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Commentary

Antioxidant Actions of Plant Foods: Use of Oxidative DNA Damage as a Tool for Studying Antioxidant Efficacy

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Plant-food-derived antioxidants and active principles such as flavonoids, hydroxycinnamates (ferulic acid, chlorogenic acids, vanillin etc.), β-carotene and other carotenoids, vitamin E, vitamin C, or rosemary, sage, tea and numerous extracts are increasingly proposed as important dietary antioxidant factors. In this endeavor, assays involving oxidative DNA damage for characterizing the potential antioxidant actions are suggested as *in vitro* screens of antioxidant efficacy. The critical question is the bioavailability of the plant-derived antioxidants.

Keywords: Plant-derived antioxidants, prooxidants, DNA damage, free radicals, dietary antioxidants, flavonoids

INTRODUCTION

Plant extracts and plant-derived antioxidants including those from oil seeds are receiving wide attention in the food industry and in biomedical research primarily as a result of their ability to stabilize bulk oils, emulsions and biological membranes against lipid peroxidation and in the context of human health and diseases, their propensity to act as prophylactic agents.[1–6] Generation of ROS are unavoidable *in vivo*.[2,7,8] For example, the most important route for the formation of nitric oxide is the oxidation of arginine by nitric oxide synthase (NOS) of which there are two types: the constitutive calcium/calmodulin-dependent forms (cNOS), which produces low levels of $NO^{\bullet}$ and, the inducible forms (iNOS). The iNOS which have tightly bound calmodulin are permanently active and are capable of generating high levels of $NO^{\bullet}$.[9,10] The phagocytes (neutrophils, monocytes, macrophages, eosinophils) that defend against foreign organisms are also involved in this good and bad scenario. They generate $O_2^{\bullet -}$, H_2O_2 and (in the case of neutrophils) hypochlorous acid

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(HOCl) as one of their mechanisms for killing foreign organisms.[11,12] Since peroxynitrite ($ONOO^-$) is implicated in the toxicity of $NO^\bullet$, the ability of plant-food components to effectively scavenge $ONOO^-$ might present an important prophylactic protection in relevant disease conditions, e.g. inflammation[13,14] and in the clinical conditions indicated in Table I. The understanding of the chemistry of free radical damage to DNA on the one hand and of the mechanisms of DNA repair processes on the other, is of fundamental importance. The level of DNA damage at a particular time point will reflect the rate of DNA damage versus the rate of repair. Oxidative damage to DNA is important in that it not only may represent an early stage of carcinogenesis but could provide a valuable biomarker of overall oxidative stress. The question of how to accurately measure products of oxidative DNA damage is the subject of the book *"DNA and Free Radicals: Techniques, Mechanism and Applications"*.[15] (This book is highly recommended to the reader.) Gas chromatography coupled with mass spectrometry is a sensitive method which permits structural identification and measurement of a wide range of base damage products. The pattern of base damage can sometimes be used to identify the species that has attacked DNA, e.g. $^1O_2$, $^\bullet OH$, $ONOO^-$ and HOCl give different DNA base damage products. Thus it should become possible to apply the technique for assessing antioxidant capacity. In order for gas chromatography/mass spectroscopy (GC/MS) to be carried out, DNA is hydrolyzed either by enzymatic means or acid in order to release nucleosides or free bases respectively. Derivatization is subsequently needed to convert the polar nucleosides/bases and internal standards to volatile, thermally stable derivatives which possess characteristic mass spectra. Recent developments in the application of

TABLE I Some clinical conditions in which a role for nitric oxide and peroxynitrite has been implicated*

Clinical condition	Reference
Diabetes	Corbett, J.A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8992–8995, Cunningham, J.M. (1994) *Growth Regul.* **4**, 173–180, Krõncke, K.-D. *et al.* (1995) *Biol. Chem. Hoppe. Seyler.* **376**, 179–185
Rheumatoid Arthritis	Kaur, H. and Halliwell, B. (1994) *FEBS Lett* **350**, 9–12
Ischemia-reperfusion	Ma, T.T. *et al.* (1995) *Gastroenterology* **108**, 463–469. Beckman, J.S. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1620–1624, Rubbo, H. *et al.* (1994) *J. Biol. Chem.* **269**, 26066–26075
Atherosclerosis	White, C. *et al.* (1994) **91**, 1044–1048, Rubbo, H. *et al.* (1994) *J. Biol. Chem.* **269**, 26066–26075
Inflammatory bowel disease	Ma, T.T. *et al.* (1995) *Gastroenterology* **108**, 463–469, Rachmilewitz, D. *et al.* (1993) *Gastroenterology* **105**, 1681–1688, Schulz, B.J. *et al.* (1995) *J. Neurochem.* **64**, 1681–1688
Amyotrophic lateral sclerosis	Shinobu, L.A. and Beal. M.F. (1998) In *Molecular Biology of Free Radicals in Human Diseases*, Eds. O.I. Aruoma and B. Halliwell, OICA International: Saint Lucia, pp. 367–395, Beckman *et al.* (1993) *Nature* **364**, 584, Bruijn, L.I. *et al.* (1997) *Proc. Natl. Acad. Sci. USA* **97**, 7607–7611
Cancer	Warren, W. *et al.* (1995) *Carcinogenesis* **16**, 1181–1189, Felley-Bosco *et al.* (1998) In *Molecular Biology of Free Radicals in Human Diseases*, Eds., O.I. Aruoma and B. Halliwell, OICA International: Saint Lucia, pp. 287–325
Acute respiratory distress syndrome	Haddad, I.Y. *et al.* (1994) *J. Clin. Invest.* **94**, 2407–2413, Kooy, N.W. *et al.* (1995) *Am. J. Respir. Crit. Care Med.* **151**, 1250–1254
Septic shock	Szabo, C. *et al.* (1995) *FEBS Lett.* **363**, 235–238, Wizemann, T.M. *et al.* (1994) *J. Leukoc. Biol.* **56**, 759–768
Neurodegenrative diseases	Schulz, B.J. *et al.* (1995) *J. Neurochem.* **64**, 1681–1688, Felley-Bosco *et al.* (1998) In *Molecular Biology of Free Radicals in Human Diseases*, Eds., O.I. Aruoma and B. Halliwell, OICA International Saint Lucia, pp. 287–325

*Excellent reviews may also be found in Anggård, E. (1994) *Lancet* **343**, 199–206, Lancaster, J. (1995) *Biological Chemistry of Nitric Oxide*, Academic Press: New York and Ischiropoulos, H. (1998) *Archives of Biochemistry and Biophysics*, **356**, 1–11. This is by no means an exhaustive list. The clinical conditions indicated are therefore illustrative.

GC/MS to the measurement of oxidized DNA bases have included the use of isotope dilution (GC/IDMS),[15] a valuable technique in establishing absolute levels of base damage products in DNA. The refinement of the technology of DNA adducts including the use of high performance chromatography with electrochemical detection for the measurement of single products and the ^{32}P-postlabeling technique would enable not only the evaluation of the role of free radicals in disease pathology but will provide the forum for establishing logical basis for therapeutic uses of plant-derived antioxidants. Figure 1 presents a working strategy for such studies.[4] Potential applications of the DNA adduct technology include the assessment of the role of diet in traumatic brain injury and stroke both of which may involve a post-injury stimulation of iron ion-dependent free radical reactions. Parkinson's disease is another example, death of cells in the substantia nigra contribute to the disease pathology.[16] It would be worthwhile to find out if the profile of DNA adducts that are being reported in neurodegenerative diseases follows a particular diet profile.

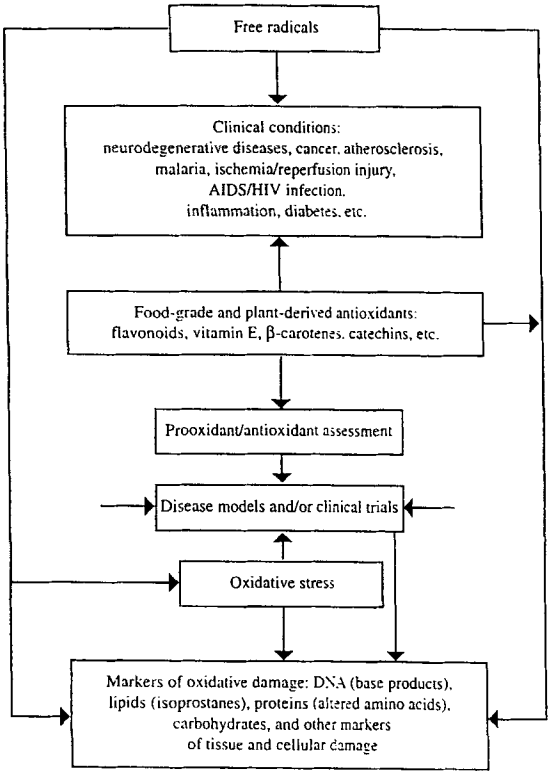

FIGURE 1 Strategy for assessing the *in vitro* and *in vivo* role of plant-food-derived antioxidants. Data from *in vitro* assays can be complemented with *in vivo* data on the levels of biomarkers in a suitable animal model or in humans following a supplementation study. In addition to the DNA adducts, products of lipid and protein oxidation will provide a better picture of efficacy.

OXIDATIVE DNA DAMAGE AND ITS USE TO ASSESS PROOXIDANT AND ANTIOXIDANT ACTIONS

DNA damage is often measured as single strand breaks, double strand breaks or chromosomal aberrations and as products arising from reactions involving Fenton chemistry, ionizing radiation and nuclease activation. Hydroxyl radicals ($OH^{\bullet}$) induce extensive damage to all the four bases in DNA to yield a variety of products.[15] The ability of antioxidants to diminish or induce this $OH^{\bullet}$-dependent base modification may be used as a tool for assessing the antioxidant or prooxidant potentials respectively of such compounds. The antioxidants are assessed for their ability to affect DNA base modifications *in vitro*. This is illustrated with the natural flavoring agent and antioxidant, vanillin (Table II). Incubation of DNA with Fe(III)-EDTA, ascorbate and H_2O_2 led to significant rises in the amounts of several oxidized bases: this is characteristic of attack by $OH^{\bullet}$. Omission of ascorbate from the reaction mixture greatly decreases the DNA base modification suggesting that this vitamin has prooxidant action towards DNA at least *in vitro*.[17] High concentrations of vanillin promoted oxidative modification of DNA bases. Hydroxyl radical scavengers decreased the levels of the DNA adducts (Table II). It is important to emphasize that a high concentration of vanillin is required for the prooxidant action shown. The concentration of vanillin *in vivo* (for example, following consumption of ice creams and/or related dairy

TABLE II Damage to DNA bases by ascorbate or vanillin at pH 7.4

Systems tested	Products monitored (nmol/mg DNA)					
	A	B	C	D	E	Total
DNA, Fe^{3+}-EDTA, H_2O_2 (RM)	0.08	0.02	0.06	0.10	0.43	0.69
RM + ascorbate	1.13	0.98	1.76	8.20	2.94	15.01
RMA + mannitol (100 mM)	0.23	0.06	0.12	0.33	0.31	1.05
RMA + Trolox C (20 mM)	0.31	0.11	0.17	0.88	0.21	1.68
RMA + DMSO	0.14	0.01	0.08	0.19	0.15	0.57
RMA + hypotaurine (20 mM)	0.19	0.04	0.18	0.41	0.42	1.24
RM + vanillin (20 mM)	0.40	0.18	0.26	1.20	0.50	2.30

Values for the products formed are the means of three determinations that agreed to within 10%. Control experiments showed that Trolox C, mannitol, hypotaurine, vanillin or dimethylsulphoxide (DMSO) did not themselves cause any base modification. RM – reaction mixture; RMA – reaction mixture plus ascorbate. *Key*: A = Thymine glycol; B = 5,6-Dihydroxycytosine; C = 4,6-Diamino-5-formamidopyrimidine; D = 2,6-Diamino-4-hydroxy-5-formamido-pyrimidine; E = 8-Hydroxyguanine. (Data from Ref. [17].)
The results of GC/MS analysis of modified bases in DNA can be expressed as nanomoles (nmols) of modified bases per milligram of DNA (equivalent to picomoles per microgram (pmol/μg) of DNA). However, it is easy to convert these data into the actual number of bases modified. Dividing the amount of nmol bases/mg DNA by 3.14 (or multiplying by 0.318) gives the number of modified bases per 10^3 bases in DNA, i.e. 1 nmol/mg DNA corresponds to about 318 modified bases per 10^6 DNA bases.

products) is not known but could reach only high micromolar levels. Nevertheless, the high millimolar concentrations of vanillin required for the prooxidant effects suggests that this action may not present adverse physiological consequences. This is important as it reflects on the way that pure dietary components may be viewed.[18] Similarly, the beneficial effects of ascorbic acid *in vivo* may not be confined to its antioxidant action. For example, ascorbate plays a role in collagen biosynthesis, tyrosine metabolism, carnithine biosynthesis. Under controlled *in vitro* conditions, ascorbic acid exhibits prooxidant action but this may have little physiological significance since proteins may provide buffering capacity against any adverse effect due to prooxidant action *in vivo*.

THE COPPER–PHENANTHROLINE DEPENDENT OXIDATION OF DNA

The copper–1,10-phenanthroline complex has nuclease activity and has been used for structural studies upon DNA[19,20] as it can induce strand breakage. Damage to DNA in the copper-phenanthroline system is confined mainly to the DNA bases with a small amount of DNA sugar damage.[21–24] Application of the method is illustrated by the ability of hydroxytyrosol found in extra virgin olive oil to inhibit oxidative DNA damage *in vitro*. The effects that hydroxytyrosol and ascorbate had on oxidative damage to DNA bases were examined using the technique of GC/MS.[25] Hydroxytyrosol was prooxidant in this system (Table III). It is interesting that a concentration of 5 mM was required to produce the same level of products as 0.25 mM ascorbate. This concentration of hydroxytyrosol produced a 62% rise (based on the number of products measured) in the number of modified bases per 10^6 DNA bases. This observation[25] just like in the case of vanillin, has implications for natural antioxidants and their bioavailability. It is not anticipated that the *in vivo* circulating concentration of the plant-derived antioxidants will be greater than 500 μM at best. Therefore the high concentrations required for the prooxidant activities *in vitro* are not physiologically relevant.

INHIBITION OF PEROXYNITRITE ($ONOO^-$)-DEPENDENT DNA DAMAGE

Exposure of DNA to peroxynitrite leads to modifications of the purine and pyrimidine bases in

TABLE III DNA base modification in the copper–phenanthroline system

Base products	Control		A		B		C	
5-OH uracil	0.04	± 0.00	0.03	± 0.00	1.24*	± 0.21	0.77*	± 0.09
5-OH, Me-uracil	0.01	± 0.00	0.02	± 0.00	0.07*	± 0.01	0.07	± 0.00
5-OH Cytosine	0.17	± 0.02	0.52	± 0.10	1.65*	± 0.47	1.71*	± 0.11
Thy Glycol (cis)	0.32	± 0.06	0.43	± 0.12	2.08*	± 0.53	1.60*	± 0.15
FAPy Adenine	0.08	± 0.01	0.07	± 0.02	0.28*	± 0.02	0.16*	± 0.01
8-OH Adenine	0.15	± 0.02	1.65	± 0.53	1.76	± 0.12	3.75*	± 1.17
2-OH Adenine	0.06	± 0.01	0.09	± 0.00	0.27*	± 0.02	0.30*	± 0.00
FAPy Guanine	0.16	± 0.06	0.44	± 0.16	2.48*	± 0.18	1.07*	± 0.28
8-OH Guanine	0.33	± 0.03	1.72	± 0.48	3.28*	± 0.98	4.98*	± 1.70
Total	1.32	± 0.21	4.97	± 1.41	13.11	± 2.54	14.41	± 3.51
Modified bases per 10^6 DNA bases	420	± 67	1580	± 448	4169	± 808	4582	± 1116

Data are the means ± standard deviations (SD) of three independent experiments. Base product yields in nmol/mg DNA.
*$p < 0.001$ versus samples that contained the copper (Cu) ions–phenanthroline (ph) complex alone. A = Plus ph/Cu (Mix). B = (Mix) Plus 5 mM Hydroxytyrosol. C = (Mix) Plus 0.25 mM ascorbate. (Data from Ref. [25].)

DNA.[26–28] Measurement of the products of peroxynitrite-dependent DNA damage is another useful method for assessing the antioxidant action of a plant-derived compound whereupon the compound maybe tested for its ability to inhibit the production of respective DNA adducts. Recent studies have shown that ergothioneine 2(2-mercaptohistidine trimethylbetaine) (see Figure 2) formed via hercynine from histidine, methionine and cysteine in micro-organisms[29–31] is a natural antioxidant. Ergothioneine illustrates the application of the DNA adduct technology to study antioxidant efficacy.

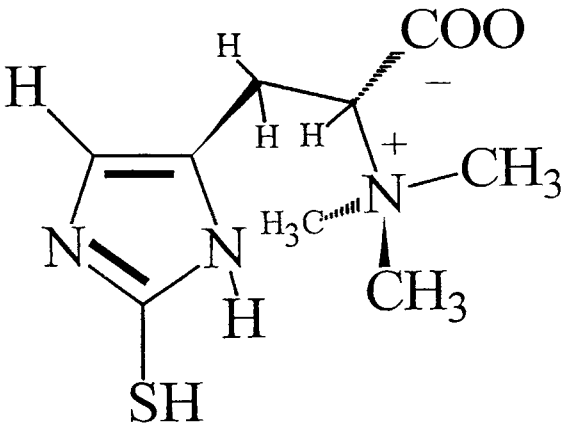

FIGURE 2 Structure of ergothioneine.

In this study, human neuronal hybridoma cells (N-18-RE-105) challenged with $ONOO^-$ were protected against oxidative damage by ergothioneine.[32] The levels of the products, 4,6-diamino-5-formamidopyrimidine, 2,6-diamino-5-formamidopyrimidine, 8-hydroxyguanine, 8-hydroxyadenine, 5-hydroxyuracil, 5-hydroxycytosine, hypoxanthine and xanthine measured by GC/MS were reduced to almost control values.[32] Some of these products are shown in Figure 3. Table IV shows typical target ions used for quantitations in selected ion monitoring mode for various products. Hydroxytyrosol also inhibited the oxidation of DNA bases by $ONOO^-$. The levels of all the products measured were again decreased to control values by hydroxytyrosol at concentrations lower than that of $ONOO^-$. It must, however, be borne in mind that the fate and action of $ONOO^-$ in biological systems would be dependent on the biological environment in which the oxidant is present. The work involving ergothioneine is the subject of a research paper in preparation. Both ergothioneine and hydroxytyrosol preferentially scavenged $ONOO^-$ (Figure 3). This can be extended to other diet-derived compounds that are suggested to possess antioxidant activity or to formulations of dietary antioxidant supplements.

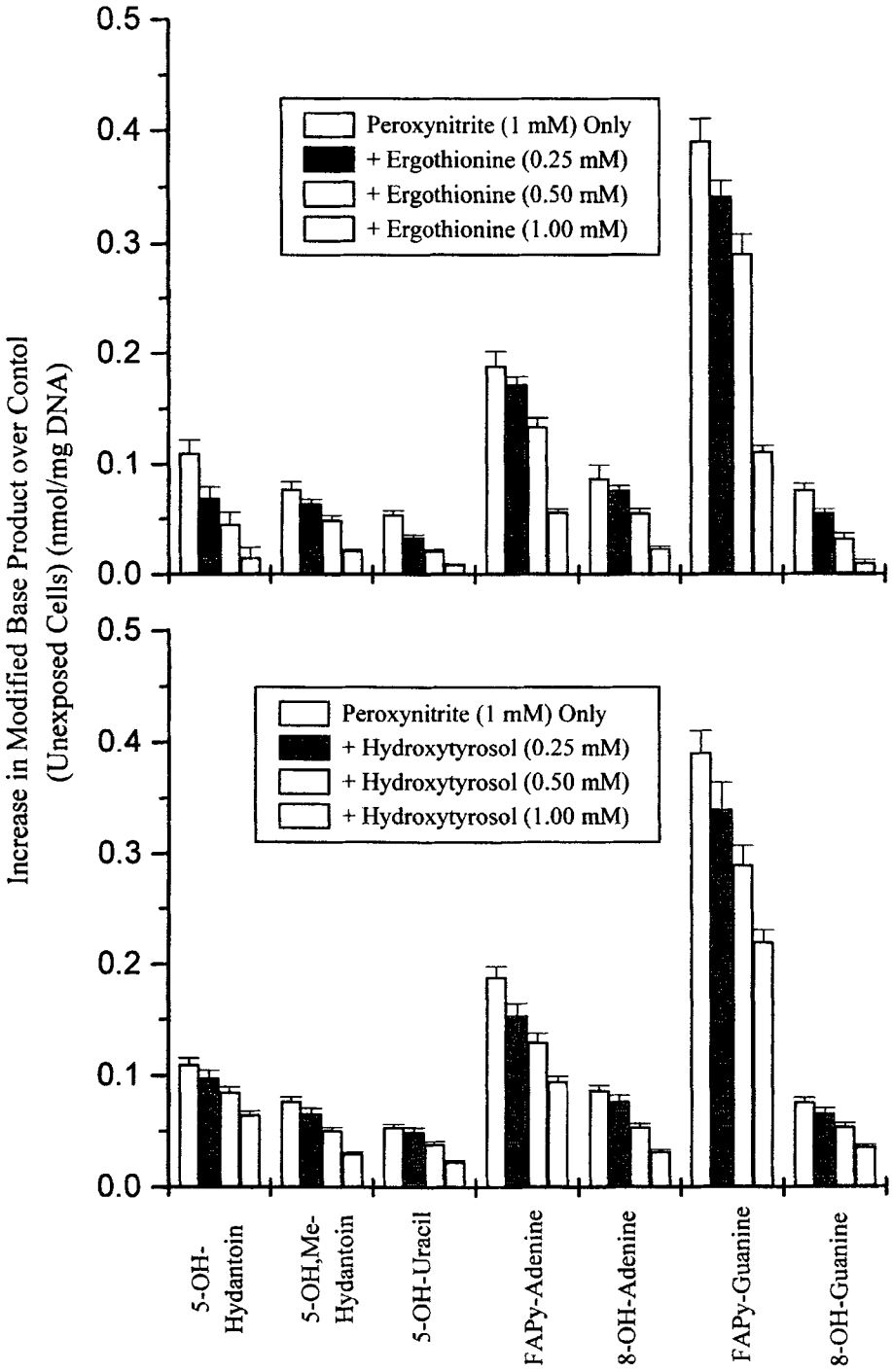

FIGURE 3 Effect of increasing concentrations of ergothioneine and hydroxytyrosol on the yield of oxidized DNA base products following the treatment of neuronal cells with peroxynitrite (1 mM). *Key*: FAPy-adenine, 4,6-diamino-5-formamidopyrimidine; FAPy-guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH guanine, 8-hydroxyguanine; 2-OH adenine, 2-hydroxyadenine; 8-OH adenine, 8-hydroxyadenine; 5-(OH,Me)-uracil, 5-(hydroxymethyl)uracil; 5-OH,Me-hydantoin, 5-hydroxy-5-methylhydantoin; 5-OH hydantoin, 5-hydroxydantoin; 5-OH uracil, 5-hydroxyuracil; 5-OH cytosine, 5-hydroxycytosine. (From Ref. [32].)

TABLE IV Target ions used for GC/MS selected ion monitoring

Base Product (trimethylsilyl derivative)	Target ion (m/z)
Thymine	255
5-(OH, Me) hydantoin	331
5-OH hydantoin	317
5-OH uracil	329
5-(OH, Me) uracil	358
5-OH cytosine	328
Thymine glycol	259
Hypoxanthine	265
FAPy adenine	354
8-OH adenine	352
Xanthine	353
2-OH adenine	352
FAPy guanine	442
Guanine	367
8-OH guanine	440
Azathymine	256
Diaminopurine	351

USE OF THE COMET ASSAY TO MEASURE OXIDATIVE DNA DAMAGE

In principle, generation of $OH^{\bullet}$ by reaction of H_2O_2 with the transition metal ions already bound onto the DNA would lead to strand breakage, base modification and deoxysugar fragmentation. In the nuclease activation mechanism, oxidative stress leads to the inactivation of Ca^{2+}-binding by endoplasmic reticulum, inhibition of plasma membrane Ca^{2+}-extrusion systems, and the release of Ca^{2+} from mitochondria results increased levels of intracellular free calcium ions.[33] The resulting endonuclease activation leads to DNA fragmentation without the base modification observed in the Fenton mechanism. DNA fragmentation suggested here could be assessed by the COMET assay.[34–36] Free radical induced strand breaks are transient and are rejoined within minutes of formation in normal cells in culture; however, oxidized bases are long-lived. Isolating DNA from cells and incubating them with certain bacterial endonucleases which have specific activity against oxidized bases, these are converted into DNA breaks which are then measured. Extensive reviews on the comet assay technology may be found in

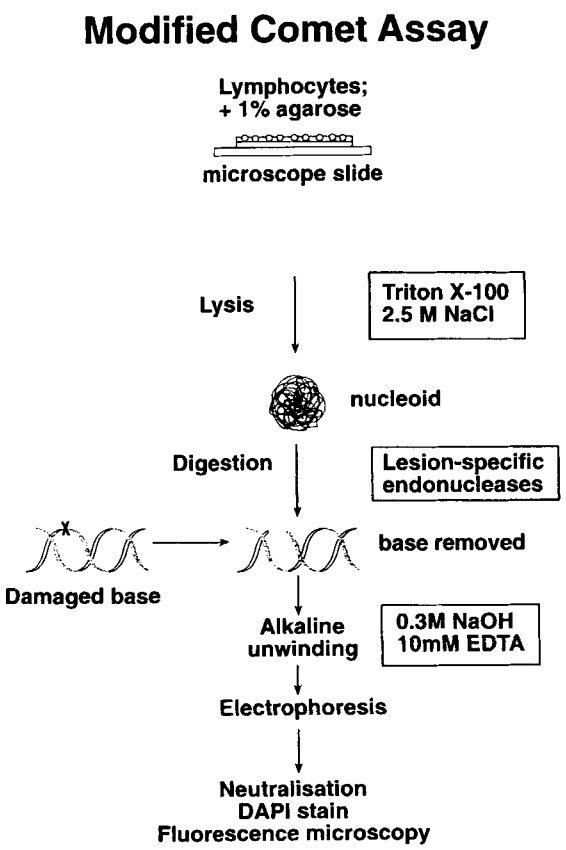

FIGURE 4 Modified strategy showing the principles of the comet assay (courtesy of Dr. Andrew Collins).

McKelvey *et al.*[37] and Fairbairn *et al.*[38] The comet assay is an extremely important tool for assessing the protective effects of dietary antioxidants. The features of a modified comet assay are illustrated in Figure 4 (courtesy of Dr. Andrew Collins). In one antioxidant supplementation study[36] volunteers received 45 mg of β-carotene, 1 g of vitamin C or 1 g of vitamin E. Lymphocytes isolated from blood taken before and several hours after the supplementation and challenged with 0.24 mM hydrogen peroxide for 5 min. The resistance of lymphocytes to H_2O_2 was assessed by measuring DNA strand breaks with the comet assay. Vitamin supplementation increased the

resistance of lymphocytes to H_2O_2. Use of the comet assay to examine antioxidant effects at the level of chromatin avoids the complications of cellular uptake and processing. Thus the comet assay is a versatile tool that is highly efficacious in human bio-monitoring of plant food antioxidants.

CONCLUSIONS

The feasibility of an active compound in plant foods exerting a direct antioxidant effect can be evaluated by *in vitro* tests that investigate how the putative antioxidant react with biologically relevant oxygen-derived species. The DNA adduct technology is one novel tool that could enable scientists to assess their potential role as prophylactic agents. The impetus for this is driven by the need to understand the functional role of plant foods in health and disease management.[18,39,40]

Acknowledgment

O.I. Aruoma is Collaborador Professor of the University of Sao Paulo-Ribeirao Preto.

References

[1] Johnson, T., Williamson, G. and Musk, S.R.R. (1994). Anticarcinogenic factors in plant foods. A new class of nutrients. *Nutrition Research Review* **7**, 1–30.
[2] Aruoma, O.I. (1998). Free radicals, oxidative stress and antioxidants in human health and disease. *Journal of the American Oil Chemist's Society* **75**, 199–212.
[3] Cook, N.C. and Samman, S. (1996). Flavonoids – Chemistry, metabolism, cardio protective effects and dietary sources. *Journal of Nutritional Biochemistry* **7**, 66–76.
[4] Aruoma, O.I. (1997). Extracts as antioxidant prophylactic agents. *INFORM* **8**, 1236–1242.
[5] Pezzuto, J.M. (1997). Plant-derived anticancer agents. *Biochemical Pharmacology* **53**, 121–133.
[6] Shahidi, F. (1997). *Natural Antioxidants. Chemistry, Health Effects and Applications*. AOCS Press: Champaign.
[7] Halliwell, B. and Gutteridge, J.M.C. (1989). *Free Radicals in Biology and Medicine*, 2nd Edition. Clarendon Press: Oxford.
[8] Sies, H. (1996). *Antioxidants in Disease Mechanisms and Therapy*. Academic Press: London.
[9] Lancaster, J. (1996). *The Biological Chemistry of Nitric Oxide*. Academic Press: New York.
[10] Moncada, S. and Higgs, H. (1993). The L-arginine-nitric oxide pathway. *New England Journal of Medicine* **329**, 2002–2012.
[11] Weiss, S.J. (1989). Tissue destruction by neutrophils. *New England Journal of Medicine* **320**, 3365–3375.
[12] Babior, M. (1984). Oxidants from phagocytes: agents of defense and destruction. *Blood* **64**, 959–966.
[13] Szabo, C., Ferrer-Sueta, G., Zingarelli, B., Southan, G.J., Salzman, A.L. and Radi, R. (1997). Mercaptoethyl-guanidine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxynitrite induced oxidative damage. *Journal of Biological Chemistry* **272**, 9030–9036.
[14] Southan, G.J. and Szabo, C. (1996). Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochemical Pharmacology* **51**, 383–394.
[15] Aruoma, O.I. and Halliwell, B. (1998). *DNA and Free Radicals: Techniques, Mechanisms and Applications*. OICA International: Saint Lucia.
[16] Olanow, C.W., Jenner, P. and Youdim, M. (1996). *Neurodegeneration and Neuroprotection in Parkinson's Disease*. Academic Press: London.
[17] Aruoma, O.I., Evans, P.J., Kaur, H., Sutcliffe, L. and Halliwell, B. (1990). An evaluation of the antioxidant and potential prooxidant properties of food additives and trolox C, vitamin E and probucol. *Free Radical Research Communications* **10**, 143–157.
[18] Aruoma, O.I. (1999). Free radicals, antioxidants and international nutrition. *Asia Pacific Journal of Clinical Nutrition* (February 1999 issue, in press).
[19] Sigman, D.S. (1986). Nuclease activity of 1,10-phenanthroline copper ion. *Accounts in Chemical Research* **19**, 180–186.
[20] Thederahn, T.B., Kuwabra, D.M., Larson, T.A. and Sigman, D.S. (1989). Nuclease activity of 1,10-phenanthroline copper: kinetic mechanism. *Journal of the American Chemical Society* **III**, 4941–4946.
[21] Dizdaroglu, M., Aruoma, O.I. and Halliwell, B. (1990). Modification of bases in DNA by copper-ion 1,10-phenanthroline complexes. *Biochemistry* **29**, 8447–8451.
[22] Aruoma, O.I., Halliwell, B., Gajewski, E. and Dizdaroglu, M. (1991). Copper-ion dependent damage to the bases in DNA in the presence of hydrogen peroxide. *The Biochemical Journal* **273**, 601–604.
[23] Que, G., Downey, K.M. and So, A.G. (1980). Degradation of deoxyribonucleic acid by a 1,10-phenanthroline–copper complex: the role of hydroxyl radicals. *Biochemistry* **19**, 5987–5991.
[24] Reich, K.A., Marshall, L.E., Graham, D.R. and Sigman, D.S. (1981). Cleavage of DNA by the phenanthroline–copper ion complex. Superoxide mediates the reaction dependent on NADH and hydrogen peroxide. *Journal of the American Chemical Society* **103**, 3582–3584.
[25] Aruoma, O.I., Deiana, M., Jenner, A., Halliwell, B., Kaur, H., Banni, S., Corongiu, F.P., Dessi, M.A. and Aeschbach, R. (1998). Effect of hydroxytyrosol found in extra virgin olive oil on oxidative DNA damage and on low density lipoprotein oxidation *in vitro*. *Journal of Agricultural and Food Chemistry* **46**, 5181–5187.
[26] Spencer, J.P.E., Wong, J., Jenner, A., Aruoma, O.I., Cross, C.E. and Halliwell, B. (1996). Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin epidermal keratinocytes exposed to peroxynitrite or 3-morpholinosydnonimine. *Chemical Research in Toxicology* **9**, 1152–1158.

[27] Douki, T. and Cadet, J. (1996). Peroxynitrite mediated oxidation of purine bases of nucleosides and isolated DNA. *Free Radical Research* **24**, 369–380.
[28] Douki, T., Cadet, J. and Ames, B.N. (1996). An adduct between peroxynitrite and 2′-deoxynitrite and 2′-deoxyguanosine: 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine. *Chemical Research in Toxicology* **9**, 3–7.
[29] Jocelyn, P.C. (1971). *Biochemistry of the SH Group*. Academic Press, New York.
[30] Hartman, P.E. (1990). Ergothioneine as an antioxidant. *Methods in Enzymology* **186**, 310–318.
[31] Melville, D.B., Horner, W.H., Otken, C.C. and Ludwig, M.L. (1955). Studies on the origin of ergothioneine. *Journal of Biological Chemistry* **213**, 61–68.
[32] Deiana, M., Aruoma, O.I., Bianchi, M.L.P., Spencer, J.P.E., Kaur, H., Halliwell, B., Aeschbach, R., Dessi, M.A., Banni, S. and Corongiu, F.P. (1999). Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. *Free Radical Biology and Medicine* **26** (in press).
[33] Halliwell, B. and Aruoma, O.I. (1991). DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian system. *FEBS Letters* **281**, 9–19.
[34] Ostling, O. and Johanson, K.J. (1984). Microelectrophorectic study of irradiation-induced DNA damage in individual mammalian cells. *Biochemical and Biophysical Research Communications* **123**, 291–298.
[35] Singh, N.P., McCoy, M.T., Tice, R.R. and Schnieder, E.L. (1988). A simple technique for quantitation of low level of DNA damage in individual cells. *Experimental Cell Research* **123**, 184–191.
[36] Collins, A.R. (1998). The comet assay: a novel approach to measuring DNA oxidation. In *DNA and Free Radicals: Techniques, Mechanisms and Applications*, Eds. O.I. Aruoma and B. Halliwell, pp. 241–260, OICA International: Saint Lucia.
[37] McKelvey, V.J., Green, M., Schmezer, P., Pool-Zobel, B.L., De-Meo, M.P. and Collins, A. (1993). The single cell gel electrophoresis (SCGE) assay (comet assay): A European review. *Mutation Research* **288**, 47–63.
[38] Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995). The comet assay: A comprehensive review. *Mutation Research* **339**, 37–59.
[39] Diplock, A.T., Charleux, J.-L., Crozier-Willi, G., Kok, F.J., Rice-Evans, C., Roberfroid, M., Stahl, W. and Vina-Ribes, J. (1998). Functional food science and defence against reactive oxidative species. *British Journal of Nutrition* **80** (Suppl 1) S77–S112.
[40] Halliwell, B. (1996). Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radical Research* **25**, 1–32.