Title: Antioxidants and the comet assay

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PII: S1383-5742(08)00075-6
DOI: doi:10.1016/j.mrrev.2008.05.002
Reference: MUTREV 7902

To appear in: *Mutation Research*

Received date: 30-8-2007
Revised date: 2-5-2008
Accepted date: 15-5-2008

Please cite this article as: E. Cemeli, A. Baumgartner, D. Anderson, Antioxidants and the comet assay, *Mutation Research-Reviews in Mutation Research* (2007), doi:10.1016/j.mrrev.2008.05.002

ANTIOXIDANTS AND THE COMET ASSAY

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Keywords: Review, Comet assay, antioxidants

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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_2O_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_2O_2 to O_2 and H_2O. At high concentrations of H_2O_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_2O_2. The reason for this is that catalase requires the reaction of two H_2O_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_2O_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_2O_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_2O_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 H_2O_2, 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 H_2O_2. 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 $O_2^{\cdot-}$ (superoxide radical) is a major factor in O_2 toxicity and that the role of SOD as a scavenger of $O_2^{\cdot-}$ by coupling and converting it to H_2O_2 is of utmost importance. The superoxide theory of O_2 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 (50μM) and H_2O_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_2O_2, being more suitable for the kinetics of disproportionation of the two $O_2^{\cdot-}$ carried out by SOD [20]. In other words, the rate of production of $O_2^{\cdot-}$ for the non-phytoestrogenic compounds is slower than their conversion to further H_2O_2. The fact that SOD accelerates H_2O_2 generation in the cellular environment obligates this enzyme to work in conjunction with enzymes that eliminate H_2O_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-nitrosoglutathione (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 H_2O_2 [49]. GPx reacts with H_2O_2 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 H_2O_2 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 $HO^{\cdot}$ 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 *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 –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 H_2O_2 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 O_2, 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 predominatly 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_2O_2 [100]. Further evidence of protection was seen against the effects of H_2O_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_2O_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 O_2 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 H_2O_2 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 B_1 (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 B_2 (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 B_3 (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 B_9 (folic acid), vitamin B_{12} (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 CoQ_{10}) 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 H_2O_2-induced oxidation but does not inhibit DNA strand breaks formation as observed in the Comet assay [129]. The formation of 8-hydroxydeoxy-guanosine (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_2O_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 H_2O_2 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 H_2O_2 [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 O_2, 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 $O_2^{\cdot-}$ quencher [142]. Among all carotenes, it is considered the most efficient at quenching singlet O_2 [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 $O_2^{\cdot-}$ production [149,150]; the low redox potentials of flavonoids thermodynamically allow them to reduce highly oxidising free radicals such as $O_2^{\cdot-}$, $RO^{\cdot}$ and $HO^{\cdot}$ [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 H_2O_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 H_2O_2 decreased, although not significantly [156]. Human

lymphocytes were pre-treated with a variety of flavonoids on their own, then challenged with H_2O_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 H_2O_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 (β-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 H_2O_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 μM) [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 μM whereas antioxidant effects occurred at 500 μM. 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 genestein 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 H_2O_2 in both cell types in the Comet assay. Both, daidzein and genestein 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 H_2O_2-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], H_2O_2 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_2O_2 to a certain extent in glioma cells although resveratrol *per se* induced a slight time and dose-dependent DNA damage. Using H_2O_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_2O_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_2O_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 O_2 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, O_2 is considered a potential mutagen, clastogen 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.

Reference number	Antioxidant (concentration)	Genotoxicant and/or disease	Target cells (Species)	Type of experiment	Antioxidant effect observed	Publication, year.
[16]	Vitamin C (0.04 – 5 mM)	H_2O_2	Lymphocytes (human)	*In vitro*	Generated DNA damage on its own, slight reduction DNA damage at low concentrations, increase at higher concentrations.	Anderson et al, 1994.
[17]	Vitamin C (100 mg/day) + vitamin E (280 mg/day) + β-carotene (25 mg/day)	Non/Smokers +/- H_2O_2	Lymphocytes (non/smokers 50-59 years old men)	*In vivo and ex vivo*	Reduction DNA damage baseline, increased resistance to DNA damage (H_2O_2)	Duthie et al, 1996.
[20]	Catalase (100-500 U/ml)	Diethylstilboestrol/ 17β-oestradiol/ nonylphenol/ equol/ genistein/ daidzein/ H_2O_2	Lymphocytes/ sperm (human)	*In vitro*	Reduction DNA damage	Anderson et al, 2003.
[20]	SOD (50-150 U/ml)	Diethylstilboestrol/ 17β-oestradiol/ nonylphenol/ equol/ genistein/ daidzein/ H_2O_2	Lymphocytes/ sperm (human)	*In vitro*	Reduction DNA damage (equol, genistein, daidzein)	Anderson et al, 2003.
[20]	Vitamin C (0.5-1 mM)	Diethylstilboestrol/ 17β-oestradiol/ nonylphenol/ equol/ genistein/ daidzein/ H_2O_2	Lymphocytes/ sperm (human)	*In vitro*	Reduction DNA damage or no effect (no apparent pattern)	Anderson et al, 2003
[21]	Catalase (100-500 U/ml)	Triiodothyronine/ noradrenaline	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Djelic and Anderson, 2003.
[22]	Catalase (100-500 U/ml)	Triiodothyronine/ L-Thyroxine sodium salt/ noradrenaline	Sperm (human)	*In vitro*	Reduction DNA damage	Dobrzynska et al, 2004.
[23]	Vitamin C (0.04-5 mM)	H_2O_2	Lymphocytes (human)	*In vitro*	Slight reduction DNA damage (up to 1 mM). Increase DNA damage (5 mM)	Anderson and Phillips, 1999.
[24]	Vitamin C (40 or 100 µM) +/- vitamin E (30 or 100 µM)	BaP	Lymphocytes (human female)	*In vitro*	Reduction DNA damage	Gajecka et al, 1999.
[39]	Catalase (20 µg/ml)	Ozone	Leukocytes (human)	*In vitro*	Prevention DNA damage	Diaz-Llera et al, 2002
[30]	Catalase (250 U/ml)	Chromium (III)/ (Vi)	Lymphocytes (human)	*In vitro*	Reduction (chromium (VI))	Blasiak and Kowalik, 2000.
[33]	Catalase (250 U/ml)	H_2O_2/ xanthine + xanthine oxidase	Cortical cells (adult mice)/ cultured astrocytes (rat)	*In vitro*	Abolition DNA damage	Cemeli et al, 2003.
[34]	Catalase (500 U/ml)	Iodoacetic acid	CHO (hamster)	*In vitro*	Reduction DNA damage	Cemeli et al, 2006.
[34]	BHA (10-100 µM)	Iodoacetic acid	CHO (hamster)	*In vitro*	Reduction DNA damage	Cemeli et al, 2006.
[35]	Catalase (250 U/ml)	Vitiligo +/- H_2O_2/ vitiligo +/- 17β-oestradiol	Lymphocytes (human)	*In vitr/, ex vivo*	Reduction DNA damage	Schallreuter et al, 2006.
[38]	SOD (200 U/ml)	SIN-1, GSNO or RBS	HIT-T15 (hamster)	*In vitro*	No effect DNA damage	Delaney et al, 1997.
[41]	SOD (144 U/ml)	Metronidazole/ dimetridazole	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Ré et al, 1997.
[42]	SOD (100 U/ml)	Benzidine	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Chen et al, 2003.
[42]	BHT (100 µM)	Benzidine	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Chen et al, 2003.

[43]	SOD	Hyperbaric oxygen	Lymphocytes (human)	*Ex vivo*	Reduction DNA damage	Muth et al, 2004.
[47]	Sodium selenite (Culture 100 nM for 10 days)	H_2O_2	Leukemic lymphoblasts (mice)	*In vitro*	Reduction DNA damage	Bouzyk et al, 1997.
[48]	Sodium selenate (600 µM)	UVA	Skin fibroblasts (human)	*In vitro*	Reduction DNA damage	Emonet-Piccardi et al, 1998.
[49]	Sodium selenate (50 nM)/ selenomethionine (200 nM)	UV	Keratinocytes (human)	*In vitro*	Prevention DNA damage (with pre-treatment)	Rafferty et al, 2003.
[50]	Sodium selenate (0.5 M)	Potassium dichromate	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Cemeli et al, 2003.
[51]	Selenomethionin (6 µg/kg/day) / high-selenium yeast (6 µg/kg/day)	Elder	Lymphocytes and prostate cells (dogs)	*In vivo supplementation*	Reduction DNA damage	Waters et al, 2003.
[52]	Kappa-selenocarrageenan (0.25 µg/ml)	Lead acetate	Blood cells (Mice)	*In vivo supplementation*	Reduction DNA damage	Yuan and Tang, 2001.
[56]	Sodium selenite (10 µM)	On its own	HepG2 (human)	*In vitro*	Generation DNA damage	Shen at al, 1999.
[59]	Ferritin (intracellular levels)	Hyperbaric oxygen	Lymphocytes (human)	*Ex vivo*	Increased expression ferritin	Rothfuss and Speit, 2002.
[60]	Deferiprone (0.05-1.6 mM)/ deforexamine (0.05-1.6 mM)	Ferric chloride or ferrous chloride	Lymphocytes (human)	*In vitro*	Reduction DNA damage (deferoxamine)	Anderson et al, 2000.
[61]	Deferoxamine (0.05-1.6 mM)	H_2O_2 + ferrous sulphate/ H_2O_2 + ferric chloride or H_2O_2 + ferrous chloride	Lymphocytes (human)	*In vitro*	Reduction DNA damage (H_2O_2 + ferrous sulphate)	Anderson et al, 2000.
[65]	Melatonin (10 mg/kg/ for 6 days) or (0.1-2.5 mM)	Adriamycin	(rats)	*In vivo and in vitro*	Reduction DNA damage	Kim et al, 2005.
[66]	Melatonin (50 µM)/ amifostine (14 mM)	Idarubicin	Lymphocytes heathy subjects, leukemic K562 or HeLa (human)	*In vitro*	Reduction DNA damage by melatonin. Reduction DNA damage only in lymphocytes healthy subjects by amifostine. Generation DNA damage in cancer cells by amifostine	Majsterek et al, 2005.
[67]	Melatonin (10-50 µM)	Amoxicillin	Lymphocytes, gastric mucosa cells (human)	*In vitro*	Reduction DNA damage	Arabski et al, 2005.
[68]	Melatonin (300 mg single-supplement)	Gamma-irradiation	Whole blood (human)	*Ex vivo*	Reduction DNA damage	Vijayalaxmi et al, 1998.
[69]	Melatonin (100 mg/kg)	Ionising radiation	Brain cells (rat)	*In vivo*	Reduction DNA damage	Undeger et al, 2004.
[70]	Melatonin (0.5-1 mM)	Magnetic fields + iron ions	Lymphocytes (rat)	*In vitro*	Reduction DNA damage at 0.5mM and abolition at 1 mM	Jajte et al, 2001.
[71]	Melatonin (100 µM)	Graves' disease	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Tang et al, 2005.
[72]	Melatonin (0.1-1 mM)	On its own, H_2O_2 or bleomycin	CHO (hamster)	*In vitro*	Slight increase DNA damage (on its own). Slight reduction DNA damage (H2O2). No effect DNA damage (bleomycin)	Festa et al, 2001.
[73]	Melatonin (100 µM)	On its own	CHO (hamster)	*In vitro*	Generation DNA damage	Anisimov et al, 2006.

[74]	Melanin (stimulated to reach 14, 93 to 164 µg melanin / mg protein)	H_2O_2	Melanocytes, HaCat keratinocytes (human)	*In vitro*	Reduction DNA damage	Hoogduijn et al, 2004.
[75]	Melanin (stimulated melanin production)	Ionising radiation	Melanoma cell line (human)	*In vitro*	Reduction DNA damage	Kinnaert et al, 2004.
[76]	Melanin (stimulated melanin production, tomato extract 50 µg/ml, palm fruit 10 µg/ml, vit E 35 µM and vit C 100 µM)	UVA	Melanocytes (human)	*In vitro*	Reduction DNA damage	Smit et al, 2004.
[77]	Melanin (stimulated melanin production)	UVA	Melanocytes, fibroblasts (human)	*In vitro*	Generation DNA damage	Marrot et al, 1999.
[78]	DHICA (0.125-2 µM)	UVA	HaCat keratinocytes (human)	*In vitro*	Generation DNA damage	Kipp and Young, 1999.
[80]	Vitamin C	H_2O_2	Lymphocytes (human non/smokers)	*Ex vivo*	Reduction DNA damage	Panayiotidis and Collins, 1997.
[81]	Vitamin C	On its own +/- H_2O_2	Lymphocytes (human)	*In vitro*	Slight reduction DNA damage (low concentrations). High increase DNA damage (high concentrations).	Harréus et al, 2005.
[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 (H_2O_2)	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)	Møller 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-dimeDBC	HepG2 (human)	*In vitro*	Reduction DNA damage (vitamin C). Slight reduction DNA damage (vitamin E)	Lazarová and Slameňová, 2004.
[86]	Vitamin E (20 µM)	Bleomycin	Colonic mucosa (human)	*In vitro*	Reduction DNA damage	Wozniak et al, 2004.
[87]	Vitamin E (10-25 µM)	Acrylamide	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Blasiak et al, 2004.
[88]	Vitamin E (50 µM, 4h pre-treatment)	Ciprofloxacin	Primary culture astrocytes (rat)	*In vitro*	Reduction DNA damage	Gürbay et al, 2006.
[89]	Vitamin E (10-50 µM)	Streptozotocin	Lymphocytes/ HeLa (human)	*In vitro*	Reduction DNA damage	Blasiak et al, 2004.
[90]	Vitamin E (300 mg/day) + vitamin C (500 mg/day)	Waste anaesthetic gases	Lymphoctes (human)	*In vivo*	Reduction DNA damage	Sardas et al, 2006.
[91]	Trolox (50-200 µM)	H_2O_2	Buccal cells (human)	*In vitro*	Reduction DNA damage	Szeto et al, 2005.
[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	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.
[94]	Vitamins A (10,000-50,000	p-dimethylaminoazobenzene	Hepatocytes	*In vivo*	Reduction DNA damage (high	Velanganni et al, 2007.

	IU/ml) + vitamin C (75-1000 mg) + vitamin E (50-500 mg) / kg body. Once a week for 6 months.		(mice)		doses each vitamin individually and low doses as mixture)	
[95]	Vitamin A (5-7 μM)	On its own	V79 lung cells (Chinese hamster)	*In vitro*	Generation DNA damage	Klamt et al, 2003.
[97]	Thiamine + vitamin C	Lead acetate	Testicular cells (mice)	*In vivo*	Reduction DNA damage (low and middle doses)	Zhang et al, 2006.
[99]	Riboflavin (50 mg/kg) +/- vitamin C (150 mg/kg). Single or multiple doses. 24h prior, during genotoxicant administration or 24h after.	Sanguinarine/ argemone oil	Bone marrow/ whole blood (mice)	*In vivo*	Reduction DNA damage (in all cases)	Ansari et al, 2006.
[101]	Nicotinamide (5 μM)	H_2O_2	Lymphocytes (human)	*In vitro*	Inhibits DNA repair. Reverses necrosis to apoptosis	Tronov et al, 2002.
[102]	Nicotinamide (200-800 mg/kg)	+/- Ionising radiation	Tumor/ thymus/ spleen/ testis/ bone marrow/ brain (mice)	*In vivo*	Extensive DNA damage. Slowed down strand break rejoining (except for brain cells)	Zheng and Olive, 1996.
[105]	Coenzyme Q10 (100 μM)	H_2O_2	Lymphocytes (human)	*In vitro*	No effect DNA damage. Reduction DNA oxidation	Tomasetti et al, 1999.
[106]	Coenzyme Q10 (3 mg/kg/day) 2 weeks.	On its own	Lymphocytes (human)	*In vivo*	Generation 8-OHdG (detected by Comet assay)	Niklowitz et al, 2007.
[110]	β-carotene (0.25-10 μM) + lycopene (0.25-10 μM)	Catechol-oestrogens	Naked plasmid DNA, V79 lung cells (Chinese hamster)	*In vitro*	Reduction DNA damage	Muzandu et al, 2005.
[111]	β-carotene (1-10 μM) + lycopene (1-10 μM)	Xanthine/xanthine oxidase	Colon adenocarcinoma HT29 (human)	*In vitro*	Reduction DNA damage (Up to 3 μM). Generation DNA damage (4-10 μM)	Lowe et al, 1999.
[112]	β-carotene (0.5-8 μM)/ lycopene (0.5-8 μM)/ lutein (0.5-8 μM)	H_2O_2	Leukemia MOLT-17 (human)	*In vitro*	Reduction DNA damage (0.5-1 μM)	Astley et al, 2004.
[112]	β-carotene/ lycopene/ lutein, (natural isolate capsules, 15 mg/d, 4 weeks)	+/- H_2O_2	Lymphocytes (human males)	*In vivo/ ex vivo*	Generation DNA damage (β-carotene). No effect DNA damage on its own (lycopene, lutein). No effect after H_2O_2 challenge (β-carotene, lycopene, lutein)	Astley et al, 2004.
[113]	β-carotene (12 mg/kg) + vitamin C (400 mg/kg) + vitamin E (200 mg/kg)	γ-radiation	Cultured lymphocytes (mice)	*In vitro*	Reduction DNA damage after 1h "liquid holding"	Konopacka et al, 1998.
[114]	β-carotene (15 mg/day)/ lycopene (15 mg/day/ lutein (15 mg/day) 1 week. Supplementation separated by 3-week wash-out periods.	H_2O_2	Lymphocyes (human)	*Ex vivo*	Rejoining DNA breaks (lycopene and β-carotene). No effect (lutein)	Torbergsen and Collins, 2000.
[116]	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-	H_2O_2	Lymphocytes (human)	*Ex vivo*	Reduction DNA damage	Porrini et al, 2005.

	tocopherol)					
[118]	Zeaxanthin (5-100 μM) + asthaxanthin (5-100 μM) + lutein (5-100 μM)	UVA	Trachea epithelial cells (rat)/ neuroblastoma cells (human)	*In vitro*	Presence carotenoids during exposure: reduction (tracheal cells) and generation (neuroblastoma) DNA damage. Addition carotenoids after exposure: generation (tracheal cells) and reduction (neuroblastoma) DNA damage	Santocono et al, 2006.
[119]	Zeaxanthin (20-40 μM)/ asthaxanthin (20-40 μM)/ lutein (20-40 μM)	Reactive nitrogen species	Neuroblastoma cells (human)	*In vitro*	Reduction DNA damage	Santocono et al, 2007.
[127]	Quercetin (50 μM)/ myricetin (1 mM)/ kaempferol and rutin (up to 10 mM)	H_2O_2	Caco-2 cells (human)	*In vitro*	Prevention DNA damage (quercitin and myriecitin). No effect (kaempferol and rutin).	Duthie and Dobson, 1999.
[128]	Quercetin (1-100 μM)	H_2O_2	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Wilms et al, 2005.
[129]	Quercetin/ quercetin-3-glucoside/ quercitrin/ luteolin/ myricitin/ apigenin/ kaempferol/ rutin/ vitamin C (all of them 7.6-279.4 μM).	H_2O_2	Lymphocytes (human)	*In vitro*	Reduction DNA damage (in all cases). Better reduction free flavonoids than conjugated ones	Noroozi et al, 1998.
[130]	Quercetin/ myricitin/ rutin (10-200 μM)	H_2O_2	Caco-2 cells/ HepG2 (human)	*In vitro*	Reduction DNA damage	O'Brien et al, 2000.
[131]	Quercitrin/ luteolin/ myricitin/ apigenin	H_2O_2	Myelogenous leukemia K562 (human)	*In vitro*	Reduction DNA damage quercetin and luteolin (20-100 μM), rutin (100-1000 μM). No effect apigenin.	Horváthová et al, 2004.
[132]	Quercetin/ luteolin/ apigenin/ rutin	H_2O_2	Leukemia L1210 (mice)	*In vitro*	Reduction DNA damage quercetin, luteolin and apigenin (600 μM). Generation DNA damage apigenin (1.2 mM). No effect rutin.	Horváthová et al, 2003.
[133]	Quercetin (0.03-6 mM)/ rutin (0.02-3.28 mM)	Mitomycin C	Lymphocytes (human)	*In vitro*	Reduction quercetin (all concentrations) and rutin (0.02-0.82 mM). Generation DNA damage rutin (1.64-3.28 mM).	Undëger et al, 2004.
[134]	(Quercitin (10-23 μM) +/- rutin (10-23 μM) +/- naringin (10-23 μM)) +/- β-carotene (20 μM)	UVA	C3H10T1/2 embryo fibroblast cells (mouse)	*In vitro*	Reduction DNA damage (quercetin, rutin and naringin) with/without β-carotene. Generation DNA damage β-carotene (on its own).	Yeh et al, 2005.
[136]	Quercetin (100-500 μM)/ Kaempferol (100-500 μM)	H_2O_2/ β-estradiol/ diethylstilbestrol/ daidzein/ genistein	Lymphocytes/ sperm (human)	*In vitro*	Reduction DNA damage (not consistently at 100 μM but consistently at 500 μM)	Cemeli et al, 2004.
[136]	Silymarin (100-550 μM)/ myricetin (100-550 μM)/ quercetin (10-500 μM)/ kaempferol (50-500 μM)/ rutin (100-500 μM)/ kaempferol-3-	Food mutagens (Trp/ IQ)	Lymphocytes/ sperm (human)	*In vitro*	Generation DNA damage at lower concentrations. Reduction or abolition (quercetin) at higher concentrations.	Anderson et al, 1997.

	rutinoside (50-500 μM)					
[137]	Silymarin (100-550 μM)/ myricetin (100-550 μM)/ quercetin (10-500 μM)/ kaempferol (50-500 μM)/ rutin (100-500 μM)/ kaempferol-3-rutinoside (50-500 μM)	Food mutagens (Trp/ IQ/ Phip)	Lymphocytes/ sperm (human)	*In vitro*	Generation DNA damage at lower concentrations. Reduction or abolition (quercetin) at higher concentrations.	Anderson et al, 1998.
[139]	Genistein and daidzein (0.01-2.5 μM in lymphocytes/24h, 2.5-20 μM in Jurkat cells/24h)	H_2O_2	Lymphocytes/ Jurkat T-cells (human)	*In vitro*	Reduction DNA damage in all cases.	Foti et al, 2005.
[140]	Genistein (0.01-100 μM)/ equol (0.01-100 μM)/ ascorbic acid (10-600 μM)/ α-tocopherol (1-150 μM)	H_2O_2	Sperm (human)	*In vitro*	Reduction DNA damage genistein and equol (0.01-100 μM), vitamin C (10-600 μM) and α-tocopherol (1-100 μM)	Sierens et al, 2000.
[141]	Genistein (10-400 μM)/ daidzein 100-400 μM)	On its own	Lymphocytes/ sperm (human)	*In vitro*	Generation DNA damage (in all cases)	Stopper et al, 2005.
[142]	EGCG (0.01-200 μM)	On its own +/- H_2O_2	HL60 cells (human)	*In vitro*	Generation DNA damage (in all cases)	Elbling et al, 2005.
[143]	EGCG (2 μM)	Bleomycin	Leucocytes (human)	*In vitro*	Reduction DNA damage	Glei and Pool-Zobel, 2006.
[144]	EGCG (250 μM)	UV	Lung fibroblasts/ skin fibroblasts/ keratinocytes (human)	*In vitro*	Reduction DNA damage	Morley et al, 2005.
[145]	EGCG (25-100 μM)	MNNG/ H_2O_2	V79 lung cells (Chinese hamster)	*In vitro*	Reduction DNA damage (in all cases)	Roy et al, 2003.
[146]	EGCG, EGC or THF (10-100 μM)	BaP	Chang liver cells (human)	*In vitro*	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	Yen et al, 2004
[147]	Resveratrol/ curcumin/ indole-3-carbinol/ ellagic acid	MNNG	V79 lung cells (Chinese hamster)	*In vitro*	Reduction DNA damage	Chakraborty et al, 2004.
[148]	Resveratrol (10-250 μM)	H_2O_2	Glioma C6 cells (rat)	*In vitro*	Reduction DNA damage	Quincozes-Santos et al, 2007.
[150]	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)	H_2O_2	Lymphocytes (human)	*In vitro*	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).	Liu and Zheng, 2002.
[150]	Resveratrol (10-100 μM)/ 4-hexylresorcinol (10-100 μM)	H_2O_2	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Yen et al, 2003.
[151]	Resveratrol (25 μg/ml)	Selenium-cisplatin conjugated	Lymphocytes (human)	*In vitro*	Reduction DNA damage	Olas et al, 2005.
[152]	Resveratrol (50-200 μM)	On its own	Lymphocytes (human)	*In vitro*	Generation DNA damage	Azmi et al, 2006.
[153]	Resveratrol (50 μM)	Cu(II)	Lymphocytes	*In vitro*	Generation DNA damage	Azmi et al, 2005.

			(human)			
[154]	BHA	MNNG	V79 lung cells (Chinese hamster)	*In vitro*	No effect DNA damage	Horváthová et al, 1999.
[155]	BHA (250 µM)	MNNG	V79 lung cells (Chinese hamster)	*In vitro*	No effect DNA damage	Slameňová et al, 2003.
[157]	Ethoxyquin-rutin (1-25 µM)/ ethoxyquin-quercetin (10-25 µM)	On its own +/- H_2O_2	Lymphocytes (human)	*In vitro*	Generation DNA damage (on its own). Reduction DNA damage (H_2O_2)	Blaszczyk and Skolimowski, 2007.