Proc. R. Coll. Physicians Edinb. 1995; **25**: 544–549

EDUCATIONAL REVIEW: ANTIOXIDANTS

J. S. Pears, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire*

Antioxidants and free radicals are boom areas for conjecture and research. Currently they are associated with a multitude of pathological disorders from atherosclerosis to carcinogenesis, from psoriatic arthropathy to microvascular complications of diabetes mellitus. One review lists over 100 diseases in which there is evidence of free radical damage.[1] A search of the Scientific Citation Index for the first two months of 1995 revealed over 350 references in which the word 'antioxidant' appears in the title, keyword or abstract. There are 3 international journals dedicated to free radical research, one of which is in its 22nd volume. The lay press is interested: antioxidants such as vitamin E have been heralded as preventing heart attacks, and where the cosmetic industry advertises anti-ageing creams containing free radical scavengers (which are antioxidants). There is a new drive by marketers aiming to sell vitamin antioxidant supplements on the back of 'educating' the population about diet and its current deficiencies which can be corrected by appropriate supplements.[2] Underlying this promotion of supplements is the assumption that most of us will resist altering our lifestyles yet again, as we have recently adjusted to high fibre, low saturated fat diets and increased exercise, and will find it easier to take supplements. As an estimated 20 per cent of the UK population take multivitamins in a growing supplement market currently worth £260 million per year this assumption appears rational.[2].

Why is there all this interest?

The hypothesis is that many diseases are caused or exacerbated by an imbalance between overproduction of free radicals and antioxidant 'activity'—so called oxidative stress. In health the free radicals that are continuously produced in the body by normal cell metabolism or by environmental insults, as for example tobacco smoking or low wavelength radiation, are rapidly mopped up ('scavenged' is the term used by those in the know) by a variety of molecular defence mechanisms whose prime purpose is to neutralise free radicals before they can cause damage. These defences are antioxidants. In some diseases, although a glance at the literature may suggest all diseases, there does appear to be evidence of oxidative stress which might be due to overproduction of free radicals and/or inefficient scavenging by antioxidants. Moderate oxidative stress results in oxidation of proteins, lipids or DNA depending upon which macromolecule is closest to the site of free radical production, whereas severe stress can lead to cell death and may be related to the process of apoptosis. Hence the projection from the hypothesis that taking appropriate, extra, antioxidants will redress this pro-oxidant/antioxidant imbalance and so ameliorate the disease process.

What are the mechanisms involved?

A free radical is an atom with an unpaired electron and is denoted by a superscript dot; $OH^{\bullet}$ is the hydroxyl radical and $O_2^{\bullet -}$ the superoxide radical: The hydroxyl radical is a very reactive chemical species and therefore has a very

*Medical Adviser.

short life (microseconds or less), but has the potential to induce chain reactions generating free radicals which cause further oxidative damage to macromolecules. The superoxide radical is much less reactive and is part of the human body's normal defence mechanism—it is produced by phagocytes to kill foreign organisms. However, in some circumstances, such as in the presence of high concentrations of free copper and iron atoms, the $OH^{\bullet}$ radical can be produced from the $O_2^{\bullet -}$ species. Indeed some of the tissue damage found in diseases associated with evidence of inappropriate phagocytic activation (such as chronic infection, rheumatoid arthritis or inflammatory bowel disease) may be due to free radical damage consequent upon high levels of production of the superoxide radical.

There are a variety of endogenous and exogenous antioxidant mechanisms which may limit the unwanted damage of free radicals. Superoxide dismutase (SOD) is a mitochondrial and cytosolic enzyme which has the superoxide radical as its specific substrate. This is converted to hydrogen peroxide which is in turn removed by the catalase family of enzymes found in peroxisomes, or by glutathione peroxidases in the mitochondria or the cytosol which catalyse the conversion of reduced glutathione (GSH) and hydrogen peroxide to oxidised glutathione (GSSG) and water. GSH can also scavenge free radicals directly and non-enzymatically. Selenium is an essential cofactor for glutathione peroxidase, hence the interest in selenium supplements. The exogenous antioxidants which have attracted most attention are vitamins C and E and beta carotene. It is assumed that the worth of these compounds to the body is largely as antioxidants. Vitamin E (in particular alpha tocopherol) is found in membranes and lipoproteins and it blocks chain reactions, scavenging peroxide radicals by converting them to the tocopherol radical which is less reactive and is converted back (recycled) to alpha tocopherol by vitamin C. Vitamin C has several physiological functions including detoxifying inhaled oxidising pollutants. When free radicals react with lipids, peroxides are formed which themselves contain, and are thus able to generate more, free radicals (the $C^{\bullet}$ radical in particular). The formation of lipid peroxides is believed to be one of the reasons that low density lipoprotein (LDL) is apparently more atherogenic in certain circumstances, such as where there is increased oxidative stress due to low antioxidant levels in LDL (alpha tocopherol is a constituent of LDL) or increased free radical production in areas of ischaemia or inflammation, or both. In these circumstances the amount of lipid peroxide measurable in LDL is increased. These lipid peroxides appear to be potent chemoattractants for phagocytes and allow the LDL particles to be more readily taken up by the phagocytes (hence forming foam cells), but high concentrations of peroxides are cytotoxic. LDL with a high lipid peroxide content is concentrated in atheromatous lesions, and may result in increased susceptibility of the lesions to rupture: atheromatous plaques with higher inflammatory cell infiltrates are more likely to fissure and rupture resulting in arterial thrombosis.[3] Oxidised LDL is also immunogenic resulting in circulating immunoglubulins to oxidised LDL being found more commonly in people with atheroma and stimulates smooth muscle hypertrophy in *in vitro* systems.

In similar environments of local ischaemia and phagocyte activity the $NO^{\bullet}$ radical can be formed. Like the superoxide radical, low concentrations do little harm but high levels may be locally toxic to endothelial cells and $NO^{\bullet}$ has been implicated in the vascular dilation which characterises septic shock.

Damage to DNA by free radicals causes oxidation of its base constituents.

The oxidised bases are excised from DNA by glycolases. With increasing age, increased numbers of these oxidised bases are found which result in point mutations in the DNA. In areas of increased oxidative stress it is postulated that these point mutations may be carcinogenic.

Glycosylation of proteins occurs in all tissues, but is much more marked in diabetes mellitus due to the higher glucose concentrations. Glycosylation is a non-enzymatic process which is augmented by and which produces free radicals. Glycosylation of SOD (reducing its activity) and LDL (resulting in a molecule with similar properties to peroxide containing LDL) can then enhance many of the mechanisms postulated for free radical induced tissue damage. Furthermore there is evidence that patients with the tissue complications of diabetes mellitus have higher levels of oxidative stress and show more evidence of free radical damage (low SOD activity, increased lipid peroxide levels and higher glycosylated protein concentrations) than patients without such complications.[4,5] Proteins with low turnover rates are converted irreversibly to glycosylated end products (AGEs) which form cross links within the protein and with neighbouring AGEs; while proteins with more rapid turnover result in ketoamines (such as glycosylated haemoglobin).

All the above reactions occur in health. It is possible to measure the results of oxidative damage in normal humans—lipid peroxides, glycated proteins and urinary excretion of free oxidised bases excised from DNA are all present. Likewise it is possible to measure both concentrations of the antioxidants which are thought to be physiologically most important and SOD activity; it is also possible to measure (*ex vivo*) the susceptibility for LDL particles separated from an individual to free radical damage; this information is a major epidemiological tool used to link oxidative stress and LDL to the development of atheroma.

One theory of normal ageing is that with time the oxidative damage caused by free radicals comes to outweigh the body's antioxidant potential or mechanisms for dealing with the oxidised macromolecules. Hence the glycosylation of collagen resulting in cross linkage with other collagen molecules and thereby the decreased elasticity which is characteristic of older collagen and is also evident in diabetes mellitus.

However one of the major problems that the proponents of the free radical theory of disease have is that it is not possible to measure free radical production either *in vivo* or *ex vivo* as they exist for too brief a time; it is only possible to infer their presence by the appearance of oxidative damage in macromolecules. It is also therefore not possible to separate the production of free radicals by the disease process (ischaemia or inflammation, for example) from the postulated increased oxidative stress which supposedly underlies that disease—i.e. the question remains, are the free radicals cause or effect or both?[6]

What is the evidence that antioxidants promote health?

The argument that free radicals cause disease depends on the balance of the oxidative stress equation, i.e. that low antioxidant levels are related to disease or that increased antioxidant concentrations prevent it. There is supportive evidence for this.

The mechanism whereby paracetamol poisoning causes hepatocellular necrosis involves the reaction of a paracetamol metabolite with GSH, thus depleting the hepatocyte of GSH and reducing the effectiveness of catalases to scavenge free

radicals. Paraquat metabolism results in formation of a self-regenerating Paraquat free radical and superoxide free radicals with the potential for causing oxidative damage. Cigarette smoking and radiation exposure both increase free radical production, the effect of the latter apparently ameliorable by high levels of antioxidant supplements in the diet. Some diseases are associated with specific abnormalities or deficiencies of endogenous antioxidant mechanisms such as the reduced cytosolic SOD activity found in patients with familial amyotrophic lateral sclerosis.[7] The human body has evolved complex protein structures for transferring pro-oxidant metal ions in such a way that they cannot react to form free radicals—both elemental forms of iron and copper are potent promoters of free radical production in *in vitro* assays of physiological models; for example, iron may produce hydroxyl radicals in conjecture with vitamin C and this may explain the severe cardiovascular decompensation which may occur if vitamin C supplements are given to people with iron overload. Furthermore in disease states where these transport mechanisms are overwhelmed or deficient (such as haemochromatosis and Wilson's disease) the increased risks of developing cancer or chronic tissue damage such as cirrhosis may be related to increased oxidative stress resulting from increased exposure of the metal ions to physiological systems.

Much of the research into antioxidants and human disease has focused on more common illnesses. The relationship between evidence of oxidative stress and the presence of the microvascular complications of diabetes mellitus has already been referred to. Other areas of interest are carcinogenesis and atheromatous cardiovascular disease. Much of the data taken as supportive for the hypothesis of increased oxidative stress being related to both of these disease areas is epidemiological and can therefore never prove a causal relationship; nevertheless there have been several trials, some very large ones, to look for associations between dietary antioxidant intake, measurable tissue (often plasma) antioxidant concentrations, susceptibility to oxidative damage (e.g. of LDL) or evidence of free radical damage (peroxidase concentrations or urinary excretions of oxidised DNA bases) and cancer and coronary artery disease.[3,8,9,10] All these trials have had to conclude that confounding variable(s) may explain the outcome other than the oxidative stress hypothesis, and there have been trials to address these confounders specifically.[11]

Associations have been shown between the risk of developing some forms of cancer with plasma concentrations and with dietary intake of beta carotene (lung disease) and vitamin C (gastrointestinal disease), but no definitive proof exists that there is not some other carcinogen in the environment or deficiency in the diet of such people which is responsible rather than the low antioxidant concentrations. Two large intervention studies examining the effect of antioxidant supplements on the incidence of cancers have given conflicting results—the Linxian intervention study, in an area of China with one of the highest incidence rates of carcinoma of the oesophagus in the world, showed a 20 per cent reduction in cancer mortality in those given supplements of beta carotene, vitamin C and selenium. The Helsinki ATBC study, however, showed an increased incidence of lung cancer in male smokers given beta carotene which is in direct opposition to the majority of the epidemiological data regarding the intake of beta carotene and the risk of developing lung cancer.[12] (This data is not supported by analysis of the trials using beta carotene supplements which looked at the incidence of coronary artery disease.) Nevertheless the Helsinki data has fuelled the contro-

versy with the school of thought which thinks that excessive antioxidant intake may have deleterious pharmacological effects and may in turn promote disease[13] and which, under certain conditions, may exhibit pro-oxidant activity as described above. However, whether such conditions occur in physiological systems remains unclear.[14]

The epidemiological evidence linking coronary artery disease and antioxidant deficiency is as inconsistent as that for cancer and has been variously reviewed.[3,10,15] So strong is the evidence to some, especially in conjunction with the postulated free radical mechanisms which may underlie the development of atherosclerosis, that large prospective randomised primary prevention trials have been set up and are still running in an attempt to try and clarify whether antioxidant supplementation reduces the risk of developing coronary arterial disease. For example the Physician's Health Study in the USA recruited 22,000 male physicians in 1982 and randomised them to beta carotene supplementation or placebo. An interim analysis after 6 years in the small subset of patients with pre-existing coronary artery disease showed a marked reduction in all coronary events in those receiving beta carotene.[3] This and other similar trials are still ongoing. Other, more cynical interpretations of the rationale for these trials would be that physicians are desperate to try anything that might reduce the incidence of coronary artery disease or that the promotions of the antioxidant supplement marketers have been successful.

Where do exogenous antioxidants come from?

The source of the antioxidants now considered important is a healthy balanced diet which is rich in fruit and vegetables which have not been overcooked. If the antioxidant theory is correct, this would support the WHO recommended diet which includes 500 g/day of fresh fruit and vegetables. There may be other sources of the known and possible other as yet unrecognised, antioxidants. Many oriental spices contain potential antioxidants[16] which may help to explain (along with a diet high in fibre and low in saturated fat) the low incidence of coronary disease in areas of the world with high consumption of spiced foods. And then there is the question of red wine. One anomaly in the epidemiological evidence for the incidence of coronary artery disease is the so-called 'French Paradox' where the population in areas of France, especially in the south, consume a high fat diet and smoke heavily and yet have a very low incidence of coronary artery disease. One possible explanation for this comes from the finding that red wine contains phenolic substances (flavonoids) which, *in vitro*, can inhibit the oxidation of LDL more potently than can vitamin E,[17] but again this may not be the whole story,[18] and there are large numbers of flavonoids; trying to find the important one(s) will be very difficult. Naturally one has to balance any possible benefit of red wine consumption (which has also been postulated to increase HDL concentrations) against potentially harmful effects of alcohol abuse.

There are also drugs marketed as antioxidants, of which probucol is perhaps the best known. Probucol does have antioxidant activity, but it also has other potential therapeutic effects such as action upon macrophage function and smooth muscle hypertrophy both of which could be beneficial in reducing the incidence of coronary artery disease. A number of other drugs have had their potential free radical scavening investigated,[19,20] although possibly stimulated by the hope of gaining potential marketing advantage over rivals.

Such is the potential influence of these antioxidant arguments upon the health of society as a whole that governments are becoming involved in debates and in research to answer the questions. In the USA the Food and Drug Administration has sponsored a conference on the topic and in the UK the Ministry of Agriculture Food and Fisheries is sponsoring major programmes of research in related topics costing over £1.5 million in 1995 alone. The involvement of large sums of money by such as government agencies will undoubtedly be required to test out any hypotheses as primary prevention studies, even in common disorders like cancer and cardiovascular disease, have to be on a large scale. The hope is that all these efforts will produce a clear resolution to some of the arguments and answers to some basic questions. Are free radicals cause or effect (or both) of disease? Will taking antioxidant supplements prevent diseases or attenuate their clinical courses? If so, which antioxidants for which disease(s)? How much antioxidant is appropriate and safe to take long-term? Finally is taking a pharmacologoical preparation of an antioxidant as good as taking the same compound as part of a healthy balanced diet?

REFERENCES

1 Gutteridge JMC. Free radicals in disease processes: a compilation of cause and consequence. *Free Radic Res Commun* 1993; **19:** 141–58.

2 Elliot R. Breaking through the language barrier—the marketer's task for antioxidants. *The Biochemist* 1995; **17:** 19–21.

3 Hoffman RM, Garewal HS. Antioxidants and the prevention of coronary heart disease. *Arch Intern Med* 1995; **155:** 241–6.

4 Jennings PE, Barnett AH. New approaches to the pathogenesis and treatment of diabetic microangiopathy. *Diabetic Med* 1988; **5:** 111–7.

5 Sinclair DJ. Free radical mechanisms and vascular complications of diabetes mellitus. *Diabetes Rev* 1993; **2:** 7–10.

6 Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause or consequence? *Lancet* 1994; **344:** 721–4.

7 Robberecht W, Sapp P, Viaene MK *et al.* Cu/Zn—superoxide dismutase activity in familial and sporadic amytrophic lateral sclerosis. *J Neurochem* 1994; **62:** 384–7.

8 Abbey M, Nestel PJ, Baghurst PA. Antioxidant vitamins and low density lipoprotein oxidation. *Am J Clin Nutr* 1993; **58:** 525–32.

9 Cerutti PA. Oxy-radicals and cancer. *Lancet* 1994; **344:** 862–3.

10 Rice-Evans C. Antioxidant nutrients in protection against coronary heart disease and cancer. *The Biochemist* 1995; **17:** 8–11.

11 Jarvinen R, Knekt P, Seppanen R *et al.* Antioxidant vitamins in the diet: relationships with other personal characteristics in Finland. *J Epidemiol and Community Health* 1994; **48:** 549–54.

12 The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. *N Engl J Med* 1994; **330:** 1029–35.

13 Herbert V. The antioxidant supplement myth. *Am J Clin Nutr* 1994; **60:** 157–8.

14 Halliwell B. Antioxidants: Elixirs of life or tonics for tired sheep? *The Biochemist* 1995; **17:** 3–6.

15 Hennekens CH, Gaziano JM. Antioxidants and heart disease: Epidemiology and clinical evidence. *Clin Cardiol* 1993; **16** (suppl. 1) I: 10–15.

16 Narasinga Rao BS. Bioactive phytochemicals in Indian foods. *NFI Bulletin* 1995; **16:** 1–5.

17 Frenkel EN, Kanner J, German JB *et al.* Inhibition of oxidation of human low density lipoprotein by phenolic substances in red wine. *Lancet* 1993; **341:** 454–7.

18 Leake D. The French paradox. *The Biochemist* 1995; **17:** 12–5.

19 Godfrey EG, Stewart J, Dargie HJ *et al.* Effects of ACE inhibitors on oxidation of human low density lipoprotein. *Br J Clin Pharmacol* 1994; **37:** 63–6.

20 Jennings PE. The potential of gliclazide, a sulphonylurea, to influence the oxidative process within the pathogenesis of diabetic vascular disease. 1995. In Press—personal communication at present.