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METHODS FOR THE DETECTION OF ANTIOXIDANTS WHICH PREVENT AGE RELATED DISEASES: A CRITICAL REVIEW WITH PARTICULAR EMPHASIS ON HUMAN INTERVENTION STUDIES

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It is well documented that reactive oxygen species (ROS) are involved in the aetiology of age related diseases. Over the last decades, strong efforts have been made to identify antioxidants in human foods and numerous promising compounds have been detected which are used for the production of supplements and functional foods. The present paper describes the advantages and limitations of methods which are currently used for the identification of antioxidants. Numerous *in vitro* methods are available which are easy to perform and largely used in screening trials. However, the results of such tests are only partly relevant for humans as certain active compounds (e.g. those with large molecular configuration) are only poorly absorbed in the gastrointestinal tract and/or may undergo metabolic degradation. Therefore experimental models are required which provide information if protective effects take place in humans under realistic conditions. Over the last years, several methods have been developed which are increasingly used in human intervention trials. The most widely used techniques are chemical determinations of oxidised guanosine in peripheral blood cells or urine and single cell gel electrophoresis (comet) assays with lymphocytes which are based on the measurement of DNA migration in an electric field. By using of DNA-restriction enzymes (formamidopyrimidine DNA glycosylase and endonuclease III) it is possible to monitor the endogenous formation of oxidised purines and pyrimidines; recently also protocols have been developed which enable to monitor alterations in the repair of oxidised DNA. Alternatively, also the frequency of micronucleated cells can be monitored with the cytokinesis block method in peripheral human blood cells before and after intervention with putative antioxidants. To obtain information on alterations of the sensitivity towards oxidative damage, the cells can be treated *ex vivo* with ROS (H_2O_2 exposure, radiation). The evaluation of currently available human studies shows that in approximately half of them protective effects of dietary factors towards oxidative DNA-damage were observed. Earlier studies focused predominantly on the effects of vitamins (A, C, E) and carotenoids, more recently also the effects of fruit juices (from grapes, kiwi) and beverages (soy milk, tea,

coffee), vegetables (tomato products, berries, Brussels sprouts) and other components of the human diet (coenzyme Q_{10}, polyunsaturated fatty acids) were investigated. On the basis of the results of these studies it was possible to identify dietary compounds which are highly active (e.g. gallic acid). At present, strong efforts are made to elucidate the different parameters of oxidative DNA-damage correlate with life span, cancer and other age related diseases. The new techniques are highly useful tools which provide valuable information if dietary components cause antioxidant effects in humans and can be used to identify individual protective compounds and also to develop nutritional strategies to reduce the adverse health effects of ROS.

INTRODUCTION

More than 50 years ago, two scientists, namely Harman and Gerschman who worked independently at the University of California (Berkeley), published a series of articles on the role of oxygen free radicals (ROS) in the pathogenesis of aging (1, 2). In subsequent years, numerous investigations were conducted in which it was shown that ROS play a key role in senescence and some of the underlying mechanisms were elucidated. For example, it was demonstrated that oxidative damage in nuclear DNA causes mutations which lead to destruction of vital molecular mechanisms (3). In addition, it was found that also telomere shortening is affected by ROS (4, 5) and it was shown that mitochondrial DNA is a target for damage caused by ROS. In this context it is notable that it was hypothesized that accumulation of point mutations in mitochondria with age might be due to oxidative damage (4, 6). Apart from damage of DNA, also oxidative damage of lipids and proteins plays an important role in aging and it was proposed that oxidatively modified proteins, which are incorrectly folded, are instable and assume structures that form aggregates which lead to a number of degenerative human diseases (7, 8).

As a result of the damage caused at the molecular level, ROS exposure leads to a number of age specific diseases. Apart of cancer, which is probably a consequence of oxidative DNA-damage and interactions with signaling pathways (3, 9), also immune functions are impaired (10, 11). Furthermore, there is convincing evidence that ROS are involved in arteriosclerosis and coronary heart disease. This assumption is also supported by the observation of beneficial effects of antioxidants towards cardiovascular diseases which were seen in a number epidemiological and animal studies (3, 12).

Strong support for the assumption that ROS are involved in aging processes comes additionally from studies that suggest that elevated levels of protective enzymes, as well as increased concentrations of exogenous and endogenous antioxidants can increase the life-span of different species (7, 10, 13-17).

In order to avoid the adverse health effects of ROS, intense efforts were made to identify protective constituents in the human diet. Already in the 1950s and 60s, the antioxidant properties of vitamins (C, A, E) were intensely studied. Subsequently, it was discovered that many other plant constituents do possess antioxidant properties and a vast number of protective compounds was discovered. Among the most important groups are carotenoids, flavonoids (comprising flavonols, isoflavones and anthocyans) as well as many other polyphenolics (18, 19). Also pigments such as chlorophyllins, phytosterines and allylsulfides were found to be ROS protective (20, 21).

Most of these antioxidants were discovered in *in vitro* trials. Although it is not known if they are protective in humans, many of these compounds are currently marketed as food supplements and are used for the production of functional foods (22). In the present article we will critically discuss the methods which are currently used for the detection of antioxidant properties and their limitations. In the last paragraphs, methods will be described which can be used for the detection of antioxidants in humans.

In vitro tests used for the detection of antioxidants

A broad variety of *in vitro* techniques has been developed for the detection of antioxidants which are based on the ability of compounds to scavenge peroxylradicals. These methods are based on the direct interaction with reactive molecules or on their reactivity with metal ions and the effects are monitored by chemical measurements (in many cases by spectrophotometry). Examples are determinations of peroxyl radical scavenging (trichlormethyl peroxyl or alkoxyl peroxyl radical) (23, 24), the ORAC assay (oxygen radical absorbance assay) (25, 26), the PLC test (phytochemoluminescence assay) (27), different forms of the TEAC test (2,2′-azino-bis/ 3-etyhlbenzthioazoline-6-sulfonic acid radical ABTS+/metmyoglobin) (28), including the TROLOX (a specific form of TEAC with manganese dioxide) (29), the TOSCA (total antioxidant scavenging assay) (30), the DPPH test (diphenyl-1-picrylhydrazyl assay), the TRAP (total radical-trapping antioxidant parameter) (31), or the FRAP method (ferric reducing ability of plasma) (32). The TBARS (thiobarbituric acid reactive substances) assay is based on the measurement of malondialdehyde (MDA) which is formed as a consequence of lipid peroxidation and can be conducted with subcellular membrane preparations or intact cells, prevention of formation of MDA can be used to assess antioxidant properties (33).

In addition to these methods, chemical approaches have been developed which allow the detection of radical specific DNA-modifications *in vitro* (34) or chemical (bleomycin mediated) damage (35). Several other techniques are described in a recent review of Aruoma (36) to which the reader is referred for more details. The same author also compared antioxidant indices of a variety of compounds obtained with different methods and found pronounced differences

indicating that the findings obtained with the different methods are not comparable. A general major problem associated with the use of chemical analytical *in vitro* methods is that they are conducted under non physiological conditions. Therefore, the results obtained with subcellular fractions can not be extrapolated to the *in vivo* situation.

A step closer to the human situation is the use of intact cells which can be challenged with ROS generating chemicals or radiation in absence and presence of putative antioxidants. Extracellular release of superoxide can be measured on the basis of superoxide dismutase (SOD) inhibition of superoxide induced reduction of exogenously supplied reactive cytochrome c by means of a plate reader assay with cells *in situ* (37). Intracellular ROS and superoxide production can be detected by means of the fluorescent probes dichlorofluorescin diacetate (DCFH-DA) and dihydroethidium (DHE), respectively, and measured by flow cytometric analysis (38, 39).

Use of endpoints such as induction of oxidative DNA-damage and mutations (40) provides information if protection can be detected in intact cells. Furthermore, some methods have been developed which provide valuable information if the antioxidant effects take place intercellularly (41).

New methods to detect antioxidative effects in humans

Many preparations and extracts contained in herbs and plants which are used as foods are currently marketed but have never been tested for protective effects in humans. Some of these supplements are probably ineffective in inner organs as the active compounds are very poorly absorbed (typical examples are supplements containing chlorophylls, curcumin or anthocyanins). These compounds are highly effective under *in vitro* conditions but it is unlikely that they cause protective effects in inner organs since they are not absorbed in the intestinal tract.

In the case of green tea, highly concentrated preparations are produced with catechin concentrations which are substantially higher than those contained in native tea. Our recent investigations indicated that elevated concentrations of (-)-epigallocatechin-3-gallate (EGCG) as well as tea condensates cause substantial oxidative DNA-damage in intact cells and intracellular generation of H_2O_2 [42]. Therefore, it is unlikely that consumption of such supplements causes protective effects in humans and it is possible that they lead to adverse health effects. In this context it is notable that many antioxidants can also act under certain conditions as prooxidants and in many protection studies U-shaped dose response curves have been obtained (40, 43). All these findings underline the importance of human studies to verify putative antioxidant effects under realistic conditions.

Chemical approaches used in in vivo studies

Some of the analytical chemical methods which can be used for the detection of antioxidative activity under *in vitro* conditions, can also be employed to assess

protective effects in human intervention studies. Some examples, as well as other endpoints which are used occasionally are listed in *Table I*.

Table I. Analytical chemical methods for the detection of antioxidative activity in humans

Endpoint	Example - Remarks	Reference
FRAP-Status	Ferric reducing ability in plasma was used to assess antioxidant effects of fruit juices in a few studies	[69] [32]
TBARS	Thiobarbituric acid reactive substances in plasma, which are formed as a consequence of lipid-peroxidation can be monitored fluorometrically or by HPLC	[33]
FOX-2	Ferrous oxidation of xylene orange is used for determination of hydroperoxides in serum	[88]
TEAC	Trolox equivalent antioxidant capacity in plasma used to study effects of fruit juices	[89]
Isoprostane levels	Can be analyzed by GC/MS to measure lipid-peroxidation in urine and blood, used e.g. in studies with alcohol consumption, ß-carotene or cocoa supplementation	[90] [91] [92]
TFR	Total free radicals in plasma measured by chemoluminescence with lucigenie, used in a study on grape juice consumption	[93]
LDL-oxidation	Oxidation of human low density lipoprotein (LDL) can be measured by monitoring the change of diene absorption, dienes are products of lipid-peroxidation in presence of Cu^{2+} ions	[94]

Detection of oxidised DNA bases in humans

When ROS attack DNA, several types of DNA-lesions are formed including small base lesions and exocyclic adducts (44). 8-OxodG is one of the most easily formed oxidised bases. It can be detected in both, urine and tissue, after oxidative stress (45) and can be measured by use of several chromatographic techniques including HPLC with electrochemical detection and tandem mass spectrometry, GC-MS, thin layer chromatography and antibody based immunoassays (46). The different methods give results which vary strongly, mainly because of the formation of artefacts during the sample preparation and strong attempts have been made to standardize and validate the different methods (47). In totals results from sixteen human intervention trials are available in which the impact of dietary factors on 8-OxodG levels in white blood cells was measured and approximately in half of them protective effects were observed (for details see review of Moller and Loft (48, 49)). Protective effects were seen in some (not all) studies with vitamin supplementation (e.g. vitamin C, E) and also after intervention with combinations of vitamins (vitamin C, E and ß-carotene), vegetables (e.g. Brussels sprouts, onions), red wine and tomato based products.

Use of single cell gel electrophoresis assays for detection of protective effects

The single cell gel electrophoresis assay is based on the determination of DNA-migration in an electric field (50). Depending on the experimental conditions, different types of lesions and effects can be monitored and recently additional methods have been developed which can be used to measure repair of oxidative damage (51). *Table II* gives an overview of different parameters which can be studied in comet assays.

Table II. Different modifications of the single cell gel electrophoresis assay

Endpoint	Procedure	Reference
Double strand breaks	DNA unwinding, under neutral conditions	[95]
Endogenously formed single strand breaks	DNA unwinding under alkaline conditions	[50]
Endogenous formation of oxidised pyrimidines	Treatment of nucleotides with formamido-pyrimidine (FPG)	[96]
Exogenous formation of oxidised purines	Treatment of cells with endonuclease III	[96]
Altered sensitivity to exogenous ROS	Treatment of the cells with ROS- generating chemicals (H_2O_2) or γ-radiation	[97, 98]
DNA repair of oxidised bases	Monitoring enzymatic repair activity in cell free extracts; not suitable to monitor compounds with putative antioxidant effects, such as reduction of oxidative stress may be interpreted as repair. In an improved version, extracts are added to gel embedded nucleotids with a fixed level of oxidised guanosine (caused by treatment with a photosensitizer RO19-8022 + light)	[99] [100]
Alteration of the sensitivity towards heterocyclic aromatic amines	Lesions are induced *ex vivo* by Trp-P-2 and PhIP in lymphocytes (up to now there is only one study with Brussels sprouts)	[101]

The first human intervention study was performed by Pool-Zobel et al. in 1997 (52). In the meantime, 45 studies have been published in total, twenty of them in the last two years (on the contrary only two papers appeared in this time period in which 8-Oxo-dG formation was used as an endpoint). The results of the individual studies are summarized in the articles of Moller and Loft (48, 49). Some newer experiments conducted in our laboratory with coffee, Brussels sprouts and with the spice Sumach (*Rhus coriaria*) are only available in abstract form (53-55). Most of the trials were carried out with normal healthy individuals, but over the last years, a number of investigations were published in with subjects under oxidative stress (i.e. healthy individuals with hyperbaric treatment, after

exercise, or patients with diabetes) were included. Most of the earlier studies were carried out with individual vitamins and combinations of vitamins, whereas more recently the interventions focused on the effects of natural foods (juices, vegetables). Taken together, protective effects were seen in about 50% of the investigations and in most cases, reduction of exogenous formation of purines and pyrimidines as well as increased resistance towards H_2O_2 was found, whereas only in three investigations a reduction of endogenous formation of single strand breaks (reduction of the size of endogenously formed comets) was detected.

The comet assay has also been used to investigate seasonal differences in DNA migration and it was shown that DNA damage is lower in summer than in the cold season. These differences correlated well with the serum levels of certain antioxidants (56).

Monitoring chromosomal alterations and micronuclei as markers of oxidative damage

It has been shown that antioxidant supplementation in humans leads to a decrease of chromosomal alterations in peripheral lymphocytes (57). Also the frequency of micronuclei (MN), which are formed as a consequence of breakage and aneuploidy, was decreased after supplementation with vitamins and selenium (58). However, it should be noted that these endpoints are not indicative *per se* for oxidative damage, in other words a decrease of their frequencies by dietary constituents is not necessarily due to protection against ROS, but may be due to other mechanisms (e.g. protection against other DNA-reactive chemicals, or induction of repair mechanisms, etc.).

Fenech et al. (59) published the results of an intervention study in which they investigated the effect of wine consumption on MN frequencies induced by ROS in peripheral lymphocytes and found pronounced protection. MN formation was also used as an endpoint in a comparative study with vegetarians and non-vegetarians (60). Quite unexpectedly it was found that the vitamin C levels correlated with MN induction in young females and that the frequency of micronucleated cells was lower in non vegetarians than in vegetarians. On the basis of this study the authors concluded that folate and vitamin B12 supply (via meat consumption) might be more important parameter for MN prevention than vitamins with antioxidant capacity (60). This assumption was also hardened by the results of a recent study in Slovakia, in which the effect of antioxidant supplementation (Vitamin C+ E+ ß-carotene+ Se) on MN-formation was monitored. Decreases in the numbers of MN were only seen in individuals with normal folate levels (61), but not in participants who had low levels. Furthermore, it was found in the same study, that MN-numbers declined in particular in those individuals who had high levels of MDA in their plasma. These latter results indicate that oxidative DNA-damage contributes to MN-formation. The MN assay was also used in a number of intervention studies with vitamins in

individuals which are exposed to increased ROS levels, for example in persons treated with X-rays (62), smokers (63), or mitochondrial disease patients (64). Also successful results from an intervention studies with workers exposed to chemical mutagens which cause oxidative DNA-damage were reported with this method (65).

A specific modification of the chromosomal aberration assay is the "mutagen sensitivity test" with bleomycin, a compound which induces DNA-damage via generation of ROS. Godmann et al. (66) found in a study with healthy volunteers that the sensitivity of peripheral blood cells is not affected by antioxidant supplementation, but in another study, a significant association was found between mutagen sensitivity and antioxidant vitamin levels (vitamin C, carotenoids) in serum (67).

Design and interpretation of human intervention studies

There is no doubt that the human trials described in the last chapter are important tools to identify dietary antioxidants. Intervention studies are definitely more adequate than comparative studies with different population groups as inter-individual variations can be minimized. A sequential design seems not adequate since biomarkers of DNA-damage show alterations over time. Moller and Loft (48, 49) emphasized the importance of the inclusion of placebo groups (which is not feasible when complex foods are tested but can be used in studies with supplements) as well as washout periods to improve the quality and predictive value of such trials.

Also the treatment schedule is an important parameter. We found in our studies that certain dietary components (gallic acid and coffee) are potent inducers of superoxide dismutase in serum, and that their protective effects are only partly due to direct scavenging of ROS (68). Such enzyme induction effects can only be detected after multiple dosing; also the induction of glutathione (an important endogenous ROS scavenger molecule) by coffee is only seen after repeated consumption.

As mentioned above, good correlations were found between the levels of individual antioxidants and DNA-stability parameters (MN induction and comet induction) in a number of studies (57, 69, 70). In a recent comprehensive study, Bub et al. (69) compared the results of TBARS, FOX2 and FRAP measurements with DNA migration (comet assay) in an intervention study with vegetable juices. Although oxidative DNA-damage was significantly reduced after consumption, only the TBARS values were decreased, whereas the other parameters were not altered, indicating that the sensitivity of the biochemical parameters is lower as that of the comet assay.

A valuable validation study in which the sensitivity of 8-Oxo-7,8-dihydroguanine (8-oxoGua) and comet formation was compared was published by Gedik et al. (67). They induced lesions by use of a photo-oxidizer in cultured

cells (Hela) and analyzed DNA damage simultaneously with HPLC and in comet assays with FPG and found identical sensitivity of the two methods. In an additional human trial they compared urinary excretion of 8-oxoGuo with 8-oxoGua in lymphocytes and DNA-migration (SCGE). Overall, they found significant correlations between 8-oxoGuo and 8-oxoGua although the individual levels and comet formation were not directly associated. The authors explain this discrepancy by the fact that FPG is not specific for 8-oxoGua but detects in addition also ring opened bases (67).

No data are at present available which answer the question if prevention of oxidative DNA damage in humans leads to an overall increase of the life span. In this context it is notable, that it is still a matter of debate if DNA-migration (monitored in the comet assay) is increasing with age. In a study of Mendoza-Nunez et al. (71) subjects ≥ 70 years had more DNA-damage than a younger group (60-69 years), but the difference was not significant. Also in a study of Betti et al. (72) no age effects were seen whereas Singh et al. (73) reported higher amounts of DNA-damage in elderly persons. In all these studies only overall (not oxidative) damage was investigated. In a very recent study (available only in abstract form) the formation of oxidized bases and MN-formation was compared in young (20-25 yrs.) and elderly (65-70 yrs.) people. No overall differences were seen in oxidative damage levels, but in man (not in women) a significant age depended increase of oxidized pyrimidines was observed. On the contrary to the comet results very pronounced differences in the MN-frequencies were seen in the two study groups, also the levels of chromosomal aberrations were significantly higher in older individuals (74).

In addition, some data on the prevention of age related diseases are available. Collins et al. (70) monitored 8-oxoGua levels in lymphocytes from five different countries and found a strong correlation between premature coronary heart disease and oxidized DNA base levels in males. No such relation was seen in women who have low heart disease mortality rates. In terms of cancer, the only positive correlation was seen for colorectal cancer in man, whereas stomach cancer in women correlated negatively. To our knowledge, no data on correlations between oxidative DNA-damage and age related diseases are available at present, but a number of human diseases such as diabetes (75), Werner syndrome (a inherited genetic disease in which individuals display premature aging) (76), asthma (77), mitochondrial diseases (64), telomere dysfunction (clearly associated with decreased life span) (78), various forms of cancer (79, 80), coronary artery disease (81), Parkinson (82) and Alzheimer (83) and chronic renal failure (84) diseases are characterized by increased DNA-migration in white blood cells.

The correlation between chromosomal aberrations and cancer is well documented in a number of epidemiological studies (85-87) and recent analyses indicated that also MN-frequencies correlate well with human cancer risks (M. Fenech, personal communication).

CONCLUSIONS

As mentioned above, numerous plant derived preparations are currently sold as supplements, which claim to have beneficial health effects (including prevention of aging) in humans due their antioxidant properties. Many of these products have only been tested in simple in vitro screening trails and no evidence is available at present which proofs that they are also effective in humans. The advantages and limitations of different approaches are summarized in *Table III*.

Table III. Comparison of advantages and disadvantages of different test systems to elevate antioxidative properties

Tests with subcellular fractions/ chemical reactions	Tests with intact cells	Human intervention trails
Fast to perform (use in screening trials)	Require cultivation of cells (time consuming)	Time consuming
Provide no information if effects take place inside the cells	Intracellular effects can be shown with specific techniques	Reflect information on intracellular (DNA stability) as well as extracellular (plasma measurements) effects
Provide no information on absorption and tissue distribution of antioxidants	Provide no information on absorption	Provide information on absorption
No relevant information on time kinetics	No relevant information on time kinetics	Provide information of time kinetics
Reflect only direct scavenging	Reflect scavenging effects and in certain cases induction of ROS protective enzymes	Reflect effects which are due to enzyme induction (multiple dosing)
Provide only "qualitative" information	Provide to a certain extent quantitative information (provided that the used exposure concentrations correlate with serum concentrations found in humans)	Provide quantitative information (i.e. comparison of the protective capacities of different antioxidants)

In the present article we focused on the current state of knowledge on the use of new methodologies which enable the detection of antioxidant effects in humans. Taken together, these approaches are highly promising and will provide information, which of the numerous dietary natural antioxidants are effective in humans. Apart of the identification of pure constituents, the results of these human studies will also facilitate the development of dietary strategies to prevent the consequences of adverse ROS effects in humans.

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