Relevance of apple consumption for protection against oxidative damage induced by hydrogen peroxide in human lymphocytes

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In a single-dosing crossover study, we investigated the ability of apple fruit consumption to protect human lymphocytes against peroxide-induced damage to DNA. Six healthy, non-smoking male volunteers were placed for 2 d on an antioxidant-poor (AP) diet. After 48 h of AP diet, the volunteers were required to consume a homogenate obtained from 600 g of red delicious unpeeled apples or water (500 ml); blood samples were collected 0, 3, 6 and 24 h post-consumption. To evaluate whether the apple intake was sufficient to restore resistance of DNA to oxidative damage, for each subject at any time point the plasma total antioxidant activity, reactive oxygen species (ROS) formation and induction of micronuclei (MN) in isolated lymphocytes following hydrogen peroxide (H_2O_2) treatment were measured. Results indicated a significant inhibition (58 %, $P < 0.05$) of H_2O_2-induced MN frequency in the plasma samples collected at 3 h after apple consumption, as compared with plasma samples collected at 0 h (4·17 (SD 1·83) *v.* 9·85 (SD 1·87) MN/1000 binucleated (BN) cells, respectively). A gradual return towards the value observed at 0 h was recorded starting from 6 to 24 h. MN frequency induced by H_2O_2 was significantly influenced by plasma total antioxidant activity ($r = -0.95$, $P < 0.05$) and by the increase of intracellular ROS formation ($r = 0.88$, $P < 0.05$). These findings suggest that the consumption of whole apple provides a useful dietary source of active scavengers to protect cells and tissue from oxidative stress and related DNA injury.

Phytochemicals: Plasma antioxidant activity: Reactive oxygen species: Genetic damage

A considerable body of evidence indicates that the risk of degenerative diseases is considerably lower in people who consume antioxidant-rich fruits and vegetables and it has been proposed that extra health benefits may derive from above-average intake of these compounds (Peto *et al.* 1981; Block *et al.* 1992; Hertong *et al.* 1995; Steinmetz *et al.* 1996; WCRF/AICR, 1997; Terry *et al* 2001; Bazzano *et al.* 2002; Knekt *et al.* 2002; Collins *et al.* 2004). Food components can modulate the maintenance of the oxidant–antioxidant balance and may thereby influence the health status. Oxidative reactions are of major significance in human pathophysiology, and oxidative stress has been linked to diseases such as atherosclerosis, diabetes, cancer and tissue damage in rheumatoid arthritis (Hertog *et al.* 1993, 1995; Knekt *et al.* 1996; Scalbert *et al.* 2005). In the field of cancer, it is accepted that both genotoxic and non-genotoxic carcinogens act through the induction of DNA oxidative damage (Ames *et al.* 1995; Duthies *et al.* 1996; Collins & Ferguson, 1998).

Epidemiological studies have shown only an association between total intake of fruit and vegetables and beneficial effects, with limited evidence linked to individual phytochemicals. Large-scale intervention trials have failed to show decreased cancer risks following selective vitamin and antioxidant supplementation (Omenn *et al.* 1996; Jacobson *et al.* 2000; Galati & O'Brien, 2004). These results confirm that the dietary intake of antioxidant-rich food (rather than single antioxidant supplementation) can be a means of improving the health of individuals and of preventing or reducing the risk of chronic diseases. The antioxidant activity of pure antioxidant compounds has been studied in cell cultures, but human studies are needed to evaluate the biological effects of the complex mixture in which they occur in real food.

Apples are one of the main sources of dietary antioxidants, the polyphenolic compounds, in the Western diet (Art *et al.* 2001; Boyer & Liu, 2004). Gallus *et al.* (2005) found a consistent inverse association between apple consumption and risk of various cancers. Fresh apples have been shown to provide greater levels of antioxidants and anticancer activity *in vitro* than are provided by dietary supplementation of vitamin C (Eberhardt *et al.* 2000), suggesting that phytochemicals (phenolic acids and flavonoids) other than ascorbic acid interact in a synergistic manner to provide biological effects. Apple polyphenol extracts were demonstrated to prevent damage to human gastric epithelial cells *in vitro* and to rat gastric mucosa *in vivo* (Graziani *et al.* 2005). Assessment of antioxidant bioactivity *in vitro*, measured in terms of

intracellular antioxidant, cytoprotective and antiproliferative activity in human colon carcinoma cells, showed strong, time-related decreases for all three parameters by whole apple homogenates (Hrelia *et al.* 2003; Tarozzi *et al.* 2004). Whether apples have a protective effect against oxidative stress-related injury in humans is still unproven.

Therefore, we performed a single-dosing crossover study to investigate the ability of whole apple fruit to prevent hydrogen peroxide-induced damage to DNA in humans. An *ex vivo* approach was adopted – utilising plasma and lymphocytes from peripheral blood of healthy volunteers – to study total plasma antioxidant activity alongside exogenously induced reactive oxygen species (ROS) formation and DNA damage in separated lymphocytes.

Materials and methods

Subjects and study design

Six healthy, non-smoking male volunteers aged 29·7 (SD 4·5) years (range: 25–37 years) with a mean BMI of 23·5 (SD 2·0) kg/m^2 (range: 21·6–27·2 kg/m^2) participated in a single-dosing crossover study, which was performed according to the Declaration of Helsinki. All participants were informed of the study aims and provided written informed consent. Each individual was interviewed in-depth by a specialised physician who filled in a structured questionnaire to exclude the following characteristics: exposure to diagnostic/therapeutic X-rays or chemotherapeutic drugs in the previous 3 months; current use of nutritional supplements, deficient diet or peculiar dietary habits; past/present smoking status; chronic or metabolic disease; and occupational exposure to genotoxic compounds. All volunteers had a normal-caloric diet, and were regular consumers of all the food categories (vegetables, fruit, meat, milk, cheese, eggs, pasta and bread). They were moderate consumers of wine and/or beer (<1 l per week), but did not consume spirits.

The experimental protocol followed a single-dosing crossover design as summarised in Fig. 1. The volunteers were initially required to provide a blood sample after an overnight fast, following which they were placed for 2 d on an antioxidant-poor (AP) diet, since the antioxidant effects of apples may be more easily observed when the status in other antioxidants is low. The foods that were allowed in the AP diet are listed in Table 1. After 48 h of AP diet, a second fasted blood sample was taken. Ten minutes later, subjects were randomly assigned (three subjects per group) to consume either a homogenate obtained from 600 g of fresh red delicious unpeeled apples (the equivalent of five pieces of fruit, obtained from a local market) or 500 ml of water. For the preparation of the homogenate, unpeeled apples were cut into small pieces, briefly blended, and then the homogenate was immediately consumed by the subjects. The amount of apples selected for the study is a large portion, that is not always easy to eat in a single intake; however, the homogenate allowed a quick and easy consumption of apples in large quantities. Further blood samples were taken 3, 6 and 24 h after the drink, while still on the AP diet. Volunteers then returned to their normal diets for a 1 week wash-out period, after which the intervention was repeated with a crossover in the type of drink taken.

Blood processing

Venous peripheral blood samples were collected by medical workers in heparinised tubes and were coded. Processing and scoring of the samples were performed blind and concurrently. At the end of the study, the data from the questionnaire were linked to the code number for data analysis. Within 30 min of collection, blood samples were centrifuged (1500 *g*, 15 min, 4 °C) to obtain the plasma and buffy-coat from each sample. Each plasma sample was removed and divided into two aliquots: one for H$_2$O$_2$ challenge and the other for biochemical analysis. The buffy coat was mixed with physiological solution (0·09 % NaCl), and carefully layered onto a Hystopaque 1077 gradient (Sigma-Aldrich, St Louis, MO, USA) to isolate the peripheral lymphocytes. After centrifugation for 30 min at 1800 r.p.m., the lymphocytes were collected, washed (with RPMI 1640 medium), centrifuged and divided into two aliquots: one for micronuclei (MN) assay, the other to determine the ROS formation induced *in vitro* by H$_2$O$_2$.

Determination of plasma total antioxidant activity

Total antioxidant activity – a marker of plasma antioxidant status – was measured as reported by Re *et al.* (1999) This method is based on the ability of the antioxidant molecules
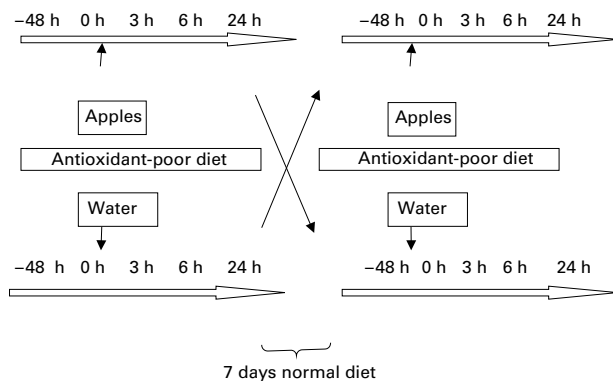

Fig. 1. Intervention crossover design showing time, in hours, relative to apples or placebo drink.

Table 1. Details of food and drink restrictions during the antioxidant-poor diet

Foods allowed	Foods not allowed
Beef, pork, chicken, ham	All fruits (fresh, fried or stewed)
Cheese	All vegetables, legumes
White bread	Nuts
White rice (not brown)	Fruit or vegetable juice
Ordinary (not whole meal) pasta	Coca-Cola, Fanta and all fizzy drinks
Butter	Wine, beer, alcoholics drinks
Water, milk	Tea and coffee
	Chocolate and chocolate drinks
	Jams, marmalades
	Tomato sauce, chutney, curry, pepper
	All breakfast cereals
	Cakes

present in plasma to reduce the radical cation of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), determined by the decolorization of ABTS$^+$ and measured as quenching of absorbance at 740 nm. Values obtained for each sample were compared with the concentration–response curve of a standard Trolox solution and expressed as Trolox equivalent antioxidant activity (TEAA) in mmol/l.

Determination of intracellular ROS formation

Formation of intracellular ROS was determined using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), as previously reported (Rosenkranz *et al.* 1992). Briefly, freshly isolated human lymphocytes were suspended at a concentration of 1×10^6 cells/ml in 2 ml of Hank's balanced salt solution (HBSS) and incubated for 30 min with DCDHF-DA (10 μmol/l) at 37°C in the dark in a multiwell plate. After removal of DCFH-DA, the lymphocytes were washed with phosphate-buffered saline and incubated with H_2O_2 (100 μmol/l) in HBSS for 30 min at 37°C. The fluorescence of the cells from each well was measured using 485 nm excitation and 535 nm emission with a microplate spectrofluorometer (VICTOR3 V™ Multilabel Counter, Perkin Elmer, Wellesley, MA, USA). The values were expressed as the percentage increase of intracellular oxidative species evoked by exposure to H_2O_2 and calculated by the formula $[100 \times (F_t - F_{nt})/F_{nt}]$, where F_t = fluorescence of treated lymphocytes and F_{nt} = fluorescence of non-treated lymphocytes.

MN assay

The cytokinesis-block MN assay in human lymphocytes is a multi-end-point assay that can measure DNA damage (chromosome damage) after oxidative stress, along with the morphological evaluation of necrosis and apoptosis, in cells that have completed nuclear division (Crott & Fenech, 1999; Fenech *et al.* 1999). MN are efficiently expressed in dividing cells when chromosome breaks are induced by hydroxyl radicals. The induced chromosome breaks lag behind at anaphase in dividing cells and are subsequently packaged within nuclear membranes to produce MN.

To measure baseline MN frequencies, aliquots of lymphocytes (1×10^6) collected and isolated at each time point were cultured directly in an RPMI 1640 medium (5 ml) containing 15 % fetal calf serum, 1 % phytohaemmaglutinin, L-glutamine 1 mmol/l, 100 U of penicillin and 100 μg/ml streptomycin (all purchased from Sigma-Aldrich). Cultures were incubated with 5 % CO_2 at 37°C for 72 h.

To test the effect of the H_2O_2 challenge, lymphocytes (1×10^6) from each collection were added to the same donor's plasma (1 ml) taken at the same time point, stored at 37°C, and then incubated for 30 min to allow metabolites in plasma and cells to equilibrate. Cells were then treated with either (1) H_2O_2 (700 μmol/l) from a freshly prepared stock solution in HBSS; or (2) the same volume of HBSS without H_2O_2.

The selection of the dose of 700 μmol/l H_2O_2 was based on preliminary dose–response studies with/without H_2O_2 challenge (dose range: from 100 to 1000 μmol/l). It has been found that 700 μM induced a significant increase of MN frequency in the lymphocytes from each volunteer without causing cytotoxicity or markedly diminishing the

frequency of the BN cells used for MN scoring (data not shown). Furthermore, the dose of H_2O_2 (700 μmol/l) has been previously used by Fenech *et al.* (1997). A higher dose of H_2O_2 was used in these experiments because the lymphocytes were much less sensitive to this agent when incubated with plasma relative to incubation in HBSS selected for the determination of intracellular ROS formation.

After 30 min, the lymphocytes were transferred into the culture medium used for MN measurement (described above) and incubated with 5 % CO_2 at 37°C for 72 h.

In all cultures, cytochalasin-B (Sigma-Aldrich) was added (final concentration 6 mg/ml) for the last 28 h. Cells were then collected and treated with a mild hypotonic treatment (RPMI1640 medium–distilled water, 50:50 v/v) and fixed with a mixture of methanol/glacial acetic acid, as previously described (Fenech & Morley, 1985). The slides were prepared by cytocentrifuge, air-dried, and stained by conventional May–Grünwald Giemsa staining (Sigma-Aldrich).

In accordance with standard criteria, MN analysis was performed on coded slides by scoring 1000 BN lymphocytes for each of the two duplicate cultures. Cell division frequency is shown as the percentage of viable (non-apoptotic/non-necrotic) cells that are binucleated (% BN). Apoptosis and necrosis are expressed as percentages of the total cell number. Total cell numbers included mononuclear cells, BNs, other multi-nucleated cells, apoptotic and necrotic cells (Fenech *et al.* 1999). All assays were performed in duplicate cultures.

Statistical analysis

Values are means and standard deviations. Paired data *t* test was performed to analyse the time–response data. Pearson's regression analysis was used to evaluated the influence of the plasma total antioxidant activity and H_2O_2-induced intracellular oxidative species formation on MN frequency induced by H_2O_2. Statistical analysis was conducted using GraphPAD Prism-3.02.

Results

Effect of apple consumption on plasma total antioxidant activity

As reported in Fig. 2, an AP diet significantly reduced the plasma total antioxidant activity in terms of TEAA (apple-assigned subjects, 1·69 (SD 0·05) (−48 h) *v.* 1·59 (SD 0·04) mmol/l (0 h); water-assigned subjects, 1·68 (SD 0·04) (−48 h) *v.* 1·58 (SD 0·04) mmol/l (0 h); all *P* <0·05). Subsequently, after ingestion of water, TEAA values at 3, 6 and 24 h remained similar to those found at 0 h. In contrast, ingestion of apples led to significantly increased plasma TEAA at both 3 h (1·64 (SD 0·02) mmol/l; *P* <0·05 *v.* 0 h) and 6 h (1·64 (SD 0·03) mmol/l; *P* <0·05 *v.* 0 h). Plasma antioxidant activity 24 h after apple intake returned toward the values observed before apple consumption.

Effect of apple consumption on H_2O_2-induced intracellular oxidative species formation

As shown in Fig. 3, an AP diet led to a significant percentage increase of levels of H_2O_2-induced intracellular oxidative
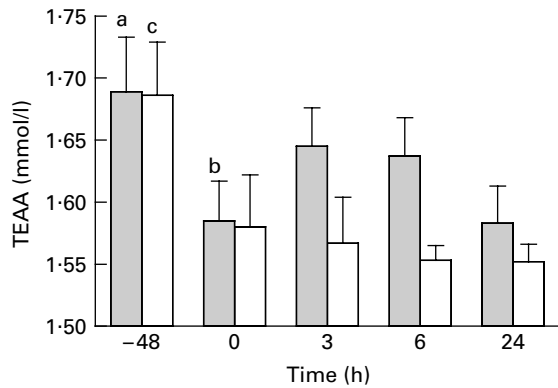
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Fig. 2. Effects of AP diet, apples or water consumption on plasma total antioxidant activity. The plasma was subjected to the ABTS radical cation decolorization assay as reported in Materials and methods, and antioxidant capacity was expressed as Trolox equivalent antioxidant activity (TEAA) in mmol/l. Values are means and standard deviations, *n* 6. The shaded bars indicate apple and the white bars indicate water. (Apple: a, −48 h *v.* 0 and 24 h, *P* <0·05; b, 0 h *v.* 3 and 6 h, *P* <0·05. Water: c, −48 *v.* 0, 3, 6 and 24 h, *P* <0·05).

species (apple-assigned subjects, 22·03 (SD 4·02) % (−48 h) *v.* 34·96 (SD 6·87) % (0 h); water-assigned subjects, 23·20 (SD 3·42) % (−48 h) *v.* 34·00 (SD 4·85) % (0 h); all *P* <0·05). Whereas ROS levels up to 24 h after ingestion of water remained similar to those recorded at 0 h, apple consumption was followed by strongly decreased intracellular ROS levels at both 3 h (17·20 (SD 4·02) %, *P* <0·5 *v.* 0 h) and 6 h (24·04 (SD 3·76) %, *P* <0·05 *v.* 0 h). Intracellular ROS levels recorded 24 h after apple consumption turned out to be the same as those recorded at 0 h.

Effect of apple consumption on H_2O_2-induced DNA damage in terms of MN frequency

We first assessed the effects of AP diet followed by ingestion of apples or water on baseline MN frequency in cultures not exposed to plasma or challenged with H_2O_2. Baseline MN frequencies and the percentage of BN cells remained similar in
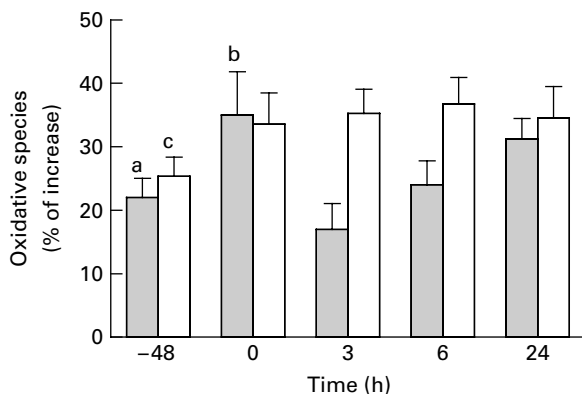

Fig. 3. Effects of AP diet, apples or water consumption on intracellular oxidative species formation in lymphocytes. Levels of intracellular ROS are expressed as the percentage increase of intracellular oxidative species evoked by exposure to H_2O_2. Values are means and standard deviations, *n* 6. The shaded bars indicate apple and the white bars indicate water. (Apple: a, −48 h *v.* 0 and 24 h, *P* <0·05; b, 0 h *v.* 3 and 6 h, *P* <0·05. Water:c, −48 h *v.* 0, 3, 6 and 24 h, *P* <0·05).

lymphocytes collected at each experimental time, regardless of apple or water consumption (data not shown). No effects on the percentage of necrotic or apoptotic cells were recorded (data not shown).

We then analysed the effect of apple consumption on peroxide-induced DNA damage, in terms of MN frequency, in cultured lymphocytes exposed to plasma with/without H_2O_2 challenge. As reported in Table 2, the data obtained for plasma in the control cultures that were not exposed to H_2O_2 after the consumption of apples or water do not suggest any effect on MN frequency. The overall data for the effects of plasma on H_2O_2 challenge indicated an induced significant increase of MN frequency with respect to the relative controls (cells incubated only with plasma) for all experimental collections.

Fig. 4 illustrates the trend observed following apple or water intake. After an AP diet (0 h) significant increases in induced MN frequency (obtained after subtraction of the relative control values) were recorded with respect to −48 h (apple-assigned subjects, 2·50 (SD 1·87) *v.* 9·85 (SD 1·87) MN/1000 BN cells, *P* <0·05; water-assigned subjects, 2·33 (SD 1·03) *v.* 9·50 (SD 1·97) MN/1000 BN cells, *P* <0·05).

The results show a significant reduction (58 %; *P* <0·05) in the induced MN frequency 3 h after apple intake (4·17 (SD 1·83) MN/1000 BN cells), with a gradual return, 24 h later (9·33 (SD 1·21) MN/1000 BN cells), toward the values observed before apple consumption (0 h). MN frequency in cells incubated with plasma from blood collected at 6 h was intermediate to that observed for plasma collected 3 h after apple consumption.

The plasma collected after the ingestion of water did not alter MN frequency induced by H_2O_2 at 3, 6 or 24 h, and the values recorded were similar to those at 0 h.

More interestingly, the increase of the frequency of MN induced by H_2O_2 was significantly influenced by the decrease of the plasma total antioxidant activity ($r = -0.95$, $P<0.05$) and by the increase of H_2O_2-induced intracellular ROS formation ($r = 0.88$, $P<0.05$). H_2O_2 challenge did not influence the proliferation of the lymphocytes, and the %BN cells was

Table 2. Effect of plasma collected before and after ingestion of apples or placebo on MN frequency induced by H_2O_2 in human lymphocytes (*n* 6)

(Mean values and standard deviation)

| | | Apples | | Water | |
| | | MN/1000 BN cells | | MN/1000 BN cells | |
	Treatment	Mean	SD	Mean	SD
−48 h	Plasma	6·83	0·98	6·83	0·75
	Plasma +H_2O_2	9·33*	2·25	9·17*	0·98
0 h	Plasma	7·00	1·10	7·33	1·03
	Plasma +H_2O_2	16·83*	3·13	16·67*	2·42
3 h	Plasma	7·67	1·03	7·50	0·84
	Plasma +H_2O_2	11·30*	2·56	15·50*	3·67
6 h	Plasma	7·50	1·22	7·50	0·55
	Plasma +H_2O_2	13·00*	3·29	16·50*	1·76
24 h	Plasma	7·33	1·02	7·83	0·98
	Plasma +H_2O_2	16·50*	1·76	18·00*	1·55

MN, micronuclei.
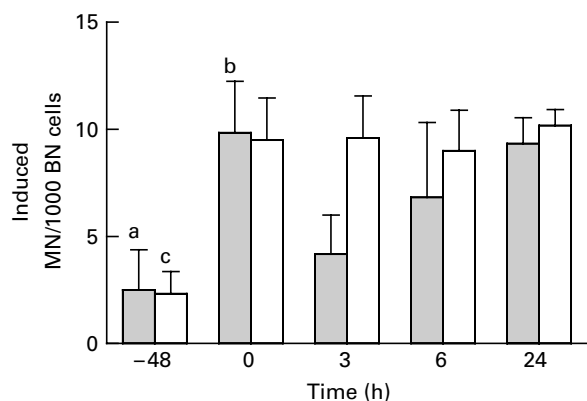* Significantly higher than the corresponding plasma value, *P* <0·05.

Fig. 4. Effects of plasma collected before and after consumption of apples or water on MN frequency induced by H_2O_2. Results were calculated for each experimental time using the formula: MN/1000 BN in lymphocytes incubated in plasma with H_2O_2 subtracted from MN/1000 BN in lymphocytes incubated in plasma without H_2O_2. Values are means and standard deviations, n 6. The shaded bars indicate apple and the white bars indicate water. (Apple: a, −48 h v. 0, 6 and 24 h, $P < 0.05$; b, 0 h v. 3 h, $P < 0.05$. Water: c, −48 h v. 0, 3, 6 and 24 h, $P < 0.05$).

similar among all the experimental conditions investigated (data not shown). No significant difference in the percentage of necrotic or apoptotic cells was observed after intake of apples or water (data not shown).

Discussion

Several studies suggest that apples might exert beneficial effects on human health due to their phytochemical content (Fang *et al.* 2002; Hassimoto *et al.* 2005). This single-dosing crossover study highlights, for the first time to our knowledge, the ability of unpeeled whole apple intake to restore the antioxidant plasma activity and to confer resistance to lymphocytes against oxidative DNA injury in humans.

Human intervention trials are important tools to identify the antioxidant activities of different foods and their biological effects on humans. Single-dosing crossover studies are adequate as inter-individual variation can be minimized. This type of study design has also been applied by other authors to assess the antioxidant activity of other fruits or drinks (Fenech *et al.* 1997; Collins *et al.* 2001). The inclusion of a placebo group (which can be used in studies with supplements) is not feasible for logistic reasons when foods such as fruit or vegetables are tested (Moller & Loft, 2002; Hoelzl *et al.* 2005). Human trials also pose the problem of the compliance of the study population in consuming the food selected in large quantities. In our study, we decided to use an apple homogenate to allow an easy and quick intake of fresh unpeeled apples (600 g) by the six volunteers enrolled in the study. Furthermore, it has been demonstrated that apple homogenate keeps the antioxidant properties (Campanella *et al.* 2003).

Apple fruit is a complex mixture containing a variety of potential beneficial compounds, the biological effect of which is dependent on a number of factors, such as bioavailability and the interaction of the various antioxidant compounds. Quercetin is the main bioavailable flavonoid, with a plasma level about 90 ng/ml at 2·5 h after intake of apple fruit, and it is more concentrated in the peel than in the pulp

(Gugler *et al.* 1975; Hollman *et al.* 1995, 1997). Apples also contain rutin and other glycosides (Manach *et al.* 2005). Also the fructose content has been proven to increase the antioxidant capacity of apples in humans (Lotito & Frei, 2004*a*). The superior antioxidant activity of apples with respect to the sum of the antioxidant activities of pure compounds has been demonstrated recently, suggesting a synergistic effect among the various phytochemical compounds (Vanzani *et al.* 2005).

Consistent with the observations of a significant decrease in blood resistance to free radicals in healthy individuals eating less than one serving of fruit per day (Lesgards *et al.* 2002; www.who.int/), our study shows that 2 d of an AP diet can lead to a significant impairment of plasma antioxidant capacity. The observed decline in plasma antioxidant activity correlates with an increased intracellular generation of ROS by an *ex vivo* exposure to H_2O_2. The antioxidant effects of apple consumption are transient, with a maximum at 3 h after ingestion. A similar effect of the antioxidant activity of plasma after consumption of apples was also found by Hollman *et al.* (1997) and Lotito & Frei (2004*b*). These data confirm the important role of repeated intake of apples for maintaining a balance between free radical production and antiradical defences in humans (Ko *et al.* 2005). This hypothesis is supported by the bioavailability of quercetin after apple consumption, which showed a long half-life of elimination, suggesting that repeated consumption of apples will cause accumulation of quercetin in blood (Holman *et al.* 1997).

Recently, it has been suggested that total plasma antioxidant activity could partly reflect the urate present in plasma (Halliwell, 2003). A flavonoid and urate antioxidant interplay in plasma oxidative stress has been demonstrated (Filipe *et al.* 2001). In particular, preservation of endogenous urate by quercetin correlated negatively with the formation of free malondialdehyde induced by copper, indicating that quercetin protects the hydrophilic antioxidant capability of plasma. The protection of plasma urate levels by quercetin and the antioxidant synergy between flavonoids and urate may contribute to the beneficial effects attributed to flavonoids. Although we have not measured plasma urate levels, the slight ($\sim 4\%$), but significant increase in plasma total antioxidant activity could reflect this synergy between urate levels and apple flavonoids.

The hydrogen peroxide challenge provided the opportunity to assess whether substances in the plasma collected after different diet conditions could quench the potential DNA-damaging effect of the generated ROS. Moreover, incubation with the plasma prior to challenge allowed potential exchange of metabolites between cells and plasma. Our results indicate that a 48 h AP diet is sufficient to increase the susceptibility of human lymphocytes to genetic damage, as shown by the main increase in MN frequency induced *in vitro* by H_2O_2 recorded at the end of the diet. This is in line with other studies indicating that a low (or lack of) consumption of fruit can alter the equilibrium status of the pro-oxidant/antioxidant system in cells, causing a pronounced impairment of cellular metabolism and significant damage to tissues (Kim *et al.* 2003). The ingestion of apple fruit restored the antioxidant defence system in all the volunteers: both plasma and lymphocytes obtained from the six subjects are able to reduce the *in vitro* genotoxic effects of H_2O_2. This phenomenon is associated with both the increase of plasma total antioxidant activity and the decrease of induced intracellular oxidative species formation.

In conclusion, although our investigation does not allow us to establish the separate contributions of different dietary components to the potential antioxidant effect of apple, the overall results confirm that the consumption of whole apple provide a useful dietary source of active scavengers to protect cells and tissues from oxidative stress and related DNA injury. However, a larger investigation with more volunteers will be required to confirm the observed effects. From both a clinical and a public health prospective, our findings also highlight the importance and utility of the dietary survey programme recommending a regular intake of fruit and vegetable (e.g. the 'five a day programme') to counteract the effect of ROS and to gain health benefit (Cox *et al.* 1998).

Acknowledgements

References

Ames BN, Gold LS & Willett WC (1995) The causes and prevention of cancer. *Proc Natl Acad Sc USA* **92**, 5258–5265.

Art IC, Hollman PC, Feskens EJ, Bueno de Mesquita HB & Kromhout D (2001) Catechin intake and associated dietary and lifestyle factors in a representative sample of Dutch men and women. *Eur J Clin Nutr* **55**, 76–81.

Bazzano LA, He J, Ogden LG, Loria CM, Vupputuri S, Myers L & Whelton PK (2002) Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study. *Am J Clin Nutr* **76**, 93–99.

Block G, Patterson B & Subar A (1992) Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer* **18**, 1–29.

Boyer J & Liu RH (2004) Apple phytochemicals and their health benefits. *Nutr J* **3**, 1–15.

Campanella L, Bonanni A, Favero G & Tomassetti M (2003) Determination of antioxidant properties of aromatic, olives and fresh fruit using an enzymatic sensor. *Anal Bioanal Chem* **376**, 467–475.

Collins AR & Ferguson LR (2004) Nutrition and carcinogenesis. *Mutat Res* **551**, 1–8.

Collins AR, Olmedilla B, Southon S, Granado F & Duthie SJ (1998) Serum carotenoids and oxidative DNA damage in human lymphocytes. *Carcinogenesis* **19**, 2159–2162.

Collins BH, Horsà A, Hotten PM, Riddoch C & Collins AR (2001) Kiwifruit protects against oxidative DNA damage in human cells and *in vitro*. *Nutr Cancer* **39**, 148–153.

Cox DN, Anderson AS, Reynolds J, McKellar S, Lean ME & Mela DJ (1998) Take five, a nutrition education intervention to increase fruit and vegetables intakes: impact on consumer chose and nutrient intakes. *Br J Nutr* **80**, 123–131.

Crott JW & Fenech M (1999) Effects of vitamin C supplementation on chromosome damage, apoptosis and necrosis *ex vivo*. *Carcinogenesis* **20**, 1035–1041.

Duthies SJ, Ma A, Ross MA & Collins AR (1996) Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. *Cancer Res* **56**, 1291–1295.

Eberhardt M, Lee CY & Liu RH (2000) Nutrition: antioxidant effect of fresh apples. *Nature* **405**, 903–904.

Fang Y, Yang S & Wu G (2002) Free radicals, antioxidants, and nutrition. *Nutrition* **18**, 872–879.

Fenech M, Crott JW, Turner J & Brown S (1999) Necrosis, apoptosis, cytostasis and DNA damage in human lymphocytes measured simultaneously within the cytokinesis-block micronucleus assay: description of the method and results for hydrogen peroxidase. *Mutagenesis* **14**, 605–612.

Fenech M & Morley AA (1985) Measurement of micronuclei in lymphocytes. *Mutat Res* **147**, 29–36.

Fenech M, Stockley C & Aitken C (1997) Moderate wine consumption protects against hydrogen peroxide-induced DNA damage. *Mutagenesis* **12**, 289–296.

Filipe P, Lança V, Silva JN, Morlière P, Santus R & Fernandes A (2001) Flavonoids and urate antioxidant interplay in plasma oxidative stress. *Mol Cell Biochem* **221**, 79–87.

Galati G & O'Brien PJ (2004) Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radic Biol Med* **37**, 287–303.

Gallus S, Talamini R, Giacosa A, Montella M, Ramazzotti V, Franceschi S, Negri E & Lavecchia C (2005) Does an apple a day keep the oncologist away? *Ann Oncol* **16**, 1841–1844.

Graziani G, D'Argenio G, Tucillo C, Loguercio C, Ritieni A, Morisco F, Del Vecchio Blamco C, Fagliano V & Romano M (2005) Apple polyphenol extracts prevent damage to human gastric epithelial cells *in vitro* and to rat gastric mucosa *in vivo*. *Gut* **54**, 193–200.

Gugler R, Leschik M & Dengler HJ (1975) Disposition of quercetin in man after single oral and intravenous doses. *Eur J Clin Pharmacol* **9**, 229–234.

Halliwell B (2003) Health benefits of eating chocolate? *Nature* **426**, 787–788.

Hassimoto NMY, Genovese MY & Lajolo FM (2005) Antioxidant activity of dietary fruit, vegetables and commercial frozen fruit pulps. *J Agric Food Chem* **53**, 2928–2935.

Hertog MG, Feskens EJ, Hollman PC, Katan MB & Kromhout D (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **342**, 1007–1011.

Hertog MG, Kromhout D, Aravanis C, *et al.* (1995) Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch Intern Med* **155**, 381–386.

Hoelzl C, Bichler J, Ferk F, Simic T, Nersesyan A, Elbling L, Ehrlich V, Chakraborty A & Knasmuller S (2005) Methods for the detection of antioxidants which prevent age related diseases: a critical review with particular emphasis on human interventions studies. *J Physiol Pharmacol* **56**, 49–64.

Hollman PCH, Devries JHM, Vanleeuwen SD, Mengelers MJB & Katan MB (1995) Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am J Clin Nutr* **62**, 1276–1282.

Hollman PCH, Van Trijp JMP, Buysman NCP, Gaag MS, Mengelers MJB, de Vries JHM & Katan MB (1997) Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett* **418**, 152–156.

Hrelia P, Tarozzi A & Cantelli Forti G (2003) Diet and risk of cancer. *Lancet* **361**, 258.

Jacobson JS, Melissa DB, Wang LW, Wang Q, Agarwal M, Norkus E, Singh VN, Young TL, Yang D & Santella RM (2000) Effects of a 6-month vitamin intervention on DNA damage in heavy smokers. *Cancer Epidemiol Biomarkers Prev* **9**, 1303–1311.

Kim HY, Kim OH & Sung MK (2003) Effects of phenol-rich diets on blood markers of oxidative stress, and urinary excretion of quercetin and kaempferol in healthy volunteers. *Am Coll Nutr* **22**, 217–223.

Knekt P, Jarvinen R, Reunanen A & Maatela J (1996) Flavonoid intake and coronary mortality in Finland: a cohort study. *Br Med J* **312**, 478–481.

Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, Hakulinen T & Aromaa A (2002) Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* **76**, 560–568.

Ko SH, Choi SW, Ye SK, Cho BL, Kim HS & Chung MH (2005) Comparison of the antioxidant activities of nine different fruits in human plasma. *J Med Food* **8**, 41–46.

Lesgards JF, Durand P, Lassarre M, Stocker P, Lesgards G, Lanteaume A, Prost M & Leucher-Micher MP (2002) Assessment of lifestyle effects on the overall antioxidant capacity of healthy subjects. *Environ Health Perspect* **110**, 479–486.

Lotito SB & Frei B (2004a) The increase in human plasma antioxidant capacity after apple consumption is due to the metabolic effect of fructose on urate, not apple-derived antioxidant flavonoids. *Free Radic Biol Med.* **37**, 251–258.

Lotito SB & Frei B (2004b) Relevance of apple polyphenols as antioxidants in human plasma: contrasting *in vitro* and *in vivo* effects. *Free Radic Biol Med* **36**, 201–211.

Manach C, Williamson G, Morand C, Scalbert A & Rémésy C (2005) Bioavailability and bioefficacy of polyphenols in humans. I Review of 97 bioavailability studies. *Am J Clin Nutr* **81**, 230S–242S.

Moller P & Loft S (2002) Oxidative DNA damage in human white blod cells in dietary antioxidant intervention studies. *Am J Clin Nutr* **76**, 303–310.

Omenn GS, Goodman GD & Hammar S (1996) Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficiency trial. *J Natl Cancer Inst* **88**, 1550–1559.

Peto R, Doll R, Buckley JD & Sporn MB (1981) Can dietary beta-carotene materially reduce human cancer rates? *Nature* **290**, 201–208.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M & Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* **26**, 1231–1237.

Rosenkranz AR, Schmaldienst S, Stuhlmeier KM, Chen W, Knapp W & Zlabinger GJ (1992) A microplate assay for the detection of oxidative products using $2',7'$-dichlorofluorescin-diacetate. *J Immunol Methods* **156**, 39–45.

Scalbert A, Manach C, Morand C & Remesy C (2005) Dietary polyphenols and the prevention of diseases. *Crit Rev Food Science Nutr* **45**, 287–306.

Steinmetz KA & Potter JD (1996) Vegetables, fruit and cancer prevention: a review. *J Am Diet Assoc* **96**, 1027–1039.

Tarozzi A, Marchesi A, Cantell Forti G & Hrelia P (2004) Cold-storage affects antioxidant properties of apples in Caco-2 cells. *J Nutr* **134**, 1105–1109.

Terry P, Terry JB & Wolk A (2001) Fruit and vegetable consumption in the prevention of cancer: an update. *J Intern Med* **250**, 280–290.

Vanzani P, Rossetto M, Rigo A, Vrhovsek U, Mattivi F, D'Amato E & Scarpa M (2005) Major phytochemicals in apple cultivars: contribution to peroxyl radical trapping efficiency. *J Agric Food Chem* **53**, 3377–3882.

WCRF/AICR (1997) *Food, Nutrition and the Prevention of Cancer: A Global Perspective*. Washington, DC: WCRF/AICR, www.who.int/dietphysicalactivity/publications/facts/fruit/en/