/ml)</td>
<td>Benzidine</td>
<td>Lymphocytes (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
<td>Chen et al., 2003.</td>
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<tr>
<td>[42]</td>
<td>BHT (100 µM)</td>
<td>Benzidine</td>
<td>Lymphocytes (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
<td>Chen et al., 2003.</td>
</tr>
<tr>
<td></td>
<td>SOD</td>
<td>Hyperbaric oxygen</td>
<td>Lymphocytes (human)</td>
<td>Ex vivo</td>
<td>Reduction DNA damage</td>
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<tr>
<td>[50]</td>
<td>Sodium selenite (6 µg/kg/day) / high-selenium yeast (6 µg/kg/day)</td>
<td>Elder</td>
<td>Lymphocytes and prostate cells (dogs)</td>
<td>In vivo supplementation</td>
<td>Reduction DNA damage</td>
<td>Waters et al, 2003.</td>
</tr>
<tr>
<td>[53]</td>
<td>Deferiprone (0.05-1.6 mM)</td>
<td>Ferric chloride or ferrous chloride</td>
<td>Lymphocytes (human)</td>
<td>Ex vivo</td>
<td>Reduction DNA damage (deferexamine)</td>
<td>Anderson et al, 2000.</td>
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<tr>
<td>[54]</td>
<td>Deferoxamine (0.05-1.6 mM)</td>
<td>H2O2 + ferrous sulphate/ H2O2 + ferric chloride or H2O2 + ferrous chloride</td>
<td>Lymphocytes (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage (H2O2 + ferrous sulphate)</td>
<td>Anderson et al, 2000.</td>
</tr>
<tr>
<td>[58]</td>
<td>Melatonin (100 mg/kg)</td>
<td>Ionsing radiation</td>
<td>Brain cells (rat)</td>
<td>In vivo</td>
<td>Reduction DNA damage</td>
<td>Undeiger et al, 2004.</td>
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<tr>
<td>[59]</td>
<td>Melatonin (0.5-1 mM)</td>
<td>Magnetic fields + iron ions</td>
<td>Lymphocytes (rat)</td>
<td>In vitro</td>
<td>Reduction DNA damage at 0.5 mM and abolition at 1 mM</td>
<td>Jagte et al, 2001.</td>
</tr>
<tr>
<td>[61]</td>
<td>Melatonin (0.1-1 mM)</td>
<td>On its own, H2O2 or bleomycin</td>
<td>CHO (hamster)</td>
<td>In vitro</td>
<td>Slight increase DNA damage (on its own). Slight reduction DNA damage (H2O2). No effect DNA damage (bleomycin)</td>
<td>Festa et al, 2001.</td>
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</table>


[78] DHICA (0.125-2 µM) UVA HaCat keratinocytes (human) In vitro Generation DNA damage Kipp and Young, 1999.


[82] Vitamin C (300-600 µM) On its own +/- H₂O₂ Sperm (human) In vitro No effect on DNA damage (on its own), reduced DNA damage (H₂O₂) Donnelly et al, 1999.

[83] Vitamin C (2x 250 mg/day) + Vitamin E (2x 91 mg/day) 4 weeks Non/Smokers Lymphocytes (human) In vivo Reduction DNA damage smokers (Vitamin C 500 mg + Vitamin E 182 mg) Meller et al, 2004.

[85] Vitamin E (10 µM, 2h pre-treatment)/ vitamin C (0.5 mM, 1h pre-treatment) Organic compounds adsorbed on to ambient particles, BAP or 5,9-dimethoxyBC HepG2 (human) In vitro Reduction DNA damage (vitamin C). Slight reduction DNA damage (vitamin E) Lazarová and Slameňová, 2004.


[92] Vitamin A (10µM/24h)/ vitamin C (0.5 mM/1h)/ vitamin E (10 µM/2h) N-nitrosomorpholine HepG2 hepatoma (human) In vitro Reduction DNA damage (vitamin E) Robichová et al, 2004.

[93] Vitamin A (5 µM)/ vitamin C (10 µM)/ vitamin E (10 µM) Imanitib Myelogenous leukemia K562 (human) In vitro Abolition DNA damage (vitamin A and C) and reduction DNA damage (vitamin E) Czechowska et al, 2005.

<table>
<thead>
<tr>
<th>IU/ml + vitamin C (75-1000 mg) + vitamin E (50-500 mg/kg body. Once a week for 6 months.</th>
<th>doses each vitamin individually and low doses as mixture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine + vitamin C</td>
<td>Lead acetate</td>
</tr>
<tr>
<td>Riboflavin (50 mg/kg) +/- vitamin C (150 mg/kg). Single or multiple doses. 24h prior, during genotoxicant administration or 24h after.</td>
<td>Sanguinarine/ argemone oil</td>
</tr>
<tr>
<td>Nicotinamide (200-800 mg/kg)</td>
<td>+/- Ionising radiation</td>
</tr>
<tr>
<td>Coenzyme Q10 (100 µM)</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>Coenzyme Q10 (3 mg/kg/day) 2 weeks.</td>
<td>On its own</td>
</tr>
<tr>
<td>β-carotene (0.25-10 µM) + lycopene (0.25-10 µM)</td>
<td>Catechol-oestrogens</td>
</tr>
<tr>
<td>β-carotene (1-10 µM) + lycopene (1-10 µM)</td>
<td>Xanthine/xanthine oxidase</td>
</tr>
<tr>
<td>β-carotene (0.5-8 µM) lycopene (0.5-8 µM)/ lutein (0.5-8 µM)</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>β-carotene (12 mg/kg) + vitamin C (400 mg/kg) + vitamin E (200 mg/kg)</td>
<td>γ-radiation</td>
</tr>
<tr>
<td>β-carotene (15 mg/day)/ lycopene (15 mg/day)/ lutein (15 mg/day) 1 week. Supplementation separated by 3-week wash-out periods.</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>Lyc-o-Mato(R)) 250 ml/daily, (provides 6 mg lycopene, 4 mg phytoene, 3 mg phytofluene, 1 mg β-carotene and 1.8 mg α-</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>Reference</td>
<td>Carotenoids</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td>[118]</td>
<td>Zeaxanthin (5-100 µM) + astaxanthin (5-100 µM) + lutein (5-100 µM)</td>
</tr>
<tr>
<td>[119]</td>
<td>Zeaxanthin (20-40 µM)/ astaxanthin (20-40 µM)/ lutein (20-40 µM)</td>
</tr>
<tr>
<td>[127]</td>
<td>Quercetin (50 µM)/ myricetin (1 nM)/ kaempferol and rutin (up to 10 mM)</td>
</tr>
<tr>
<td>[128]</td>
<td>Quercetin (1-100 µM)</td>
</tr>
<tr>
<td>[129]</td>
<td>Quercetin/ quercetin-3-glucoside/ quercitin/ luteolin/ myricitin/ apigenin/ kaempferol/ rutin/ vitamin C (all of them 7.6-279.4 µM)</td>
</tr>
<tr>
<td>[130]</td>
<td>Quercetin/ myricitin/ rutin (10-200 µM)</td>
</tr>
<tr>
<td>[131]</td>
<td>Quercetin/ luteolin/ myricitin/ apigenin</td>
</tr>
<tr>
<td>[132]</td>
<td>Quercetin/ luteolin/ apigenin/ rutin</td>
</tr>
<tr>
<td>[133]</td>
<td>Quercetin (0.03-6 mM)/ rutin (0.02-3.28 mM)</td>
</tr>
<tr>
<td>[134]</td>
<td>(Quercetin 10-23 µM) +/- rutin (10-23 µM) +/- naringin (10-23 µM) /+/- β-carotene (20 µM)</td>
</tr>
<tr>
<td>[136]</td>
<td>Quercetin (100-500 µM)/ myricetin (100-500 µM)</td>
</tr>
<tr>
<td>[136]</td>
<td>Silmyarin (100-550 µM)/ myricetin (100-550 µM)/ quercetin (10-500 µM)/ kaempferol (50-500 µM)/ rutin (100-500 µM)</td>
</tr>
</tbody>
</table>

Duthie and Dobson, 1999.
Wilm et al, 2005.
Yeh et al, 2005.
<table>
<thead>
<tr>
<th>Ref.</th>
<th>Chemical Compound(s) (Concentration)</th>
<th>Cell or Tissue Type</th>
<th>Concentration</th>
<th>Effect</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>[137]</td>
<td>Silymarin (100-550 µM) / myricetin (100-550 µM) / quercetin (10-500 µM) / kaempferol (50-500 µM) / rutin (100-550 µM) / kaempferol-3-rutinoside (50-500 µM) / Food mutagens (Trp, IQ, Phip)</td>
<td>Lymphocytes / sperm / Jurkat T-cells (human)</td>
<td>In vitro</td>
<td>Generation DNA damage at lower concentrations. Reduction or abolition (quercetin) at higher concentrations.</td>
<td>Anderson et al., 1998.</td>
</tr>
<tr>
<td>[139]</td>
<td>Genistein and daidzein (0.01-2.5 µM in lymphocytes/24h, 2.5-20 µM in Jurkat cells/24h)</td>
<td>H₂O₂</td>
<td>Lymphocytes / sperm (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage in all cases.</td>
</tr>
<tr>
<td>[140]</td>
<td>Genistein (0.01-100 µM)/ equol (0.01-100 µM)/ ascorbic acid (10-600 µM)/ α-tocopherol (1-150 µM)</td>
<td>H₂O₂</td>
<td>Sperm (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage genistein and equol (0.01-100 µM), vitamin C (10-600 µM) and α-tocopherol (1-100 µM)</td>
</tr>
<tr>
<td>[141]</td>
<td>Genistein (10-400 µM)/ daidzein (100-400 µM)</td>
<td>On its own</td>
<td>Lymphocytes / sperm (human)</td>
<td>In vitro</td>
<td>Generation DNA damage (in all cases)</td>
</tr>
<tr>
<td>[142]</td>
<td>EGCG (0.01-200 µM)</td>
<td>On its own +/- H₂O₂</td>
<td>HL60 cells (human)</td>
<td>In vitro</td>
<td>Generation DNA damage (in all cases)</td>
</tr>
<tr>
<td>[144]</td>
<td>EGCG (250 µM)</td>
<td>UV</td>
<td>Lung fibroblasts / skin fibroblasts / keratinocytes (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
</tr>
<tr>
<td>[145]</td>
<td>EGCG (25-100 µM)</td>
<td>MNNG / H₂O₂</td>
<td>V79 lung cells (Chinese hamster)</td>
<td>In vitro</td>
<td>Generation DNA damage (in all cases)</td>
</tr>
<tr>
<td>[146]</td>
<td>EGCG, EGC or THF (10-100 µM)</td>
<td>BaP</td>
<td>Chang liver cells (human)</td>
<td>In vitro</td>
<td>Generation DNA damage EGCG, EGC and THF (100 µM) when on its own. Reduction DNA damage EGCG, EGC and THF (10-50 µM) when combined with BaP</td>
</tr>
<tr>
<td>[147]</td>
<td>Resveratrol / curcumin / indole-3-carbinol / ellagic acid</td>
<td>MNNG</td>
<td>V79 lung cells (Chinese hamster)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
</tr>
<tr>
<td>[148]</td>
<td>Resveratrol (10-250 µM)</td>
<td>H₂O₂</td>
<td>Gloma G5 cells (rat)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
</tr>
<tr>
<td>[150]</td>
<td>Resveratrol (1.56-25 µM) / coumarin (1.56-25 µM) / quercetin (1.56-25 µM) / 7,8-dihydroxy-4-methyl coumarin (1.56-25 µM) / vanillin (1.56-25 µM) / curcumin (1.56-25 µM) / rutin (2.5-50 µM)</td>
<td>H₂O₂</td>
<td>Lymphocytes (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage quercetin, 7,8-dihydroxy-4-methyl coumarin (3.1-25 µM), curcumin, resveratrol and vanillin (6.25-25 µM). No effect rutin and 7-hydroxy-4-methyl coumarin (up to 50 µM).</td>
</tr>
<tr>
<td>[150]</td>
<td>Resveratrol (10-100 µM) / 4-hexylresorcinol (10-100 µM)</td>
<td>H₂O₂</td>
<td>Lymphocytes (human)</td>
<td>In vitro</td>
<td>Reduction DNA damage</td>
</tr>
<tr>
<td>Reference</td>
<td>Treatment</td>
<td>Cell Lines</td>
<td>In Vitro</td>
<td>DNA Damage Effect</td>
<td>Remarks</td>
</tr>
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<tr>
<td>[154]</td>
<td>BHA</td>
<td>MNNG</td>
<td>In vitro</td>
<td>No effect</td>
<td>Horváthová et al., 1999.</td>
</tr>
<tr>
<td>[155]</td>
<td>BHA (250 µM)</td>
<td>MNNG</td>
<td>In vitro</td>
<td>No effect</td>
<td>Slameňová et al., 2003.</td>
</tr>
<tr>
<td></td>
<td>Ethoxyquin-quercetin (10-25 µM)</td>
<td></td>
<td></td>
<td>DNA damage (on its own).</td>
<td>Reduction DNA damage (H2O2)</td>
</tr>
</tbody>
</table>