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ORIGINAL CONTRIBUTION

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DNA stability and lipid peroxidation in vitamin E-deficient rats *in vivo* and colon cells *in vitro*

Modulation by the dietary anthocyanin, cyanidin-3-glycoside

Summary *Background* Fruit and vegetable consumption protects against cancer. This is attributed in part to antioxidants such as vitamin E combating oxidative DNA damage. Anthocyanins are found in significant concentrations in the human diet. However, it remains to be established whether they are bioactive *in vivo*. *Aim* To investigate the consequence both of vitamin E deficiency on oxidative damage to DNA and lipids and the cytoprotective effect of nutritionally relevant levels of cyanidin-3-glycoside both *in vivo* in rats and *in vitro* in human colonocytes. *Methods* Male Rowett Hooded Lister rats were fed a diet containing less than 0.5 mg/kg vitamin E or a vitamin E supplemented control diet containing 100 mg dα-tocopherol acetate/kg. Half of the controls and vitamin E-deficient rats received cyanidin-3-glycoside (100 mg/kg). After 12 weeks endogenous DNA stability in rat lymphocytes (strand breaks and oxidised bases) and response to oxidative stress *ex vivo* (H_2O_2; 200 μM) was measured by single cell gel electrophoresis (SCGE). Tissue levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-Oxo-dG) were measured by HPLC with EC detection. Dα-tocopherol and lipid peroxidation products (thiobarbituric acid reactive substances; TBARS) were measured by HPLC. Rat plasma pyruvate kinase and the production of reactive oxygen by phagocytes were detected spectrophotometrically and by flow cytometry respectively. Immortalised human colon epithelial cells (HCEC) were preincubated *in vitro* with the anthocyanins cyanidin and cyanidin-3-glycoside and the flavonol quercetin (all 50 μM) before exposure to H_2O_2 (200 μM). DNA damage was measured by SCGE as above. *Results* Plasma and liver dα-tocopherol declined progressively over 12 weeks in rats made vitamin E deficient. Lipid peroxidation was increased significantly in plasma, liver and red cells. Reactive oxygen levels in phagocytes and plasma pyruvate kinase were increased. Vitamin E deficiency did not affect DNA stability in rat lymphocytes, liver or colon. Cyanidin-3-glycoside did not alter lipid peroxidation or DNA damage in rats. However, it was chemoprotective against DNA damage in human colonocytes. DNA strand breakage was decreased 38.8 ± 2.2 % after pretreatment with anthocyanin. *Conclusion* while it is accepted that vitamin E alters lipid oxidation *in vivo*, its role in maintaining DNA stability remains unclear. Moreover, whereas cyanidin-3-glycoside protects against oxidative DNA damage *in vitro*, at nutritionally relevant concentrations it is ineffective against oxidative stress *in vivo*.

Key words cyanidin-3-glycoside – vitamin E deficiency – DNA stability – lipid peroxides – rat

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Introduction

There is convincing population-based evidence that a high intake of fruit and vegetables decreases the risk of death from diseases where oxidative stress is believed to be a factor. Individuals in the highest quartile for fruit and vegetable intake are afforded significant protection against pathologies such as heart disease, cancer and other age-related degenerative diseases compared with matched individuals in the lowest quartile of consumption [1, 2]. This beneficial effect against specific diseases is ascribed in part to antioxidants in these foods that act either directly or indirectly to reduce oxidative damage to structures such as membranes, lipids and DNA, although the role of oxidative DNA damage as a precursor for cancer remains contentious [3]. Conventional antioxidants, such as vitamin C, E and carotenoids do appear to protect against vascular diseases such as coronary artery disease and stroke [3]. However, little credible evidence exists for a similar protection for these individual dietary components against cancer [3]. Attention, therefore, has turned to other potential cytoprotective micronutrients in the human diet, such as the polyphenols. Many of these compounds are present in food in significant quantities and are potent antioxidants *in vitro* [4].

One class of polyphenols, the anthocyanins, is found in antioxidant-rich soft fruits, such as raspberries, blackberries and cranberries and in certain vegetables and beverages [5]. Moreover, they are used increasingly in the food industry as natural colorants. It has been estimated that daily intake of anthocyanins (approx. 200 mg/day) may be comparable with that of the more familiar nutritional antioxidants [5]. Anthocyanins and anthocyanin-rich extracts exhibit a broad spectrum of antioxidant activity in chemical and cellular systems when assessed against differing targets for oxidative damage [6–9]. For example, an anthocyanin-rich extract from black rice, comprising cyanidin 3-glucoside and peonidin 3-glucoside, suppresses peroxyl and hydroxyl radical induced supercoiled DNA strand scission, reduces oxidative modification of low density lipoprotein and inhibits nitric oxide production in murine macrophages [6]. In addition, certain anthocyanins are effective at suppressing cancer cell growth *in vitro* by modification of cell signal pathway activity [10, 11]. However, little is understood about the bioavailability of anthocyanins and whether they can act as protective agents against oxidative damage *in vivo*.

This study investigates primarily the consequence of vitamin E deficiency (as a model of oxidative stress) on several biomarkers of DNA and lipid damage in rats *in vivo* and the potential cytoprotective effect of nutritionally relevant levels of the dietary anthocyanin, cyanidin-3-glycoside, to modulate endogenous and induced oxidative damage in this model system. In addition, to investigate further the mechanisms through which cyanidin-3-glycoside acts as a potential genoprotectant, its ability to modulate DNA stability *in vitro* was determined in normal human colon cells.

Materials and methods

Routine culture of HCEC

The human colon epithelial cells (HCEC) were a gift from Dr E. Offord at Nestle (Lausanne, Switzerland). This cell line (immortalised by SV40 T antigen transformation) retains several intestine-specific characteristics and functions and has been used previously to investigate the impact of nutrition on genomic stability [12]. HCEC were maintained in A52 medium [Biofluids, Rockville, MD, USA] supplemented with L-glutamine (2 mM), retinoic acid (100 nM), dexamethasone (1nM), vitamin C (38 µg/ml), and bovine pituitary extract (30 µg/ml). Cells were passaged at a split ratio of 1:5 into 75 cm^2 flasks precoated with human connective tissue matrix (Matrigel) and incubated at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2.

Flavonoid-mediated inhibition of DNA strand breakage in HCEC

The cytoprotective effects of the anthocyanins cyanidin and cyanidin-3-glycoside and the flavonol quercetin [Sigma, Poole, UK] against hydrogen peroxide (H_2O_2)-induced DNA damage were investigated. HCEC were subcultured using trypsin-ethylenediaminetetraacetic acid (EDTA) solution [0.25 % trypsin in 0.02 % EDTA] into 25 cm^2 flasks precoated with Matrigel [12]. Cells [1x10^6/flask] were allowed to attach for 18 h, washed twice in PBS and incubated with 50 µM flavonoid in culture medium (flavonoid stock dissolved in DMSO) for 4 h at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2. Control cells were incubated with DMSO alone [max 2 % final conc., in culture medium]. The cells were removed from the flasks using trypsin/EDTA, washed twice with PBS and incubated in plastic microtubes with H_2O_2 (200 µM in PBS) for 5 min on ice. This concentration of hydrogen peroxide was chosen to induce DNA strand breakage based on previous experiments [13]. The cells were pelleted by centrifuging at 200 g for 3 min at 4 °C and resuspended in LMP agarose for analysis of DNA strand breakage by SCGE as described previously [12, 13]. Replicate gels were prepared from each treatment within a single experiment and experiments were repeated independently on 3–4 occasions.

Animals and experimental diets

All procedures were approved by the Rowett Research Institute Ethical Review Committee and were carried out in strict accordance with the requirements of UK Animals (Scientific Procedures) Act 1986.

Group housed weanling male rats of the Rowett Hooded Lister strain were offered *ad libitum* for 12 weeks, a standard semisynthetic diet [14] containing less than 0.5 mg/kg vitamin E (E–) or a vitamin E supplemented control diet (E+) containing 100 mg dα-tocopherol acetate/kg. Half of the controls and vitamin E-deficient rats received cyanidin-3-glycoside [100 mg/kg, A+, Polyphenols Laboratories, Sadnes, Norway]. This is equivalent to each rat consuming the equivalent of 8–11 portions of soft fruit/day depending on the fruit species. Food intake and weight were recorded weekly. Weight gain throughout the experiment was similar for rats fed all diets (data not shown). Subgroups of rats were killed immediately before starting the diet (time 0) and at 4-week intervals under terminal anaesthesia and plasma and tissue samples collected. Week 0 plasma was not available from weanling rats. After 12 weeks, half of the final groups of rats received an additional oxidative stress by i. p. injection of iron sulphate ($FeSO_4$; 15 mg/kg body weight) whilst under anaesthesia [Hypnorm 0.4 ml/kg, Jansen Animal Health, Buckinghamshire, U.K.] and were killed 1 h later. Blood was recovered by cardiac puncture into heparinised tubes. Plasma was obtained by centrifugation (2400 g for 15 min at 4 °C) and snap frozen in liquid nitrogen. The lymphocyte-containing-buffy coat was removed into RPMI 1640 medium [Gibco Life Technologies Inc, Paisley, UK] supplemented with EDTA (4 mM), layered onto an equal volume of lymphocyte separation medium [LSM, Nycomed UK, Birmingham, UK] and centrifuged at 700 g for 30 min at room temperature. The lymphocytes were washed twice in RPMI 1640 medium (700 g for 20 min at room temperature), resuspended in heat-inactivated fetal calf serum [FCS, Globepharm Ltd, Surrey, UK] containing 10 % DMSO (v/v) and frozen at –1 °C/min for DNA stability analysis. Red blood cells were washed twice, resuspended in PBS to the original volume and snap frozen in liquid nitrogen. The liver was perfused *in situ* with chilled KCl (0.15 M) and immediately snap frozen in liquid nitrogen. The colon was removed and washed twice in chilled PBS, longitudinally spread flat on a glass plate over ice and the mucosa gently scraped with a glass microscope slide into a cryovial and snap frozen. All tissues, plasma and blood cells were stored at –80 °C before analysis.

DNA stability biomarkers

Endogenous DNA strand breakage and oxidised purines (detected using the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase) were measured in cryopreserved rat lymphocytes by SCGE as described previously [15]. To determine the susceptibility of lymphocytes to exogenous oxidative stress, cells were incubated with H_2O_2 (200 μM in PBS) for 5 min at 4 °C on ice before analysis of strand breaks by SCGE. In all cases, DNA stability was measured in lymphocytes on replicate gels prepared from two animals from each treatment group per experiment with analyses repeated on 3 different occasions. Fluorescently stained nucleoids (5 μg/ml DAPI stock solution) were scored visually [16]. This method of classification has been extensively validated using computerised image analysis (Komet 3.0, Kinetic Imaging Ltd, Liverpool, UK) [16].

DNA was isolated from liver and colon samples using a protocol designed to minimise artifactual oxidation of DNA [17]. 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-Oxo-dG) was determined by HPLC with electrochemical detection [17].

Biochemical analysis

Plasma [18] and hepatic [19] vitamin E concentrations (from liver homogenate) were determined by reverse phase HPLC with fluorimetric detection using the protocols and guidelines of the U. S. National Institute of Standards Quality Assurance Scheme for fat-soluble vitamins. The ferric reducing antioxidant potential (FRAP) of rat plasma was measured as the ability of sample to reduce Fe(III)–2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) to the highly coloured Fe(II)-TPTZ complex as described previously [20]. Colour formation (detected at 593 nm) is linearly related to the amount of reductant (antioxidant) in the sample [20]. Plasma pyruvate kinase (PK), as an indicator of cell membrane damage, was measured using a commercially available diagnostic kit [Boehringer Mannheim, Lewes, UK]. Reduced glutathione (plasma) was measured by reverse phase HPLC using a DS30 analyser [15]. Protein (homogenate and erythrocyte) was measured by the Biuret method [21].

Basal levels of reactive oxygen species (ROS) were determined in quiescent peripheral phagocytes. Fresh whole rat blood was incubated with dihydrorhodamine 123 (DHR; 5 μM) for 10 min at 37 °C, lysed with FACS lysing solution, washed with PBS and subjected to flow cytometric analysis (FACS Calibur, Becton Dickinson, Oxford, U.K.; excitation 488 nm, emission 530 nm). Signals were processed using a logarithmic amplifier and fluorescence distributions plotted on a 4 decade logarithmic scale (1024 channels). Forward and side scatter characteristics were used to "gate" monocyte and granulocyte populations and median fluorescence intensity (MFI) was determined using Cell Quest software (Becton Dickinson, Oxford, U.K.). DHR is converted to rho-

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damine in the presence of hydrogen peroxide and peroxynitrite [22]. Non dye-loaded phagocytes were used to determine background fluorescence.

Endogenous lipid peroxidation (detected as thiobarbituric acid reactive substances (TBARS)) was measured in plasma and liver homogenate by HPLC with fluorescence detection [23]. The sensitivity of erythrocytes to peroxidation was measured as the formation of TBARS following incubation of red cells with H_2O_2 (1 % solution in 0.9 % saline) for 1 h at 37 °C [23].

All biochemical analysis was carried out at least in duplicate for each sample.

Statistical analysis

Results are presented as mean ± SEM. Number of rats or cell replicates are indicated in individual tables and figures. Data were analysed using ANOVA in combination with Tukey's honestly significant test and Students' t-test in SPSS version 8.0 as appropriate. All results were considered significant if the p value of the relevant statistical test was < 0.05.

Results

Vitamin E deficiency and oxidative stress in vivo

Plasma and liver dα-tocopherol concentrations showed a progressive and sustained decline in rats fed the vitamin E deficient diet (Fig. 1). After 12 weeks, the relative level of dα-tocopherol detected in the liver and plasma of deficient rats was 1:24 ($P < 0.001$) and 1:16 ($P < 0.001$) respectively compared with rats given dα-tocopherol acetate. Both plasma and hepatic vitamin E levels remained relatively constant in the vitamin E sufficient rats (Fig. 1). Vitamin E deficiency was associated with increased indices of lipid peroxidation (Fig. 2). TBARS concentrations at the end of the experiment were increased 50 % in plasma (Fig. 2A; $P < 0.01$) and 2-fold in liver (Fig. 2B; $P < 0.01$). Moreover, the susceptibility of erythrocytes to oxidise *in vitro* was increased 7-fold in vitamin E-deficient rats (Fig. 2C; $P < 0.05$). In addition, phagocyte ROS concentrations [as determined by rhodamine fluorescence] and plasma pyruvate kinase were significantly higher in the vitamin E-deficient rats (Table 1; $P < 0.05$). Plasma glutathione and the plasma antioxidant capacity (measured as FRAP) were alike for all groups (Table 1). Vitamin E deficiency did not alter DNA stability in this experiment (Table 1). Despite a highly significant change in plasma and hepatic vitamin E content, endogenous DNA strand breakage (lymphocytes) and oxidised bases (FPG-reactive sites in lymphocytes and 8-Oxo-dG in liver and colon) were similar for all groups, as was the ability of lymphocytes to resist oxidant-induced strand breakage (H_2O_2) ex vivo.

Injection of $FeSO_4$ to induce additional oxidative stress (at week 12) caused a significant increase in plasma glutathione levels (ranging from 22–65 %; $P < 0.02$) across all groups. However, iron injection did not affect any other biomarkers of oxidative stress (vitamin E level, FRAP, lipid peroxidation, ROS generation, cell membrane integrity or DNA stability; data not shown).

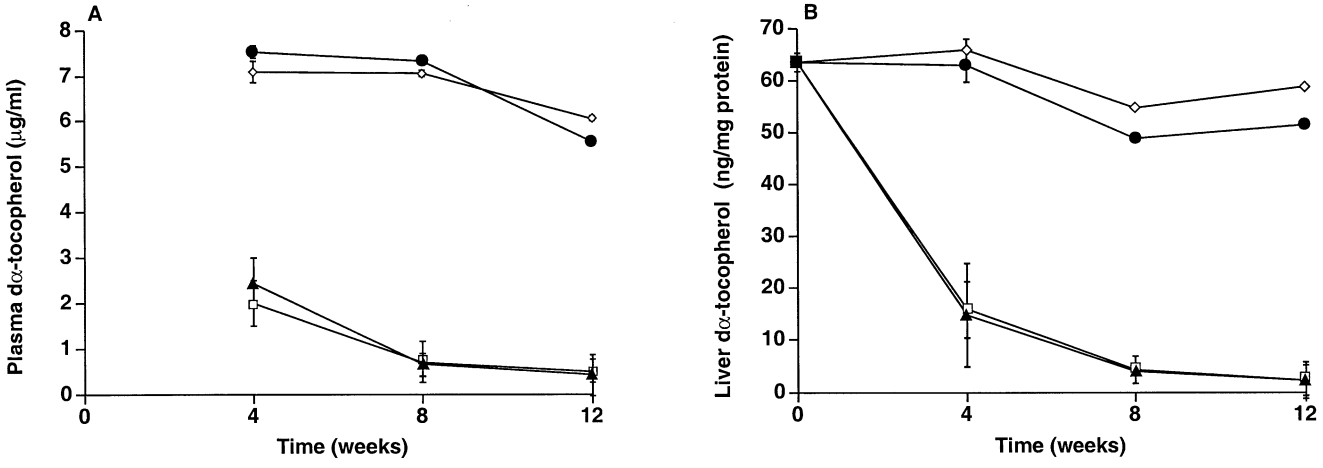

Fig. 1 Effect of vitamin E deficiency and anthocyanin supplementation over 12 weeks on rat plasma (**A**) and liver (**B**) dα-tocopherol concentrations. Rats were fed a vitamin E deficient diet (squares and triangles) containing less than 0.5 mg/kg vitamin E or a vitamin E supplemented control diet (circles and diamonds) containing 100 mg dα-tocopherol acetate/kg. Half of the controls and vitamin E-deficient rats received cyanidin-3-glycoside (100 mg/kg; closed symbols). Results are mean ± SEM, 6 rats per group

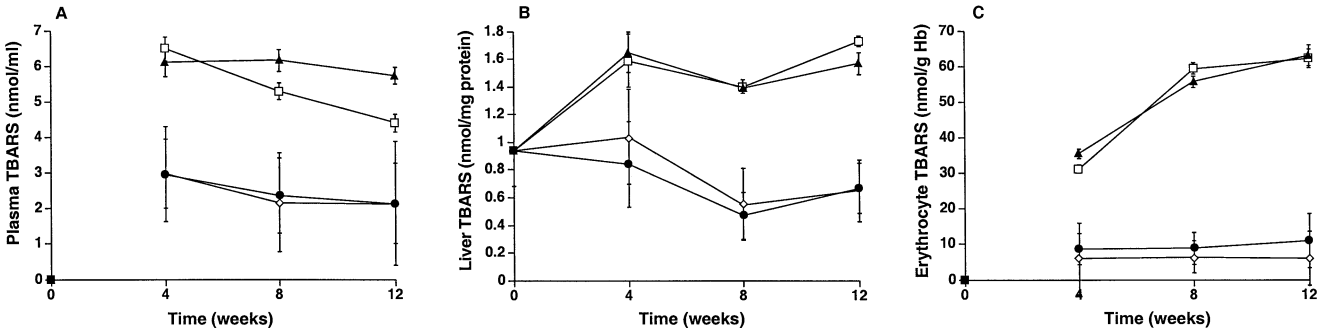

Fig. 2 Effect of vitamin E deficiency and anthocyanin supplementation over 12 weeks on TBARS formation in rat plasma (**A**), liver (**B**) and erythrocytes (**C**). Rats were fed a vitamin E deficient diet (squares and triangles) containing less than 0.5 mg/kg vitamin E or a vitamin E supplemented control diet (circles and diamonds) containing 100 mg dα-tocopherol acetate/kg. Half of the controls and vitamin E-deficient rats received cyanidin-3-glycoside (100 mg/kg; closed symbols). Results are mean ± SEM, 6 rats per group

Table 1 The effect of vitamin E deficiency and dietary cyanidin-3-glycoside on indices of oxidative stress and DNA stability in rats *in vivo*

	Group			
	(E–)	(E+)	(E– A+)	(E+ A+)
Biochemical biomarkers				
FRAP (mM)	0.43±0.02	0.47±0.02	0.45±0.03	0.50±0.02
PK (mU/ml)	409.8±38.7	65.8±9.3 *	405.8±44.2	46.9±1.8 *
GSH (μM)	31.2±3.6	36.9±2.5	36.2±1.2	33.9±2.1
Basal ROS (MFI)	75.4±10.4	44.4±5.1 *	63.7±12.3	44.6±6.2 *
Endogenous DNA damage				
strand breakage (lymphocyte) (arbitrary units)	116.8±20.7	115.0±12.9	123.8±24.8	98.9±22.0
oxidised purines (lymphocyte) (arbitrary units)	96.9±12.9	85.7±6.7	68.8±8.5	77.7±4.9
8-Oxo-dG (liver) (8-Oxo-dG/10^6dG)	4.35±0.56	3.35±0.59	3.78±0.89	3.78±1.37
8-Oxo-dG (colonic mucosa) (8-Oxo-dG/10^6dG)	2.21±0.27	1.73±1.72	3.24±0.13	1.91±0.73
Induced oxidative DNA damage				
H_2O_2-induced strand breakage (arbitrary units)	88.6±11.2	84.6±14.1	88.8±7.1	71.6±7.4

Results are mean ± SEM for 6 rats at week 12 (except for Group 2, 8-Oxo-dG in colonic mucosa, where both values for n = 2 are presented). *FRAP* ferric reducing antioxidant potential; *PK* plasma pyruvate kinase; *GSH* reduced glutathione; *ROS* reactive oxygen species; *8-Oxo-dG* 8-Oxo-deoxyguanosine, H_2O_2 hydrogen peroxide (200 μM *ex vivo*). * $P < 0.05$, where significance refers to differences between rats fed vitamin E sufficient or deficient diets

Cyanidin-3-glycoside and oxidative stress in vivo and in vitro

Consumption of vitamin E deficient diets containing cyanidin-3-glycoside did not affect vitamin E levels in plasma or liver (Fig. 1), nor did it modulate lipid peroxidation induced by vitamin E deficiency in plasma, liver or erythrocytes (Fig. 2). The antioxidant potential of the plasma (FRAP) was unchanged following anthocyanin supplementation as was the decrease in cell membrane integrity (measured as increased PK in plasma) and the increase in phagocyte-generated ROS caused by vitamin E deficiency (Table 1). Supplementation with cyanidin-3-glycoside failed to affect either endogenous DNA strand breakage and oxidised bases in lymphocytes or 8-Oxo-dG in liver and colon from non-stressed rats. Lymphocytes isolated from vitamin E deficient rats fed cyanidin-3-glycoside were as susceptible to oxidative stress (strand breaks resulting from incubation with H_2O_2) as rats fed control diet. Similar results were observed in rats injected with $FeSO_4$ (data not shown).

Conversely, cyanidin-3-glycoside was chemoprotective against H_2O_2 induced DNA damage in colonocytes *in vitro* (Fig. 3). DNA strand breakage was decreased 38.8 ± 2.2 % in cells pretreated with anthocyanin for 4 h. Indeed, cyanidin-3-glycoside was as effective in decreasing DNA damage *in vitro* as its aglycone and the model cytoprotectant quercetin (Fig. 3).

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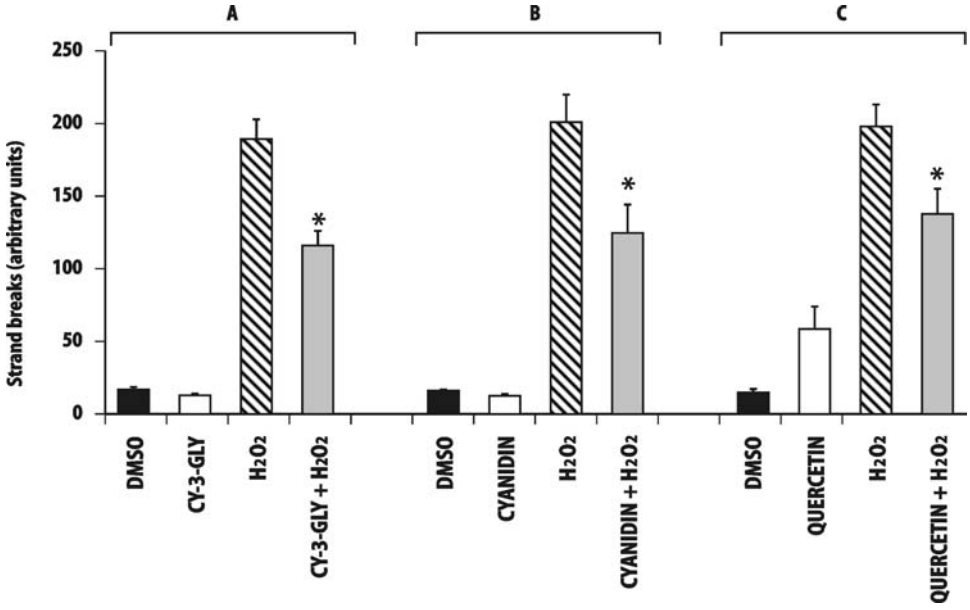

Fig. 3 Cyanidin-3-glycoside (**A**), cyanidin (**B**) and quercetin (**C**)-mediated cytoprotection against H_2O_2-induced DNA strand breakage in HCEC. Results are mean ± SEM, for 5–8 flasks per experiment. * $P < 0.01$ refers to a significant difference in oxidant-induced DNA strand breakage in HCEC preincubated with or without polyphenol

Discussion

In this study we set out primarily to answer two questions: (1) can vitamin E modulate endogenous and iron-induced oxidative DNA damage and lipid peroxidation *in vivo* and (2) will feeding the anthocyanin cyanidin-3-glycoside influence any of these biomarkers?

In order to address both questions rats were made vitamin E deficient. This allows for the affects of dα-tocopherol on genomic stability and lipid peroxidation to be investigated, and in addition, provides a nutritional model of oxidative stress against which to assess the cytoprotective properties of the anthocyanin.

Low dietary vitamin E was associated with increased non-exogenously stimulated phagocyte ROS levels, enhanced indices of lipid peroxidation (TBARS) in all of the tissues studied (plasma, liver and erythrocytes) and with elevated plasma pyruvate kinase, presumably resulting from free-radical-mediated cell membrane damage and decreased cell integrity. This is consistent with findings from previous studies [25–27]. However, vitamin E deficiency did not modulate DNA stability in this study. Endogenous DNA strand breaks and oxidised purines were alike in lymphocytes isolated from rats in all groups. Similarly, the ability of lymphocytes to resist oxidative attack (induced DNA strand breakage in response to hydrogen peroxide-generated hydroxyl radicals) was independent of vitamin E status. Moreover, 8-Oxo-dG, a widely used biomarker of oxidative damage to DNA that is directly implicated in carcinogenesis was comparable in liver and colon from all groups, confirming a lack of effect of vitamin E on DNA stability in this experiment. Alterations in long-term vitamin E intake (ranging from 0–1 g/kg diet for 50 weeks) fail to affect sister chromatid exchange or micronuclei frequencies in murine peripheral blood cells and bone marrow [26]. Similarly, 8-Oxo-dG in guinea pig liver was comparable in animals fed different intakes of vitamin E (15–1500 mg/kg diet) for 5 weeks [28]. Nonetheless, whether vitamin E modulates DNA stability *in vivo* remains controversial. In a previous study we found that hepatic 8-Oxo-dG was increased in response to vitamin E deficiency *in vivo* [25]. However, plasma and liver α-tocopherol were undetectable following 12 weeks on a vitamin E deficient regime. Here, residual tissue and blood vitamin E may have afforded protection against endogenous and induced DNA damage.

Radical-induced DNA damage may be site-specific, occurring at metal-binding sites within the DNA molecule itself. It has been proposed that generation of reactive oxygen species so close to the target DNA allows little scope for intervention by membrane or water-soluble antioxidants [28]. Moreover, lack of effect of vitamin E on oxidative DNA damage may be due to efficient endogenous DNA repair maintaining a steady-state level of nuclear 8-Oxo-dG [29]. DNA repair may be induced in response to oxidative stress [23]. These data suggest that in general, DNA is less susceptible and better protected against oxidative attack compared with lipids.

In an attempt to further increase oxidative stress, rats were injected with $FeSO_4$ prior to tissue collection. Only reduced glutathione (GSH) was affected by acute iron overload. Similarly, 8-Oxo-dG remains unchanged in testes of rats fed chow supplemented with 25 mg carbonyl-iron/g diet for 6 weeks [30]. In contrast, 3 h after exhaustive exercise, 8-Oxo-dG is increased in rat blood cells [31]. However, this increase in 8-Oxo-dG was tran-

sient and returned to baseline (presumably as a result of DNA repair) 6 h post-exercise [31]. Dietary vitamin E has been shown to protect DNA (8-Oxo-dG) against induced oxidative stress in rats subjected to total body irradiation [27]. Here, DNA damage was measured several hours or even days following prolonged oxidative stress. In the present study it is possible that only a mild oxidative stress was induced by iron sulphate treatment which failed to elicit further damage or, alternatively, that the sampling regime was too short to detect a response.

Polyphenols from plant-based sources are found in significant quantities in the human diet [32]. While anthocyanins are potent antioxidants in simple chemical systems [33] their chemopreventive activity in mammalian cells *in vitro* and most especially *in vivo* is less well understood. Anthocyanins are incorporated into the cellular membrane of aortic endothelial cells where they directly decrease lipid peroxidation and toxicity from reactive oxygen species generated by hydrogen peroxide and inflammatory cytokine production in response to TNFα [34, 35]. Anthocyanins also act indirectly by inhibiting the release of superoxide radicals from human granulocytes *in vitro* [36]. However, few studies have investigated the effect of anthocyanins on DNA stability. Here, cyanidin and cyanidin-3-glycoside significantly decreased oxidant-induced DNA strand breakage in immortalised normal human colonocytes in vitro. Both were as affective genoprotectants as the flavonol quercetin, which is a potent dietary antioxidant *in vitro* [13]. Anthocyanins from *Aronia melanocarpa* are antimutagens in both the Ames and SCE genotoxicity tests [36]. While anthocyanins obviously act as chemoprotectants via several mechanisms their antimutagenic activity has been linked to their ability to limit free radical attack on DNA. However, while anthocyanins decrease oxidant-induced DNA strand breaks in both normal colon and tumour cells [this study and 33], they afford no protection against oxidative base damage [33]. It may be that anthocyanins act directly to prevent breakage of DNA by forming an anthocyanin-DNA complex, which stabilises the molecule against oxidative attack [37].

In the present study, cyanidin-3-glycoside was not effective against lipid peroxidation, ROS generation, or cell membrane damage in vitamin E deficient rats in vivo. Endogenous TBARS levels were similar in plasma, liver and red blood cell membranes in rats from all groups. PK and reduced glutathione levels were also consistent between experimental groups. Significantly, the antioxidant potential of the plasma was unchanged following anthocyanin feeding (FRAP). Moreover, feeding anthocyanin did not alter plasma or tissue α-tocopherol levels. Several studies have reported that complex anthocyanin extracts are active in different model systems. Survival rate is extended in tumour-bearing mice intubated with camellia and hibiscus anthocyanins [38]. Hibiscus extract similarly protects against oxidative cell membrane damage and lipid peroxidation in rats treated with tertbutylhydroperoxide [39], while rats given anthocyanin extracts of rosasinensis petals are protected against radical-mediated carbon tetrachloride-induced hepatotoxicity [40]. Certain classes of polyphenols can also influence DNA stability in vivo. Feeding complex polyphenol and tannin mixtures from red wine, substantially and specifically decreased endogenous oxidative DNA base damage in rat colon mucosal cells without altering basal DNA strand breakage or the capacity of colonocytes to withstand oxidative attack from hydrogen peroxide ex vivo [41]. In this study, polyphenol intake was calculated to be 10 times higher than would be expected for average human consumption. However, DNA stability was unaffected by feeding rats tea polyphenols [41]. Likewise, we have previously reported that DNA stability is maintained in vitamin E-deficient rats fed a complex anthocyanin extract [25]. These data suggest that certain polyphenols, at relatively high doses, can modulate oxidative DNA damage in vivo, while others cannot. However, few studies have investigated the bioactivity of single anthocyanins at nutritionally relevant concentrations. In contrast with our findings from this study, endogenous and induced lipid peroxidation (TBARS) is inhibited in vitamin E deficient rats fed cyanidin-3-o-β-δ-glucoside (2 g/kg diet) for 14 days [42]. Moreover, elevated liver TBARS and serum GSH and enzyme activities resulting from ischemic-reperfusion injury are suppressed after feeding anthocyanin [43]. However, in these two studies, the cyanidin glycoside was fed at 20 times the level we employed here.

In conclusion, while it is generally accepted that vitamin E strongly modulates lipid oxidation *in vivo*, its role in maintaining DNA stability remains unclear. We have shown that dietary anthocyanins at relatively high levels protect against oxidative DNA damage in simple cellular systems *in vitro*, but that cyanidin-3-glycoside is ineffective in vivo. This discrepancy between the genoprotective effects observed in vitro and in rats may result either from poor bioavailability or subsequent metabolism of the anthocyanin, and clearly indicates that cyanidin-3-glycoside, at a level relevant to the human diet, does not act against endogenous and induced oxidative stress *in vivo*.

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