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REVIEW

The use of the comet assay in the study of human nutrition and cancer

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The influence of diet on carcinogenesis is a hugely complex area; not only is the consumption of major dietary factors such as meat, fat and fruits and vegetables associated with increased or decreased risk of a range of cancers but also an increasing number of specific nutrients such as vitamins, minerals and phytochemicals are being proposed as the next 'superfoods' to combat the development of cancer. As well as epidemiological studies to determine the association of these dietary factors with cancer risk, it is also essential to investigate the underlying mechanisms through which these factors may causally influence carcinogenesis. The comet assay provides a relatively simple, cheap and rapid method to examine DNA damage and repair and is, therefore, an ideal biomarker for the study of the effects of nutrition on cancer. This review focuses on the use of the comet assay in studies involving human subjects or human cell lines, which investigate the effects of various nutrients on biomarkers relevant to carcinogenesis, and discusses the potential of the comet assay and its various modifications for use as cancer-related biomarkers suitable for use in nutritional studies.

Introduction

With 30–40% of cancers estimated to be caused by dietary factors (1,2), the study of the effects of nutrition on carcinogenesis is of huge importance to public health. Many individual nutrients have been implicated in carcinogenesis or alleged to have protective effects against the development of cancer. However, most of this evidence comes from epidemiological studies, which can only provide evidence of a correlation between certain nutrients/diets and carcinogenesis and not whether there is truly a causal relationship between them. Also, nutritional studies using cancer as an end point generally require huge numbers of subjects and very long time periods to carry out. Therefore, the development and use of intermediate biomarkers of carcinogenesis is most important in elucidating the role of nutrition in carcinogenesis and in revealing the mechanisms involved. The use of the comet assay as one such biomarker has been rapidly expanding in recent years. The standard alkaline comet assay and its various modifications provide a relatively simple, sensitive and rapid method of analysing DNA damage and repair. As DNA damage or failure of repair, leading to mutations, is an important step in carcinogenesis, the comet assay can provide useful information on the molecular effects of diet. The comet assay has several advantages for use as a biomarker in nutritional studies: it is economical, simple and fast; it requires only a small number of cells, which is a huge benefit when working with human samples, particularly cells from biopsies; and it can be modified to detect specific types of DNA damage such as oxidative damage or uracil misincorporation.

This review will focus on human trials using the comet assay as a biomarker relevant to nutritional carcinogenesis. It will also discuss briefly *in vitro* studies using human cell lines.

Antioxidants

The vast majority of nutritional studies carried out to date using the comet assay have focused on antioxidants. Reactive oxygen species are generated in the body through normal metabolism and, if not repaired, can lead to mutations. Epidemiological studies have provided us with strong evidence that the consumption of fruits and vegetables can protect against the development of cancer, and one mechanism through which this protective effect might be exerted is the protection of DNA from oxidative damage by the antioxidants present in fruits and vegetables. Many different chemicals present in plant foods have been shown to act as antioxidants including the carotenoids, flavonoids, non-flavonoid phenolics and vitamins E and C. Oxidative damage has been implicated as playing an important role in carcinogenesis (3) but the role of dietary antioxidants in preventing carcinogenesis is more controversial and requires further investigation (4).

The comet assay can be used to study the effects of antioxidants in several different ways. Firstly, the standard alkaline comet assay can be used to assess basal levels of DNA damage in the form of strand breaks in a cell type. However, this gives no specific information on oxidative damage, and generally, endogenous levels of DNA damage in a cell type will be too low to show small protective effects of dietary components. The most widely used approach, therefore, has been to determine whether the test nutrient protects cells from strand breaks caused by an oxidative damage-inducing agent (most commonly hydrogen peroxide). A better approach, however, is to use lesion-specific enzymes to specifically detect oxidative damage, e.g. endonuclease III, which detects oxidized pyrimidines, or formamidopyrimidine glycosylase (FPG), which detects oxidized purines (5). The use of the comet assay in the study of the effects of antioxidants on DNA damage and repair has recently been reviewed by Wong *et al*. (6), so this review will focus on more recent work. Table I summarizes many of the recent human trials which have taken place examining the effects of various antioxidant dietary factors using the comet assay while Table II summarizes the human trials mentioned in this review involving other nutrients.

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Table I. Comet assay studies examining the effects of antioxidant dietary factors on DNA damage using human subjects

Dietary factor examined	Subjects	Assay type	Results	Reference
Case–control—carotenoids: α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, zeaxanthin	423 Bladder cancer patients, 467 healthy controls	Alkaline on lymphocytes	Increased damage in cases. High damage and low carotenoid intake = highest risk	10
Intervention: 4 mg each β-carotene, lutein, lycopene; 12 mg single carotenoid or placebo for 56 days	37 Healthy post-menopausal women randomly assigned	Alkaline on lymphocytes	Carotenoids reduced damage	11
Cross-sectional: vitamin C, vitamin E and carotenoids	164 Healthy, non-smoking African-Americans and whites	Alkaline on lymphocytes	Inverse association between lycopene and damage. Positive association between vitamin E and damage	12
Intervention: 300 mg/day genistein for 28 days, then 600 mg/day genistein for 56 days	20 Prostate cancer patients	Alkaline on lymphocytes	No effect	32
Intervention: 540 ml green tea	10 Healthy subjects	Alkaline on leukocytes	Green tea reduced UV-induced damage	36
Intervention: 2 × 250 mg vitamin per day (plain or slow release) + 2 × 91 mg vitamin E or placebo; 4 weeks	48 Male smokers	Alkaline and oxidative comet on mononuclear cells	Antioxidants reduced oxidative damage	38
Intervention: single dose vitamin C (500 mg) and vitamin E (400 IU)	12 Healthy volunteers	Alkaline on lymphocytes	No effect	39
Intervention: 400 mg alpha-lipoic acid, 200 mg co-enzyme Q10, 12 mg manganese, 600 mg vitamin C, 800 mg *N*-acetyl cysteine, 400 μg selenium, and 400 IU α-tocopherol per day; 7 days	14 Healthy subjects	Alkaline on lymphocytes	Exercise increases damage but antioxidants have no effect on this damage	40
Intervention: vitamin C (2 × 500 mg/day) and vitamin E (2 × 100 mg/day); 12 weeks	100 Patients on long-term proton pump inhibitors	Alkaline on oesophageal cells	No effect	41
Cross-sectional: vitamin C, α- and γ-tocopherol, carotenoids, zinc, copper and selenium	11 Males	Oxidative on lymphocytes	Damage decreased in summer. Selenium, vitamin C and lycopene correlate negatively with damage; zinc correlates positively	45
Cross-sectional: selenium	43 Subjects at high risk for prostate cancer	Alkaline on leukocytes	Lower selenium levels correlate negatively with damage	46
Intervention: one glass of red wine, dealcoholized red wine or water; or one glass daily for 6 weeks	74 Healthy subjects	Alkaline in leukocytes	Regular red wine consumption decreased endogenous damage but not H_2O_2-induced damage	54
Intervention: meal of fried onions or meal of fried onions and cherry tomatoes	Six healthy subjects	Alkaline and oxidative on lymphocytes	Onions decreased damage; onions and tomatoes decreased oxidative damage	55
Intervention: 500 ml kiwifruit juice	Six healthy subjects	Alkaline and oxidative on lymphocytes	No effect on basal damage. Reduced H_2O_2-induced oxidative damage	56
Intervention: One, two or three kiwifruits per day; 3 weeks	14 Healthy subjects	Alkaline and oxidative on lymphocytes	Reduced basal and oxidative damage	57
Intervention: 113 g cruciferous and legume sprouts; 14 days	20 Healthy subjects	Alkaline on lymphocytes	Decreased H_2O_2-induced damage	58
Intervention: 85 g raw watercress; 8 weeks	30 Smokers and 30 non-smokers	Alkaline and oxidative on lymphocytes	Decreased basal, oxidative and H_2O_2-induced damage	59
Intervention: bread + inulin, linseed and soy flours ± selenium-rich wheat, tomato extract, green tea and spice extract; 5 weeks	38 Healthy subjects	Alkaline and oxidative on buccal and peripheral lymphocytes	Bread decreased damage in smokers	60
Intervention: single dose 300 ml blood orange juice or 300 ml juice + 150 mg vitamin C or placebo	Seven healthy subjects	Alkaline on mononuclear cells	Orange juice decreased H_2O_2-induced damage; vitamin C had no effect	43
Intervention: 240 ml green vegetable drink per day; 8 weeks	20 Smokers	Alkaline on lymphocytes	Decreased basal damage	61
Intervention: 3 oz or 6 oz almonds per day; 4 weeks	30 Smokers	Alkaline on lymphocytes	Decreased basal damage	62
Intervention: 1 litre unfiltered coffee per day; 5 days	10 Healthy subjects	Alkaline on lymphocytes	Coffee decreased BPDE-induced damage	63
Intervention: 700 ml red mixed berry juice or polyphenol depleted juice; 4 weeks	18 Healthy male probands	Oxidative on lymphocytes	Red berry juice decreased oxidative damage	64
Intervention: blueberry/apple juice; 4 weeks	Eight healthy female subjects	Alkaline on lymphocytes	Juice decreased H_2O_2-induced damage	23
Intervention: 25 g tomato puree per day; 14 days	Nine healthy female subjects	Alkaline on lymphocytes	Decreased H_2O_2-induced damage	65
Intervention: 250 ml tomato drink/day; 26 days	26 Healthy subjects	Alkaline on lymphocytes	Decreased H_2O_2-induced damage	66
Intervention: one tomato product/day (puree, sauce or raw tomatoes); 3 weeks	12 Healthy subjects	Alkaline on lymphocytes	Decreased Fe^{2+}-induced damage	67
Intervention: 250 ml tomato drink/day; 26 days	26 Healthy subjects	Alkaline on lymphocytes	No effect	68
Intervention: 600 g fruits and vegetables/day or supplement containing equivalent vitamins and minerals or placebo; 24 days	43 Healthy subjects	Alkaline and oxidative on mononuclear blood cells	No effect	69

Table II. Comet assay studies examining the effects of other dietary factors on DNA damage using human subjects

Dietary factor examined	Subjects	Assay type	Results	Reference
Case–control: folate	64 Breast cancer patients, 30 controls with benign breast disease	Alkaline and oxidative on mononuclear blood cells	Increased basal and oxidative damage in cases. Red cell folate correlated negatively with oxidative damage	102
Intervention: 400 μg folate/day; 4 weeks	19 Healthy females	Alkaline and uracil misincorporation comet on leukocytes	Folate only decreased uracil misincorporation when vitamin B12 was also high. No correlation between serum folate and basal damage	103
Intervention: human milk or cows milk; 9–12 months	70 Healthy infants; 35 per group	Alkaline on lymphocytes	Human milk led to less basal damage than cows milk	109
Intervention: dairy-free versus dairy-rich diet; 1 week	18 Healthy males and females	Faecal water genotoxicity on colon adenocarcinoma cells	No difference in either diet	112
Intervention: Low meat, fat and sugar, high wholemeal and vegetables diet versus high meat, fat and sugar, low wholemeal and vegetables diet; 12 days	Seven healthy subjects	Faecal water genotoxicity on colon adenocarcinoma cells	High meat, fat and sugar, low wholemeal and vegetables diet increased faecal water-induced damage	113
Intervention: diets with varying amounts of meat, protein, heme and iron; 10, 13 or 15 days	21 Healthy subjects	Faecal water genotoxicity on colon adenocarcinoma cells	No effect of different diets	114
Intervention: probiotic versus normal yogurt; 7 weeks	Nine healthy females	Faecal water genotoxicity (alkaline and oxidative) on colon adenocarcinoma cells	Probiotic decreased basal damage caused by faecal water but increased oxidative damage. Overall damage was reduced	118

Carotenoids

There are over 600 known carotenoids, including compounds such as lutein, alpha-carotene and beta-carotene and lycopene; they are commonly found in many red, yellow and orange fruits and vegetables. Carotenoids are only synthesized in microorganisms and plants, where they are involved in photosynthesis (7). They are important dietary sources of vitamin A (8). Epidemiological studies have indicated that the carotenoids may play a protective role against carcinogenesis, particularly in lung cancer (9); however, there is still a lack of definitive mechanistic evidence. Several human studies have used the comet assay as a biomarker to look at the effects of various carotenoids on DNA damage and repair. Schabath *et al*. (10) carried out a case–control study looking at levels of carotenoids and endogenous DNA damage in lymphocytes of bladder cancer patients and healthy controls. They found an increase in DNA damage in cases, with high DNA damage combined with low carotenoid intake being associated with the highest risk. Zhao *et al*. (11) also used the alkaline comet assay to look at endogenous DNA damage in their intervention study, involving 37 healthy post-menopausal women supplemented with carotenoids (lutein, lycopene and beta-carotene). This study showed a decrease in DNA damage with carotenoid supplementation. Watters *et al*. (12) found an inverse association between DNA damage in the lymphocytes of 164 healthy subjects and the carotenoid lycopene, using the alkaline comet assay.

In vitro studies looking at the effects of carotenoids on DNA repair have examined a range of human cell types including leukaemia cells [where beta-carotene decreased peroxynitrous acid-induced damage (13)], hepatoma cells [lycopene decreased oxidative lesions (14)], melanocytes (lycopene caused a reduction in UVA-induced damage (15)) and neuroblastoma cells [lutein and zeaxanthin increased UVA-induced DNA damage (16)].

Flavonoids

Flavonoids include plant-derived compounds such as quercetin, epicatechin and kaempferol and are found in a wide variety of foodstuffs including fruits, vegetables, tea and red wine. They are polyphenolic compounds and have a wide variety of biological effects including a strong antioxidant capacity (17). Interest in the potential anticarcinogenic properties of flavonoids has grown in recent years with a number of epidemiological studies suggesting that they may be protective against lung cancer in particular (reviewed in ref. 17). The strongest evidence for a cancer-preventive effect of flavonoids comes from the study of quercetin, which is found in a variety of foods including onions, apples, tea and wine. Most comet assay data on quercetin suggests that it protects against oxidative damage in a variety of cell types—lymphocytes (18–24) colon, liver and epithelial cells (25), melanoma cells (26), macrophages (27) and hepatoma cells (28). There is also evidence from *in vitro* studies using the comet assay that other flavonoids are protective against DNA damage; Horvathova *et al*. (26) found that luteolin protects against hydrogen peroxide-induced damage in melanoma cells; Kanupriya *et al*. (27) showed that catechin and epicatechin reduce tert-butylhydroperoxide damage in macrophages, and luteolin was found to reduce tert-butylhydroperoxide-induced strand breaks in hepatoma cells by Lima *et al*. (28) and hydrogen peroxide-induced strand breaks in Jurkat cells by Melidou *et al*. (21).

The soy isoflavones, and in particular genistein and daidzein, have also been reported to prevent certain types of cancer (29). Magee *et al*. (30) investigated the effect of equol, a product of the soy isoflavone, daidzein, on 2-hydroxy-2-nonenal and menadione damage in human breast cells using the comet assay and found that it reduced DNA damage. Genistein was shown to reduce hydrogen peroxide-induced damage in human prostate cells (31). However, in a trial on 20 prostate cancer

patients, Miltyk *et al*. (32) found that genistein had no effect on strand breaks in lymphocytes, and Pool-Zobel *et al*. (33) showed that genistein caused increased DNA strand breaks in the human tumour cell line, HT29.

Teas, and especially green tea, are another important source of flavonoids. Epigallocatechin3-gallate (ECGC), extracted from green tea, has been investigated in several studies using the comet assay but the results are equivocal. Erba *et al*. (34) showed that green tea extract containing approximately 15 μM ECGC decreased oxidative lesions and strand breaks caused by bleomycin in human lymphocytes, but Elbling *et al*. (35) found that higher levels of ECGC (>20 μM) and green tea extracts actually increased DNA damage in human leukaemia cells. Johnson *et al*. (20) treated human lymphocytes with ECGC and found that low concentrations (10 μM) decreased hydrogen peroxide and 3-morpholinosydnonimine (SIN-1, a peroxynitrite generator) generated strand breaks while higher concentrations (100 μM) increased damage. It appears, therefore, that ECGC is most effective at protecting against oxidative DNA damage at concentrations of 10–15 μM with higher concentrations actually introducing strand breaks.

Studies looking at green tea itself have been more promising with green tea being shown to protect human leukaemia cells against strand breaks caused by Fe^{2+} (34) and to reduce UVA-induced damage to lymphocytes extracted from 10 healthy subjects after drinking 540 ml of green tea (36). Green coffee extracts were also shown to reduce peroxide-mediated damage in human colon and liver cells (37).

Vitamins C and E, selenium and other antioxidants

The antioxidant vitamins C and E have been investigated in a number of trials using the comet assay as a biomarker. Moller *et al*. (38) showed a protective effect of 2 × 250 mg vitamin C daily for 4 weeks against the development of oxidative lesions in the mononuclear blood cells of 48 male smokers. Choi *et al*. (39) supplemented 12 healthy volunteers with single doses of 500 mg vitamin C and 400 IU vitamin E and studied DNA damage in their lymphocytes but found no effect. Davison *et al*. (40) also found that a range of antioxidants including vitamin C (in combination with alpha-lipoic acid, co-enzyme Q10, manganese, *N*-acetyl cysteine, selenium and alpha tocopherol) had no protective effect against DNA damage, this time in lymphocytes of healthy subjects made to undergo exercise, which was shown to increase strand breaks. In another human trial, 100 patients undergoing surveillance for Barrett's oesophagus on long-term proton pump inhibitors were supplemented with 2 × 500 mg vitamin C and 2 × 100 mg vitamin E per day for 4 weeks (41). This study also showed no effect of vitamin C and E supplementation on strand breaks. Smit *et al*. (15) looked at the effects of the vitamins C and E in an *in vitro* trial and showed that both vitamins reduced UVA-induced damage in melanocytes.

In *in vitro* studies, Harreus *et al*. (42) did show a protective effect of vitamin C and zinc against hydrogen peroxide-induced damage in lymphocytes; but vitamin C alone was not sufficient to prevent strand breaks caused by peroxide in mononuclear blood cells (43). Vitamin C was shown to protect against benzenetriol-induced damage in lymphocytes (44) and against peroxide-mediated damage in lymphoma cells (22), but in this study, it was less effective than a range of flavonoids tested.

The antioxidant capacity of the mineral selenium has also been studied using the comet assay. Dusinska *et al*. (45) looked at seasonal variations in antioxidant levels and oxidative lesions in 11 Slovakian males and showed that selenium, vitamin C and lycopene correlated negatively with both strand breaks and oxidative damage whereas zinc showed a positive correlation. Selenium levels and leukocyte DNA damage were also assessed in 43 subjects identified as being at high risk of prostate cancer by Karunasinghe *et al*. (46) and lower selenium levels were found to correlate negatively with DNA damage. Emonet-Piccardi *et al*. (47) showed that *N*-acetyl cysteine, selenium and zinc caused a reduction in UVA-induced damage in human fibroblasts whereas Rafferty *et al*. (48) found that selenium did not affect cyclobutane pyrimidine dimer (CPD) formation (measured by combining the comet assay with the CPD specific enzyme, T4 endonuclease VII) or repair excision (measured by combining the comet assay with repair inhibitors) after UV irradiation but did decrease UV-induced oxidative lesions in primary keratinocytes. Seo *et al*. (49) also demonstrated that selenium in the form of selenomethionine decreased UV-induced strand breaks in human fibroblasts. However, the results for selenium are complicated by the fact that many selenium compounds (including sodium selenite, sodium selenate and selenous acid) themselves have been found through studies using the comet assay to have a genotoxic effect (50–52).

Other compounds with known antioxidant capacity have also been tested using the comet assay. The polyphenol gallic acid (extracted from the *Pistacia lentiscus* fruit), demonstrated a protective effect against hydrogen peroxide-induced DNA damage in erythroleukaemia cells (53).

Other micronutrients

Folate

The B vitamin folate is known to be involved in a wide variety of reactions within the cell, including the methylation of DNA, proteins and lipids and the synthesis of nucleotides. Reduced folate levels in the cell are believed to lead to several consequences which may have implications for the development of tumours including reducing DNA methylation, which may interfere with gene expression, and increasing the misincorporation of uracil into DNA, leading to DNA instability. Suboptimal folate levels have been implicated in the development of a range of cancers, including colorectal and breast (54). A small number of trials have used the comet assay as a biomarker to study the effects of folate on carcinogenesis. The development by Duthie *et al*. (55) of a modification of the comet assay, using the enzyme uracil glycosylase to study the misincorporation of uracil into DNA, and by Wasson *et al*. (56) of a further variation on the comet assay protocol, using methylation-sensitive restriction enzymes to assess global and gene-specific DNA methylation, should allow further research into the specific molecular effects of suboptimal folate levels.

Hussein *et al*. (57) used the standard alkaline comet assay, as well as the comet assay incorporating the oxidative lesion-specific enzymes FPG and endonuclease III, in a case–control study to examine DNA damage in the mononuclear blood cells of 64 breast cancer patients and 30 control subjects with benign breast disease. A range of folate-related measurements were also examined. The results showed an increase in both endogenous and oxidative damage in cases, with red cell folate correlating negatively with oxidative damage. Kapiszweska *et al*. (58) used the uracil misincorporation-sensitive comet

assay to investigate the effects of a 4-week folate supplementation (400 μg/day) trial on 19 healthy females. They found that folate supplementation only reduced uracil misincorporation into DNA when vitamin B12 levels were also high (i.e. above 400 pg/ml), suggesting that adequate levels of both vitamin B12 and folate are essential to prevent the imbalance in deoxyribonucleotide pools that leads to the misincorporation of uracil. They also found no correlation between serum folate levels and strand breaks measured using the standard alkaline comet assay. In contrast, Duthie *et al*. have used standard and oxidative lesion-specific comet assays to show increased DNA damage in HeLa cells and lymphocytes and decreased hydrogen peroxide damage repair after folate depletion (55, 59). They have also used the uracil misincorporation-sensitive comet assay to show increased uracil misincorporation in folate-deprived lymphocytes (55, 59). Courtemanche *et al*. (60) also showed that folate deficiency leads to increased DNA damage in primary lymphocytes and Wasson *et al*. (56) found, using the methylation-sensitive comet assay, that folate deprivation induced both global and p53 gene region-specific DNA hypomethylation in human colon carcinoma cells.

Whole foods

The comet assay has also been used to study the effects of a large range of whole foods known or thought to be rich in antioxidants. Arendt *et al*. (61) carried out a study on the effects of red wine consumption in 74 healthy subjects. Red wine is a rich source of flavonoids, and in this study, regular red wine consumption was shown to reduce the number of endogenous strand breaks, but had no effect on hydrogen peroxide-induced damage. The effects of the flavanoid-rich foods, onions and tomatoes, on endogenous and oxidative DNA damage were studied in the lymphocytes of six female subjects by Boyle *et al*. (62). Onions were shown to cause a decrease in endogenous DNA damage, and onions and tomatoes given together to decrease oxidative damage. Collins *et al*. have investigated the effect of kiwifruit and kiwifruit juice on strand breaks and oxidative lesions in lymphocytes from healthy volunteers. Both kiwifruit and kiwifruit juice were shown to prevent the development of oxidative lesions, with kiwifruit also decreasing basal levels of DNA damage (63, 64). Gill *et al*. showed that supplementing the diets of healthy men and women with either cruciferous and legume sprouts (65) or watercress (66) led to a decrease in hydrogen peroxide-induced damage in lymphocytes. Glei *et al*. (67) looked at the effect of bread supplemented with the prebiotics inulin, linseed and soy flours, and the antioxidant ingredients selenium-rich wheat, tomato extracts, green tea and spice extract, and showed that it reduced DNA damage in smokers. Orange juice was shown to be more effective than vitamin C in protecting mononuclear blood cells of seven healthy subjects from peroxide-induced damage (43). An 8-week supplementation with a green vegetable drink was shown to reduce endogenous DNA strand breaks in the lymphocytes of 20 smokers (68) and almonds were found to have a protective effect against strand breaks in lymphocytes of 30 healthy males receiving 3 oz or 6 oz of almonds a day for 4 weeks (69). Unfiltered coffee was shown to reduce benzo(a)pyreme diol epoxide (BPDE)-induced damage in lymphocytes in a study on 10 participants consuming 1 litre unfiltered coffee per day over 5 days (70). A mixed red berry juice high in flavonoids was found to protect against the development of oxidative lesions in 18 healthy male probands (71), while a blueberry/apple juice reduced peroxide-induced damage in the lymphocytes of healthy female volunteers (23). Porrini *et al*. found that both tomato puree (72) and a tomato drink (73) (both rich in carotenoids) decreased peroxide-mediated damage in the lymphocytes of healthy volunteers, while tomato products were also shown to protect against Fe^{2+}-mediated oxidative damage in the lymphocytes of 12 healthy females by Riso *et al*. (74). In contrast, Riso *et al*. (75) also showed that a tomato-based drink consumed by 26 healthy subjects for 26 days had no effect on strand breaks in lymphocytes and Moller *et al*. (76) showed no effect on strand breaks or oxidative damage of either a diet high in fruits and vegetables or supplemented with the corresponding vitamins and minerals, in the mononuclear blood cells of 43 healthy volunteers.

Other foodstuffs shown to have a protective effect against DNA damage in *in vitro* studies include garlic organosulphur compounds [against a range of damaging agents including hydrogen peroxide in hepatoma cells (77)], olive oil phenol derivatives [hydrogen peroxide-induced damage in whole blood cells (78)], mustard sprouts [benzo(*a*)pyrene-mediated damage to hepatoma cells (79)], apple phenolics [endogenous strand breaks in colon cancer cell lines (80)] and reconstituted mixtures of apple juice phenolics [menadione-induced oxidative lesions (81)]. Extracts of the herbs rosemary, oregano, sage and Echinacea decreased strand breaks caused by hydrogen peroxide in human colon cells (82) and O'Brien *et al*. (83) showed that grapeseed polyphenols and bearberry (*Arctostaphylos uva-ursi*) reduced hydrogen peroxide and tert-butylhydroperoxide-induced damage in lymphoma cells. Infrared-irradiated rice hull (84), brown seaweed extracts (85), cherry blossom extracts (86), South African herbal extracts (87), mushroom extracts (88) and electrolyzed-reduced water (89) were all shown to protect against hydrogen peroxide-induced damage in lymphocytes. However, Sage (*Salvia officinalis*) extracts were shown to have no effect on tert-butylhydroperoxide damage in hepatoma cells (90).

Although the majority of studies have found a protective effect of antioxidants against DNA damage, there have been a number of studies which have shown that higher concentrations of antioxidants may actually have a pro-oxidant effect, causing or exacerbating DNA damage. Vitamin C and caffeic acid have both been shown to increase strand breaks in lymphocytes (91, 92). Vitamin C and zinc were also shown to increase hydrogen peroxide-induced damage in lymphocytes at high doses, while being protective at low doses (42). Resveratrol, a flavonoid commonly found in grapes and wine, has been shown by comet assay to cause strand breaks at higher concentrations (93) or in conjunction with Cu(II) (94). Blasiak *et al*. (95) studied the effects of the vitamins C and E and amifostine on DNA damage in lymphocytes and found that whereas amifostine and vitamin C decreased damage caused by idarubicin, vitamin E increased it. The flavourings vanillin and cinnamaldehyde induced strand breaks in human colon cancer cells (96) and the spice curcumin, known to be an antioxidant, had no effect on damage in human hepatoma cells at low doses but increased strand breaks at higher doses (97). A similar trend was also found with olive oil-related compounds with low doses protecting against hydrogen peroxide-induced damage but higher doses causing strand breaks (98). Szeto *et al*. (99) showed that extracts of fruits and vegetables used to treat human lymphocytes were protective against endogenous DNA damage and peroxide-induced damage at lower concentrations, but this protective effect disappeared with higher concentration. As worrying as these results appear, most of the

higher concentrations used in these studies would be unlikely to be reached through normal dietary intake of antioxidants. However, it demonstrates the need for further testing of these compounds at a range of doses before making recommendations for their consumption as cancer-protective agents.

Cooked meat

Another area of nutrition, which has been studied using the comet assay as a biomarker, is the influence of cooked meat on the risk of cancer. When meat is cooked to medium and well-done states, a group of chemicals known as heterocyclic amines are formed. These chemicals have been shown to be bacterial mutagens and rodent carcinogens, and epidemiological studies have suggested that the consumption of well-done meat products may be carcinogenic in humans also (100).

Using the comet assay, a number of studies have demonstrated that heterocyclic amines and polycyclic aromatic hydrocarbons increase the levels of endogenous strand breaks in a variety of human cell types including lymphoblasts (101, 102), breast cells (103) and prostate cells (104). Wilson *et al.* (103) showed that this genotoxic effect of the heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), can be reduced using diallyl sulphide, a component of garlic and onions. Aydin *et al.* (105) looked at the effect of thyme oil volatiles on the DNA damage induced by the heterocyclic amine, 2-amino-3-methylimidazo[4,5-f]-quinoline (IQ) and found it to be protective against IQ-induced strand breaks in human lymphocytes. Comet assay studies on heterocyclic amines, therefore, confirm their genotoxicity in human cells and suggest that other nutritional compounds may provide a protective effect against the mutagenic effects of cooked meats.

Breast milk

A surprising discovery has arisen from a set of studies using the comet assay to examine the effects of human breast milk on DNA damage. Martin *et al.* have shown, using the alkaline comet assay and the comet assay combined with the repair inhibitors hydroxyurea and cytosine arabinoside (araC), that some human breast milk extracts can cause strand breaks in both lymphoblasts and exfoliated breast cells extracted from breast milk (102,106,107). This genotoxic effect of some breast milk extracts was found to be consistent throughout the breast-feeding period [i.e. from within 4 weeks of birth to more than 4 months after birth, (106)] and to be more common in the UK versus Singapore, India and Hong Kong (107). The nature of the agents responsible for this apparent genotoxicity is unknown although the authors note that potentially mutagenic substances such as organochlorines and aromatic amines have been detected in breast milk (102). Martin *et al.* have also shown that exfoliated breast cells extracted from breast milk can be used with the comet assay to examine the DNA damaging potential of other potential genotoxins (106). The heterocyclic amines 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2) were shown to increase DNA damage in breast cells (106). This study also looked at the effects of the polycyclic aromatic hydrocarbon benzo[*a*]pyrene (B[*a*]P); a nitro-polycyclic aromatic hydrocarbon, 1-nitropyrene (1-NP); and the aromatic amines *o*-toluidine and *p*-chloroaniline and found that they all caused an increase in strand breaks in exfoliated breast cells (106). The oestrogens, beta-oestradiol, oestrone and oestriol, were also shown to induce strand breaks in both breast cells and lymphocytes (108). A study by Dundaroz *et al.* (109) examining DNA damage in the lymphocytes of infants fed either cows milk (n = 35 infants) or human breast milk (n = 35) concluded that there were less strand breaks in lymphocytes from babies fed human milk.

Faecal water studies

There is considerable evidence of a strong dietary basis to many cases of colorectal cancer (1) with diets containing high concentrations of fat and animal proteins and low amounts of fibre, fruits and vegetables considered to be high risk (110). The development of a method to examine the genotoxicity of faecal water using the comet assay has offered a promising and non-invasive biomarker to study the effects of various potential nutritional risk factors and anti-cancer dietary components for colorectal carcinogenesis. The aqueous phase of faeces (faecal water) is prepared by high-speed centrifugation of faecal samples and has been shown to contain biologically active substances that are cytotoxic to mammalian cells (111). By carrying out dietary interventions on human subjects, extracting their faecal water and using this faecal water as a damaging agent to treat cells in the comet assay, it is possible to determine whether various nutritional factors increase the genotoxicity of faeces, thereby potentially increasing the risk of colorectal cancer.

Glinghammer *et al.* (112) carried out one of the first intervention trials using the comet assay and faecal water as a biomarker. In this study, 18 healthy male and female volunteers were put on either a dairy-rich or dairy-free diet for 1 week each. Faecal water genotoxicity was examined in colon adenocarcinoma cells but no significant difference in either diet was found. Rieger *et al.* (113) looked at the effects of a high meat, fat and sugar, low wholemeal and vegetables diet versus a low meat, fat and sugar, high wholemeal and vegetables diet on the genotoxicity of faecal water in seven healthy subjects. The high meat, fat and sugar diet was shown to increase the amount of damage induced by faecal water in human colon adenocarcinoma cells. However, Cross *et al.* (114) showed no effect on faecal water genotoxicity of a diet rich in meat, protein, heme and iron in two studies on a total of 21 individuals.

As well as examining the effects of large dietary changes, the comet assay and faecal water method have also been used to look at the effects of probiotics and prebiotics in intervention trials and *in vitro* studies. Probiotics are dietary supplements containing potentially beneficial bacteria, usually of the lactic acid-producing bacteria type, and are hypothesized to have beneficial effects by improving the intestinal microflora balance (115). Prebiotics have been defined as 'non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health' (116). Probiotics and prebiotics are believed to have anticarcinogenic effects in the colon (117), potentially through the protection of the colonic mucosa from DNA damage. The genotoxicity of faecal water to colon adenocarcinoma cells after dietary intervention with either a standard yogurt or a probiotic yogurt was examined in nine healthy volunteers (118). Endogenous strand breaks and

oxidative lesions were also investigated by comet assay in this study. Intervention with the probiotic yogurt, containing the strains *Lactobacillus acidophilus* 145 and *Bifidobacterium longum* 913, significantly lowered faecal water genotoxicity compared to standard yogurt but increased oxidative damage (although overall, damage was reduced) (118). Glei *et al*. (67) carried out an intervention using bread supplemented with prebiotics and antioxidants on 38 healthy males (smokers and non-smokers) and examined both endogenous DNA damage in lymphocytes and faecal water genotoxicity to colon cells. The bread intervention was shown to decrease lymphocyte DNA damage in smokers but not non-smokers and to decrease faecal water genotoxicity in non-smokers but not smokers. Therefore, smokers and non-smokers appear to be affected differently from intervention with the bread, but in both cases, it had a beneficial effect (67). An *in vitro* study by Burns *et al*. (117) also showed a beneficial effect of probiotics and prebiotics on faecal water genotoxicity. Faecal water from a human subject was incubated with six strains of lactic acid-producing bacteria and the resultant samples were used to treat HT29 human adenocarcinoma cells. Five of the six strains showed a decrease in strand breaks (with *Streptococcus thermophilus* being the exception). In a second study, the HT29 cells were treated with the supernatants of probiotic cultures incubated with various prebiotics. The cells were then subjected to faecal water, and strand breaks were measured using the comet assay. The combination of the prebiotic bacteria *Lactobacillus plantarum* with the fructooligosaccharide-based prebiotics Inulin, Raftiline, Raftilose and Actiligh were found to be the most effective in reducing DNA damage. These initial studies indicate that probiotics and prebiotics may be effective at reducing DNA damage to the colonic mucosa, but more studies are required before any definitive conclusions can be drawn.

Parikka *et al*. (119) have also used the comet assay to look at faecal water genotoxicity and peroxide-induced damage in human colon cells exposed to 5-n-alkylresorcinols, phenolic lipids found in cereal grains. They found that these phytochemicals reduced both faecal water genotoxicity and strand breaks caused by hydrogen peroxide.

Conclusions

The interaction between diet and risk of cancer is a hugely important research area, and even a brief review of the literature indicates the thousands of foodstuffs, micronutrients, phytochemicals and other dietary factors being investigated for their effect on carcinogenesis. Yet in many cases, the molecular mechanisms through which various nutrients might enhance or protect against carcinogenesis are still unknown. In this respect, the development and optimization of biomarkers suitable for use to investigate the molecular effects of dietary factors in human trials, animal studies and *in vitro* studies is of great importance. One such biomarker that has been emerging in importance over recent years is the comet assay.

From the studies outlined above, it is clear that the most common use of the comet assay in nutritional studies has been the investigation of oxidative damage, generally through studying the protective or exacerbating effects of various dietary components on damage induced in cell lines by hydrogen peroxide. Probably due to this, there have been a number of reviews published previously dealing with the use of the comet assay to study the effects of antioxidants on DNA damage (6,120–124).

The comet assay allows the study of the effects of nutrients of which anti- or pro-oxidant capacities are already known on different cell types and in different concentrations. In fact, these studies have revealed a seeming paradox, or at least a hormetic effect, in the effects of many of these known antioxidant compounds, in that they seem to protect against DNA damage at low doses while actually causing DNA damage at higher doses. The comet assay also allows the rapid screening of large numbers of compounds to investigate their antioxidant potential. Further modification of the assay by the incorporation of oxidative lesion-specific enzymes can provide more information on the exact nature of the DNA damage being induced or protected against and increase the specificity of the assay.

Other enhancements to the specificity of the assay include the use of the enzymes uracil glycosylase [to assess uracil misincorporation (100)], uvrABC [to detect DNA damage on bulky lesions (5)], methyladenine DNA glycosylase II [to identify 3-methyladenine sites (125)] and methylation-sensitive restriction enzymes [to assess epigenetic modification by DNA methylation (56)]. The comet assay can also be used to determine replicative integrity, a known factor in carcinogenesis, through labelling newly replicated DNA with bromodeoxyuridine (BrdUrd) and visualizing it in the comet with an anti-BrdUrd antibody (126,127). The combination of the comet assay with fluorescent *in situ* hybridization (comet-FISH) also offers an opportunity to increase the specificity of the assay, allowing the investigation of gene region-specific DNA damage and repair (128,129). As the standard comet assay reveals only strand breaks and alkali-labile sites, types of DNA damage which are generally easily repaired and therefore perhaps less biologically important in the development of carcinogenesis, a more widespread use of these modified comet assays should be encouraged.

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