Flavonoids: a re-run of the carotenoids story?

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Abstract. Flavonoids have powerful antioxidant activities *in vitro*, but the evidence that they act as antioxidants *in vivo* in humans is equivocal at best. However, they may be able to help protect the gastro-intestinal tract against reactive oxygen species.

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Setting the scene

Oxygen is toxic, and we only survive its presence because we have evolved a plethora of antioxidant defence systems. These systems minimize the levels of oxygen radicals and other reactive oxygen species (ROS) but do not eliminate them completely, since some ROS are useful (Halliwell & Gutteridge 2007). Thus some ROS-dependent 'oxidative damage' occurs continually in the human body, measurable by various 'biomarkers' such as F_2-isoprostanes (products of oxidation of lipids) and oxidized DNA bases, such as 8-hydroxy-2′-deoxyguanosine (8OHdG) (Halliwell & Whiteman 2004). This oxidative damage is thought to contribute to the age-related development of cancer, cardiovascular and neurodegenerative diseases, and several other disorders, and perhaps even to the ageing process itself (Beckman & Ames 1997, Sohal et al 2002, Butterfield & Boyd-Kimball 2004, Halliwell & Gutteridge 2007). To some extent this may be a product of evolution—ROS are involved in a network of signalling processes that mount the body's response to infection, and they can aid killing of bacteria and viruses (Babior 2004). When humans first gathered together in cities, infectious disease was rampant, driving the evolution of powerful immune responses to which ROS contribute (Babior 2004, Fang 2004). Thus the ability to make a lot of ROS might be selected for, keeping young people alive to reproduce. It doesn't matter to evolution if ROS give you cancer or other diseases in your later (post-reproductive) years (Halliwell 2004).

The position of plants

Green plants have a special problem with O_2 toxicity, being exposed to the full force of the pure O_2 that they produce during photosynthesis. One therefore expects them to be loaded with antioxidants and indeed they are: vitamin C, carotenoids, tocopherols, tocotrienols and the multitudinous polyphenols, such as the flavonoids. All these molecules seem to be important antioxidants in plants, although of course they have other metabolic roles as well (Halliwell & Gutteridge 2007). Humans must obtain vitamins E and C, carotenoids and flavonoids from plants, since we cannot make them ourselves.

Epidemiological studies in the 1980s and 1990s revealed that humans with high intakes (or blood levels) of vitamin C, α-tocopherol, β-carotene and other carotenoids from their diet are less likely, on average, to suffer myocardial infarctions, other vascular disease, diabetes and many forms of cancer (Gey 1995). These studies coincided with intense research on the biological importance of oxygen radicals, other ROS (such as H_2O_2 and peroxynitrite) and antioxidant defences *in vivo*. It was discovered that increased oxidative damage accompanies most, if not all, human diseases and contributes to the pathology of several, e.g. cigarette smoke-induced lung cancer, chronic inflammation, atherosclerosis and Alzheimer's disease (Beckman & Ames 1997, Butterfield & Boyd-Kimball 2004, Halliwell & Gutteridge 2007). Putting two and two together, it was widely assumed that these antioxidants were protective agents—taking them in the diet or as supplements or in fortified foods should decrease oxidative damage and diminish disease incidence.

The gold standard of epidemiology is the double-blind placebo-controlled intervention trial. That's when it started to go wrong. The ATBC (α-tocopherol/β-carotene) study in Finland revealed that α-tocopherol supplements had no effect on lung cancer incidence in heavy smokers, but β-carotene supplements increased the risk (Virtamo et al 2003). Several other studies on various populations revealed little or no effect of vitamins C, E and β-carotene on disease prevention in well-nourished subjects, and a few suggestions of harm from high doses taken for long periods (Virtamo et al 2003, Bjelakovic et al 2004, Neuhouser et al 2004, Miller et al 2005, Lee et al 2004b, Blacker 2005, Lawlor et al 2004).

So how do we explain this? It is widely agreed by nutritionists that diets rich in vegetables and fruits are associated with lowered incidence of cardiovascular disease, dementia, diabetes, stroke and certain types of cancer, especially lung and oral cancers. The more vegetables and fruits you eat, the greater will be your body content of antioxidants. However, plants contain a huge range of agents that might protect against disease (reviewed in Halliwell 2006). In addition, a fruit- and vegetable-rich diet is often low in fat, and high fat intake is a risk factor for cardiovascular disease, diabetes and some cancers, and it promotes oxidative stress

(Morrow 2005). Thus it could be anything in the dietary plants that protects against disease, and high body antioxidant levels could be a 'biomarker' of a good diet. If so, reproducing these levels with supplements may not give the same benefit (Halliwell 1999).

Antioxidants and oxidative damage

Does the failure of vitamins E, C and β-carotene to protect against the development of age-related diseases mean that ROS are unimportant as contributors to disease pathology? Actually, no. Almost all studies assumed that feeding antioxidants would decrease oxidative damage without measuring such damage to prove that it did decrease. Yet we now know that these 'antioxidants' often do not decrease oxidative damage *in vivo*. For example, 60 mg daily of ascorbate seems sufficient to minimize oxidative DNA damage in humans, and more has no further effect (Halliwell & Gutteridge 2007). High-dose α-tocopherol is poorly-effective at decreasing levels of lipid peroxidation in healthy humans, when measured by reliable biomarkers (Meagher et al 2001). It is much better at decreasing lipid peroxidation in mice, and its anti-atherosclerotic effects are correspondingly greater (Pratico et al 1998). Studies in Denmark showed that urinary excretion of 8OHdG was decreased about 28% by feeding Brussels sprouts to volunteers (Verhagen et al 1997), but not by supplementing these subjects with β-carotene, vitamin C, or α-tocopherol (Prieme et al 1997). We found that a mixture of antioxidants could sometimes transiently increase oxidative DNA damage (Halliwell 1999). Indeed, plasma F_2-isoprostane levels respond better to weight loss, good diet and lowering plasma cholesterol levels than they do to antioxidant supplements (Morrow 2005, Meagher et al 2001). Overall, for healthy subjects, there seems to be little benefit (and possible harm) of consuming high-dose supplements of single antioxidants. Never consume β-carotene supplements if you smoke.

Does this mean that fruits and vegetables are beneficial for reasons other than their antioxidant content, or that the most important antioxidants in them have not yet been identified? Probably both are true. Plants contain multiple agents protective against disease that are not antioxidants. Indeed, some may be mild pro-oxidants, increasing the levels of endogenous defence systems by creating some degree of oxidative stress (Laughton et al 1991, Velayutham et al 2005). Several authors have shown that consumption of antioxidant-rich foods decreases levels of oxidative damage *in vivo* in humans (Lee et al 2006; reviewed by Halliwell et al 2005). Others have found little effect (e.g. McAnulty et al 2005), and a few studies registered increases in biomarkers of oxidative protein damage, such as 2-aminoadipic and γ-glutamyl semialdehydes (Dragsted et al 2004). One must be very cautious in such studies to rule out confounding effects of refeeding fasted individuals, as opposed to the effects of antioxidants in the food, on biomarkers

of oxidative damage (Lee et al 2004a, 2006, Richelle et al 1999). Nevertheless, the bulk of evidence does suggest that antioxidant effects do contribute to the benefits of a high intake of fruits and vegetables (Halliwell et al 2005), although these effects cannot be reproduced by supplements of ascorbate, vitamin E, or β-carotene.

So now to the flavonoids

Flavonoids and other polyphenols have powerful antioxidant activities *in vitro*, being able to scavenge a wide range of ROS. Many can chelate transition metal ions such as iron and copper, decreasing their ability to promote oxidative damage *in vitro* (Rice-Evans 2000). Two observations drew attention to their potential importance. First, flavonoids in red wine were shown to be able to inhibit the oxidation of low density lipoproteins, and this was suggested as an explanation of the 'French paradox' (Frankel et al 1993). Second, the Zutphen study, an epidemiological study in the Netherlands, suggested an inverse correlation between the incidence of coronary heart disease and stroke and the dietary intake of flavonoids, especially quercetin (Hertog et al 1993). Since then multiple other epidemiological studies have confirmed similar associations, although a few have not, and there is little evidence of protection against cancer (Neuhouser 2003).

Thus could flavonoids be major contributors to the disease-protective effects of fruits and vegetables? Many polyphenols are absorbed, although rarely completely, and the remainder metabolized in the colon to generate high levels of monophenols (Manach & Donovan 2004, Jenner et al 2005). But are they better antioxidants than vitamins C, E and β-carotene *in vivo*? Again, studies with biomarkers have given a mixture of results, and a review by Halliwell et al (2005) concluded that the balance of evidence overall did not support significant systemic antioxidant effects of absorbed flavonoids. Indeed, since plasma levels of unconjugated flavonoids rarely exceed 1 μM and the metabolites tend to have lower antioxidant activity because of the blocking of radical-scavenging OH groups by methylation, sulfation or glucuronidation (Williamson et al 2005), it seems difficult to imagine a powerful antioxidant effect *in vivo*. Some studies have shown effects of flavonoid-rich foods in raising plasma total antioxidant capacity (TAC). But one must be cautious here; many such foods can increase plasma uric acid levels, and urate is detected by several TAC assays. Since elevated urate may be a risk factor for some diseases, the alleged 'antioxidant benefit' may not be what it seems (Halliwell 2003a, Lotito & Frei 2004). Finally, flavonoids and other phenols are complex molecules that have multiple actions *in vivo*, including inhibiting telomerase, affecting signal transduction pathways, inhibiting cyclooxygenases and lipoxygenases, decreasing xanthine oxidase, matrix metalloproteinase, angiotensin-converting enzyme, proteasome, and sulphotransferase activities, and interacting with sirtuins. Flavonoids may

also interact with cellular drug transport systems, compete with glucose for transmembrane transport, interfere with cyclin-dependent regulation of the cell cycle, inhibit protein glycation, and affect platelet function (e.g. Howitz et al 2003, van Hoorn et al 2002, Laughton et al 1991, Spencer et al 2001, Naasani et al 2003). Again, it is uncertain whether these effects can happen systemically.

Aiding the gastro-intestinal (GI) tract

Halliwell et al (2000) proposed that antioxidant and other protective effects of flavonoids and other phenolic compounds could occur before absorption, within the stomach, intestines and colon. This could account for the suggested ability of flavonoid-rich foods to protect against gastric, and possibly colonic, cancer, although again it must not be assumed that any protective effect of flavonoid-rich foods is attributable to antioxidant actions of the flavonoids, or to flavonoids at all, rather than to other components in the foods. However, ingestion of green tea was reported to rapidly decrease prostaglandin E_2 concentrations in human rectal mucosa, consistent with inhibition of cyclooxygenase activity (August et al 1999), a potential anticancer mechanism.

The logic behind this hypothesis is that phenolic compounds present in plasma at $\leq 1\,\mu M$ concentrations are present in the stomach and intestines at much higher concentrations after consumption of foods and beverages rich in such compounds (Jenner et al 2005). Because absorption of phenolic compounds is incomplete, they enter the colon, where they and their products of bacterial metabolism can exert beneficial effects. Indeed, faecal water contains micromolar levels of flavonoids, and much higher levels of monophenols, and levels of flavonoids in the stomach and intestines will be even higher (Jenner et al 2005). Why should this be important? The gastro-intestinal (GI) tract is constantly exposed to ROS, both endogenously produced and from the diet. The stomach is especially affected by the latter; indeed, Kanner and Lapidot (2001) referred to the stomach as a 'bioreactor'. Sources of ROS include the mixtures of ascorbate and Fe^{2+} in the stomach (dietary iron, dietary ascorbate, and ascorbate normally present in gastric juice), haem proteins (also potential powerful pro-oxidants), lipid peroxides, cytotoxic aldehydes, and isoprostanes in the diet. Nitrite in saliva and in foods is converted to HNO_2 by gastric acid, forming nitrosating and DNA-deaminating species. There are also high concentrations of H_2O_2 in certain beverages, which can contain oxidizable, pro-oxidant, phenolic compounds such as hydroxyhydroquinone. Activation of immune cells naturally present in the GI tract by diet-derived bacteria and toxins can also increase ROS production (Halliwell & Gutteridge 2007).

Flavonoids and other phenolic compounds might exert direct protective effects in the GI tract, by scavenging ROS. They can inhibit haem protein-induced peroxidation in the stomach (Kanner & Lapidot 2001). They are able to inhibit DNA

base deamination by HNO_2-derived reactive nitrogen species (Zhao et al 2001), up-regulate toxin-metabolizing or antioxidant defence enzymes in the GI tract and chelate transition metal ions to decrease their pro-oxidant potential (Halliwell et al 2005). Dietary iron is usually not completely absorbed, especially among subjects on Western diets. Unabsorbed dietary iron enters the faeces, where it could represent a pro-oxidant challenge to the colon and rectum (Babbs 1990). Indeed, diets rich in fat and low in fibre may aggravate this pro-oxidant effect. Phenolic compounds, by chelating iron, may help to alleviate pro-oxidant actions of colonic iron.

Artefacts of cell culture

Many studies examining the cytotoxic and other effects of flavonoids on malignant, and other, cells in culture may have been led astray by artefacts. Flavonoids oxidize readily in many commonly-used cell-culture media, generating H_2O_2, quinones and semiquinones that can contribute to cytotoxicity (Long et al 2000, Wee et al 2003, Halliwell 2003b). For example, the apparent cytotoxicity of green tea to PC12 cells was purely artefactual (Chai et al 2003). If flavonoids really are anticancer agents, more experiments demonstrating this *in vivo* are required.

Conclusion

To the author, flavonoids are typical xenobiotics, metabolized as such and rapidly removed from the circulation. High levels may even be toxic, but low levels of toxins can sometimes be good for you by raising levels of xenobiotic-metabolizing and antioxidant defence enzymes. Thus stick to flavonoid-rich foods; red wine (alcohol in moderation is good for you), tea, fruits and vegetables. Don't start taking high-dose supplements or foods heavily fortified with flavonoids until we know more; we do not want a repeat of the β-carotene error (Hercberg 2005). As I said a while ago, 'a protective effect of diet is not equivalent to a protective effect of antioxidants in diet' (Halliwell 2000).

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DISCUSSION

Katan: Do you think flavonols from chocolate lower blood pressure in humans?

Halliwell: I'd have to go back and look at the design of studies. The reason is that the act of eating lowers blood pressure, because blood goes to the gut.

Katan: There have been placebo-controlled studies, but they were mostly funded by Mars. It would be good to have some independent studies.

Aggett: I like a good bit of iconoclasm and we have had a good dose of that this morning. There is an important generic message from your presentation, and that is we should be using simple tests, assays and biomarkers that are well validated. The antioxidant field is plagued by a whole variety of tests that can be selectively chosen to get the required result. Can you comment on the overall quality of the assays and biomarkers of susceptibility to oxidative damage, and also the protective effect?

Halliwell: This question could support a symposium on its own! Let's take low density lipoprotein (LDL) oxidizability, as an example. You feed people vitamin E, isolate LDL, add some copper in the test-tube and you get a lag period that is lengthened with increasing dose of vitamin E. LDL oxidation in the test-tube doesn't really get going until the vitamin E is gone. Roland Stocker showed us

nicely that in human atherosclerosis, lipid oxidation is going on and the vitamin E is still there. This tells me that these LDL oxidation studies are not representative of what is going on in the body. The free radical field has been plagued with people using ill-defined concepts such as oxidative damage, pro-oxidants and antioxidants. You have to really get down to the specific molecular level, both in terms of antioxidant action and in terms of what some of the reactive species do. Different molecular forms of oxidized lipids do very different things to cells. For example, if you take Alzheimer's disease brain and look for increased protein damage, this damage is focused on certain proteins. It is not random damage. Most of these damaged proteins are involved in energy metabolism, which fits nicely with the idea that in Alzheimer's neuronal energy metabolism is impaired. We have to get to this mechanistic mode instead of talking about total antioxidant capacity or vaguely about oxidative damage.

Stocker: With regard to general or systemic lipid oxidation, the best biomarker is now generally regarded to be F_2-isoprostane. If analysed properly, this is a useful maker. But 'analysed properly' refers to the need for a mass spectrometry-based method.

Aggett: How generalizable is F_2-isoprostane?

Stocker: There are two major issues. If you deal with a disease that is lipid-driven, such as atherosclerosis, it is important to standardize the F_2-isoprostane concentration to that of the lipid it is derived from, i.e., arachidonic acid, because the amount of that lipid may also change as a result of the disease. Some of the studies that have shown a benefit of vitamin E or other antioxidants on lipid oxidation *in vivo* have not done so in a scenario where lipid changes did occur. With regard to specificity, F2-isoprostanes are commonly measured in biological samples without distinction between different classes of lipids, and after the lipids have been hydrolysed. As a result, potentially important information is lost, such as the class of lipid (e.g. phospholipids or cholesteryl esters) the isoprostanes derived from.

Azzi: Barry Halliwell, I appreciate your courage in abandoning the traditional oxidants-antioxidant concepts. Similarly, one of the chief researchers in one of the major companies producing vitamins and carotenoids even proposed a symposium to be entitled 'Why the antioxidants have failed'. The antioxidant concept has been inflated and over-used, and there is very little evidence for this famous paradigm involving the bad guys (the radicals) and good guys (the antioxidants). I think it is appropriate to add a further comment regarding other molecules that have been considered antioxidants, such as flavonoids and polyphenols: their function as antioxidants is in most cases insignificant, due to their very low absorption and the lack of apparent recycling mechanisms to restore them after the modification produced by the radicals (Manach et al 2005). However, they are able to show other biological activities like modulation of gene expression or signal transduction (Rushmore & Kong 2002).

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Halliwell: I agree with you. Some polyphenols are absorbed quite well, some quite badly. Many of them are COX2 inhibitors or can inhibit signal transduction. It's easy to see how this can happen in the gut. The big issue is whether people absorb enough into their body tissues for any effects to be significant, antioxidant or otherwise.

Manach: There is also a lot of interest in flavonoids as antioxidants. We must be suspicious of all *in vitro* studies in the field of flavonoids. These have almost always been done with high concentrations, and using compounds that are not present in the body. These compounds have no chance of getting to target tissues, and the doses used are much higher than the plasma concentrations achieved in the body (~1 μM). The tissue concentrations are even lower. I think that polyphenols probably don't have antioxidant action. Rather, we must look for gene transcription effects, signalling effects and induction of antioxidant defence systems by these compounds. To study this we must use small, physiologically relevant concentrations. We need to reinvestigate the *in vitro* studies on flavonoids, and we can't generalize to all polyphenols.

Boobis: If we look at the origins for some of the hypotheses of why these compounds might be effective, it is from observational studies on the effects of diet on health outcomes. The consumption of fruit of vegetables seems to be protective. But if one examines the evidence for effects of specific agents it becomes weaker. We have to keep in mind two entirely different possibilities. The first is that there is something present in our diet that is biologically active at the levels consumed, and it might be of advantage to identify this and develop it as a supplement. Such a compound would impact on physiological mechanisms. The evidence that we have identified specific agents with these properties is not very strong so far. There is a whole other dimension, though, which we shouldn't lose sight of. There are compounds in plants used as foods that we ingest but which have no effect at all on a normal individual. But if we take this same agent and give it at a pharmacological dose, it has a biological effect that is beneficial. It is quite different on the one hand to identify something from natural sources to develop as a supplement and on the other to produce something that can be given at a pharmacological dose. High levels might have an effect that is nothing to do with normal physiology but which may still be protective. The caveat here is this is essentially a drug and should be tested as such: demonstration of safety and efficacy is needed.

Halliwell: You raise an interesting point. When you go from the levels found in food to selling a 5 g supplement it may well be a drug.

Russell: Along those lines, in the ATBC study a pharmacological dose of β-carotene was used. Since then an animal model—a ferret exposed to smoke—has been studied. The ferret metabolises carotenes in much the same way as humans. High-dose β-carotene produced squamous metaplasia in smoke exposed ferrets. The metaplasia was much worse with the β-carotene plus smoke animals than in

animals exposed to smoke alone. But with a physiological dose of β-carotene there was no effect at all.

Manach: In addition to the dose we should also consider the complexity of the food. It may be that a component doesn't have the same effect when it is isolated.

Azzi: We should ask ourselves a fundamental question. There are theories of disease and ageing based on radicals. If all these theories were valid why don't we find antioxidants capable of preventing all these diseases, including ageing?

Halliwell: Because most of the antioxidants used don't decrease free radical damage *in vivo*. This could be why they don't work!

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