Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1740 (2005) 340–349
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

The effect of short-term kaempferol exposure on reactive oxygen levels and integrity of human (HL-60) leukaemic cells

Charles S. Bestwick*, Lesley Milne, Lynn Pirie, Susan J. Duthie

Phytochemical and Genomic Stability Group, Cellular Integrity Programme, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB UK

Received 25 November 2003; received in revised form 30 August 2004; accepted 15 October 2004
Available online 3 November 2004

Abstract

Flavonoids may be a principal contributor to the cancer preventative activity of fruit- and vegetable-rich diets and there is interest in their use as dietary supplements. However, there is potential conflict between the cytoprotective and cytotoxic activities of flavonoids, and their efficacy as anti-cancer agents is unresolved. Here, the integrity and survival of HL-60 promyelocytic leukaemia cells following short-term (90 min) exposure to the dietary abundant flavonoid kaempferol (1–100 μM) is reported. Supplementation initially decreased reactive oxygen levels but, paradoxically, a dose-dependent increase in single-strand DNA breakage occurred. However, there was no increase in oxidised DNA purines or membrane damage. Following a 24-h recovery period in non-kaempferol supplemented media, DNA single-strand breakage had declined and kaempferol exposed and control cultures possessed similar reactive oxygen levels. A reduction in ^{3}H-thymidine incorporation occurred with ≥10 μM kaempferol. One hundred micromolar kaempefrol increased the proportion of cells in G_2-M phase, the proportion of cells with a sub-G_1 DNA content and enhanced 'active' caspase-3 expression but only induced a loss of mitochondrial membrane potential within a minority of cells. The relevance of induced DNA damage within a non-overtly oxidatively stressed environment to the disease preventative and therapeutic use of kaempferol is discussed.

Keywords: Cell cycle; DNA damage; Flavonoid; HL-60; Kaempferol; Oxidative stress

1. Introduction

Fruit- and vegetable-rich diets are correlated with a lower incidence of degenerative diseases including various cancers and coronary heart disease [1–4]. However, whether individual phytochemicals or groups of compounds specifically confer protection remains unresolved (e.g., Refs. [5,6]).

The flavonoids represent a group of polyphenols abundant in fruits, vegetables and also in beverages such as wines, coffee and tea and are categorised into various subclasses including flavones, flavonols, flavanones, flavanols, isoflavones and anthocyanidins [7–9]. Dietary flavonoid intake varies widely depending on regional dietary habits, e.g., flavonol and flavone combined intake of 3–6 mg/day in Finland and 49 mg/day in Croatia [8].

An array of activities including anti-allergenic, anti-inflammatory and anti-viral as well as anti-carcinogenic and anti-atherogenic effects are variously attributed to flavonoids [8–10]. This diversity of patho-prevention is reflected in a wide range of effects on cellular function and integrity, which includes, but are not confined to, the mitigation of oxidative damage, antiproliferative effects, promotion of differentiation, induction of apoptosis and inhibition of malignant transformation [8–10].

In particular, the involvement of reactive oxygen species (ROS; a collective term for radicals and nonradical but reactive species derived from oxygen) in the aetiology of many degenerative conditions [11,12] has implicated phytochemicals with antioxidant activity as the potential source of, or contributors to, patho-prevention [8]. Flavonoids exhibit a broad spectrum of antioxidant activity,

* Corresponding author. Tel.: +44 1224 712751; fax: +44 1224 716629.
E-mail address: C.Bestwick@rowett.ac.uk (C.S. Bestwick).

0925-4439/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2004.10.005

reacting with superoxide anion (O_2^{-}), hydroxyl ($HO^{\bullet}$), peroxyl and alkoxyl radicals [8,13–16]. Flavonoids also act, to varying degrees, as iron/copper chelators and thus alter the propensity for Fenton Reaction generated $HO^{\cdot}$ [13]. Correspondingly, flavonoids mitigate oxidative-stress-induced DNA damage and prevent or terminate lipid peroxidation within a number of cell types [8,13].

However, flavonoids may also exhibit pro-oxidant activity [17,18]. While such activity may contribute to cytotoxicity against tumour cells, it may be damaging to normal cell integrity and act as a mechanism of the mutagenicity correlated with the addition of certain flavonoids in cultured cells [19–21]. Additionally, while being able to induce apoptosis (e.g., Refs. [19,22]), flavonoids may also inhibit apoptosis, including that directly induced by chemotherapeutics, through a combination of antioxidant and signal transduction kinase inhibitory effects [23–25]. The potential antagonism between antioxidant activity and cytotoxicity poses an intriguing dilemma in deciphering the relevance of such compounds to disease prevention.

Here, we examine the temporal relationship between reactive oxygen levels, membrane and DNA integrity and survival of promyelocytic leukaemia HL-60 cells following short-term exposure to the flavonol kaempferol. Kaempferol is found in berries, tea, *Brassica* and *Allium* species and represents a significant proportion of dietary flavonol intake, e.g., 17% of dietary flavonol/flavone in The Netherlands [8].

2. Materials and methods

2.1. Cell culture

HL-60 cells (European Cell Culture Collection, 98070106) were cultured, in a humidified atmosphere of 5% CO_2 in air at 37 °C, in RPMI medium (Gibco-BRL, Paisley, UK) supplemented with 5 mL L^{-1} 100× non-essential amino acids, 2 mM glutamine, 50 μg mL^{-1} streptomycin and 50 units mL^{-1} penicillin (Sigma Chemical, Poole, Dorset, UK) and 10% (v/v) foetal calf serum (Cyclone, Gibco-BRL). Cells were seeded at a density of 5×10^5 cells mL^{-1} in 75-cm^2 flasks (Greiner, Gloucestershire, UK).

2.2. Cell incubations

Kaempferol (Sigma) was prepared in dimethylsulfoxide (DMSO; Sigma). Cells (5×10^5 mL^{-1}) were incubated for 90 min either with water, solvent vehicle (0.1% v/v DMSO) or kaempferol (1–100 μM) within the culture medium. Following 90-min incubation, cells were washed by centrifugation (300×*g*, 5 min, RT) and resuspended in fresh serum supplemented media and cultured for a further 24 h in the absence of kaempferol. In certain experiments, to provide a positive control for apoptosis or general cyotoxicity, cells were also incubated under an identical regime, with either 1 μM etoposide, 4 μM camptothecin or 10 μM menadione (Sigma) prepared in DMSO. All incubations were conducted in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.3. Reactive oxygen detection

To detect a broad spectrum of ROS, cells were loaded with dihydrorhodamine 1,2,3 (DHR; Molecular Probes, Leiden, Netherlands). DHR reacts indirectly with H_2O_2 in a peroxidase-like reaction, and is also sensitive to peroxynitrite, to yield rhodamine 123, which localises to the mitochondria [26].

Cells were washed by centrifugation at 300×*g* for 3 min and resuspended, at 5×10^5 cells mL^{-1}, in PBS comprising 5 μM DHR prepared in DMSO (final concentration 0.1% [v/v]). Cells were incubated with DHR for 30 min at 37 °C. To prevent light accelerated oxidation, samples were maintained in the dark prior to and during analysis by flow cytometry (FACS Calibur; Becton Dickinson, Oxford, UK). The excitation wavelength was 488 nm and emission at 530 nm (FL-1 detector voltage 450). Signals were processed using a logarithmic amplifier and fluorescence distributions plotted on a 4 decade logarithmic scale (1024 channels). 15,000 events were counted and the median linear fluorescence values were calculated using Cell Quest Software (Becton Dickinson). Non-dye-treated cells incubated with kaempferol were used to determine changes in and correct for background fluorescence. Addition of hydrogen peroxide (100 μM) or menadione (10 μM) confirmed dye responsiveness to changes in ROS levels [27]. Fluorimetric determination (Perkin-Elmer, Wellesley, MA, USA) of oxidised dye fluorescence in the presence of kaempferol did not reveal any quenching of the fluorescence emission [27].

2.4. DNA damage

DNA single-strand breaks were determined by both hydroxyapatite chromatography and by single cell gel electrophoresis (SCGE). For the chromatographic determination of single-strand breaks, 1.8×10^7 cells mL^{-1} were incubated with 0.1 μCi ^{3}H-thymidine mL^{-1} for 16 h at 37 °C, 5% CO_2 within a humidified atmosphere. Cells were then washed by centrifugation (300×*g*, 5 min, RT) and resuspended (5×10^5 cells mL^{-1}) in fresh culture media for treatment with kaempferol. Following cell lysis, the proportion of DNA that was single stranded, as a measure of strand breaks, was estimated following alkali treatment and hydroxyapatite chromatography as described in Burkitt et al. [28]. For SCGE, control and kaempferol-treated cells were prepared and analysed as described in Duthie et al. [29]. Oxidised purines were detected specifically using a modified SCGE assay incorporating the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (FpG) as reported previously [30]. Following SCGE,

nucleoids were stained with DAPI (5 μg mL^{-1} stock) and were scored visually. One hundred images per gel (with duplicate gels per slide) were classified according to the intensity of fluorescence in the nucleoid tail (comet) and assigned a value of 0, 1, 2, 3 or 4 with 0 representing no damage and 4 maximum damage. Thus, the total damage score ranges from 0 to 400. Oxidized purines were measured by subtracting the damage score in the absence of FpG (buffer treated) from that obtained after treatment with FpG. This method of classification has been extensively validated using computerised image analysis [30].

2.5. Caspase-3 detection

The presence of the active fragment of caspase 3 was analysed by flow cytometry following fixation and permeabilization and labelling of cells with phycoerythrin(PE)-conjugated polyclonal rabbit anti-active caspase-3 antibody according to manufacturer's recommendations (Becton Dickinson). Cultures treated with kaempferol alone were used to assess any interference in the fluorescence emission. Fluorescence within labelled and non-labelled cells was determined with excitation at 488 nm and emission at 585 nm (FL-2 detector voltage 400). Camptothecin treatment (5 μM) was used to provide a positive control for caspase activation and the percentage of cells labelled relative to control, non-apoptotic, cells was used to define regions of positive active casapse-3 expression. The proportion of cells with active caspase-3 was expressed as a percentage of total (10,000) recorded events.

2.6. Mitochondrial membrane potential

Changes to mitochondrial membrane potential ($\Delta\Psi_m$) was analysed by flow cytometry using a commercial assay (ApoAlert Mitochondrial Membrane Sensor; Clonetech, BD Biosciences, Oxford, UK) according to the manufacturer's instructions.

2.7. Cell cycle analysis

For analysis of cell cycle distribution, cells were centrifuged at 600×*g* for 5 min, the supernatant discarded and the pellet resuspended in 100 μL of PBS. Nine hundred microliters of 70% (v/v) ice-cold ethanol was added and cells incubated at 4 °C for 30 min. The cells were then centrifuged at 10,000×*g* for 5 min, the supernatant decanted and the pellet resuspended in 1 mL PBS followed by re-centrifugation at 10,000×*g* for 5 min. The pellet was resuspended in 0.5 mL of DNA extraction buffer (4 mM citric acid, 0.2 M Na_2HPO_4, pH 7.8) and 0.5 mL of PBS and incubated at RT for 5 min. The extract was then centrifuged at 10,000×*g* for 5 min, the supernatant removed, the pellet resuspended in 0.5 mL of DNA staining solution (20 μg mL^{-1} PI and 0.2 mg mL^{-1} DNAse-free RNAse in PBS) and incubated at RT for 30 min in the dark. Flow cytometry of propidium iodide stained nuclei was performed with a flow rate of 12 μL min^{-1} and cell cycle distribution selected from linear FL-2 area vs. width plots with doublet discrimination in FL-2. Singlet events, excluding debris, were gated and 20,000 events were acquired within the gated region. The percentage of cells with DNA content <2 N (sub-G1) and cell cycle phases was calculated from histograms of linear FL-2 area plots of the singlet gated region using Cell Quest Software (Becton Dickinson) and Mod Fit LT software (Verity Software House, ME, USA). For verification of instrument linearity, doublet discrimination and cytometer alignment, a DNA QC Particle Kit (Becton Dickinson) was utilised according to manufacturer's instructions.

2.8. Membrane integrity and proliferation

Membrane integrity was routinely assessed by flow cytometric analysis of the retention of fluorescein fluorescence, following staining with fluorescein diacetate (FDA; Molecular Probes) [31] and by microscopical determination of trypan blue (Sigma) uptake. For determination of trypan blue uptake, cells were collected by centrifugation (300×*g* for 5 min), culture media removed and the pellet resuspended in PBS. An aliquot of cell suspension was diluted and incubated in PBS containing trypan blue (0.2% w/v final concentration) and cells were counted using a haemocytometer (Neubauer). In addition, as a measure of membrane integrity immediately post kaempferol treatment, the percentage of LDH release into the culture medium was determined, with correction for volume and serum-LDH as previously described [32]. The consequences of short-term kaempferol exposure on cell survival was also analysed by the delayed thymidine incorporation assay as described in Ref. [33]. Briefly, following the 90-min kaempferol treatment and subsequent recovery in fresh media, cells were exposed to 1 μCi ^3H thymidine mL^{-1} (Amersham Biosciences, UK) for 30 min at 37 °C, then centrifuged (350×*g*, 5 min), washed twice with PBS, fixed with 1 mL 5% (w/v) trichloroacetic acid (30 min at RT) and again washed twice (PBS and centrifugation; 350×*g*, 5 min). Samples were warm air-dried and 1 M NaOH (250 μL) added to digest acid insoluble material for 14 h. The digest (100 μL) was added to 5 mL of scintillation fluid (Packard Ultima Gold; Packard1900TR Liquid Scintillation Counter, Packard Bioscience, Gronigen, The Netherlands). The mean radioactivity count from treated cells was expressed as a percentage of radioactivity from control (water) treated [32].

2.9. Statistical analysis

All experiments consisted of a minimum of three independent replicates and all experiments were conducted a minimum of three times. Comparison between means of control vs. treatment was analysed via Student's *t*-test using SigmaStat (SPSS) software.

3. Results

3.1. Effect of kaempferol exposure on intracellular reactive oxygen

Cells incubated for 90 min with 1–100 μM kaempferol exhibited a decreased intracellular rhodamine fluorescence intensity distribution, indicating a general decline in basal ROS levels within the HL-60 cell population (Fig. 1A). The apoptosis inducer etoposide did not induce any such major change to rhodamine fluorescence after 90-min incubation.

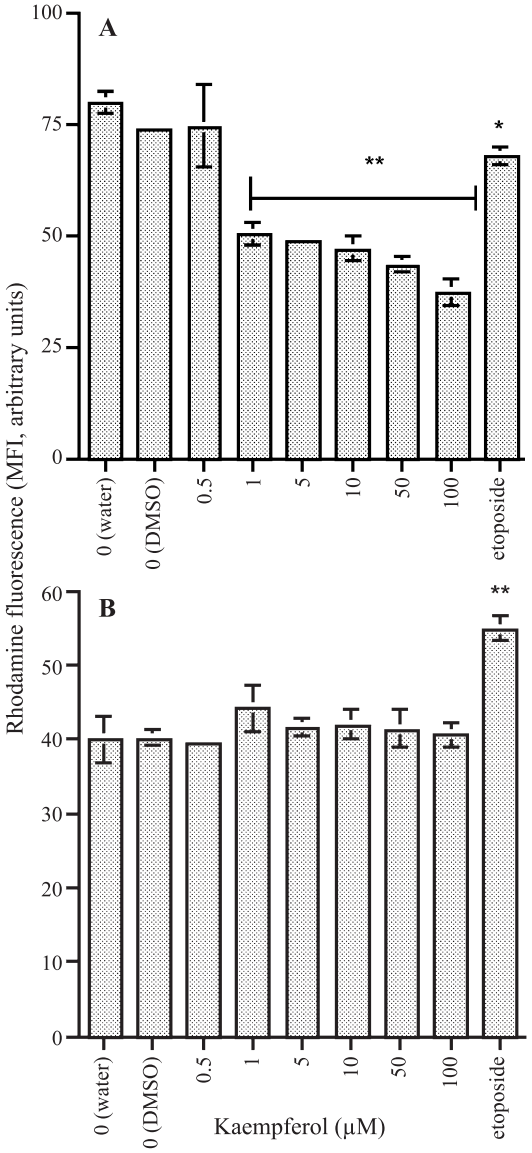

Fig. 1. Influence of kaempferol on reactive oxygen levels in HL-60 cells. HL-60 cells were incubated with water, DMSO or kaempferol for 90 min (A) and then cultured in the absence of kaempferol supplementation for 24 h (B). Following these treatments, cells were washed and loaded with DHR 123 to detect ROS. Oxidised dye fluorescence was assessed by flow cytometry (15,000 events recorded) and expressed as the median fluorescence intensity (MFI). Data are means±SD ($n \geq 6$). Small errors are contained within the histograms. $^{**}P<0.001$, $^{*}P=0.006$ vs. DMSO control.

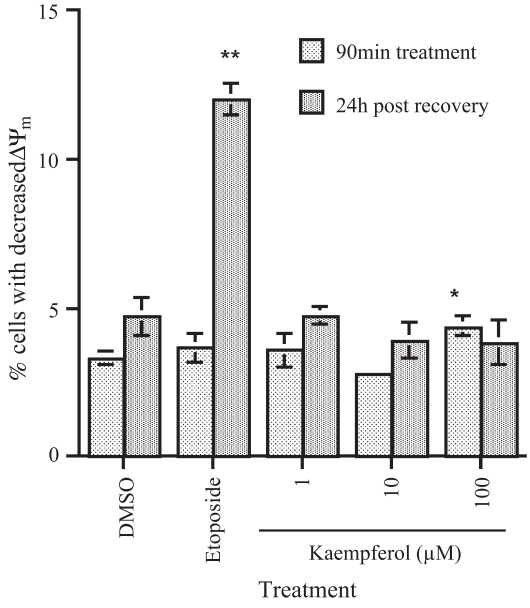

Fig. 2. Loss of mitochondrial membrane potential. Cells with decreased $\Delta\Psi_m$ were detected by flow cytometric measurement of the increase in green (FL-1) fluorescence and decrease in red (FL-3) fluorescence resulting from the inability of the mitosensor dye to aggregate within the mitochondria. Ten thousand events were recorded. Data are means±SD ($n=3$). $^{*}P=0.011$, $^{**}P<0.001$ for cultures exhibiting a decreased $\Delta\Psi_m$ vs. respective DMSO control.

We have observed cyclical fluctuations in ROS levels with prolonged culture (Bestwick and Milne, unpublished observations). Following the 24-h recovery in non-kaempferol supplemented media, negative control cultures (water and DMSO) exhibited lower fluorescence levels relative to negative control cultures examined immediately after the 90-min incubations (Fig. 1A,B). Nevertheless, after the recovery period, no significant difference in intracellular rhodamine fluorescence was observed in response to kaempferol relative to the 'post recovery' control treatments (Fig. 1B). However, fluorescence within etoposide-treated cultures was higher relative to negative control treatments after the 24-h recovery period (Fig. 1B).

3.2. Effect of kaempferol on mitochondrial membrane potential

Under conditions of maintained $\Delta\Psi_m$, the mitosensor dye aggregates within mitochondria and exhibits red fluorescence. However, no such aggregation occurs following dissipation of $\Delta\Psi_m$ and the dye remains in a monomeric form within the cytoplasm, exhibiting green fluorescence. On this basis, flow cytometric analysis revealed no major change to $\Delta\Psi_m$ after 90-min incubation with 1–100 μM kaempferol or etoposide. Following the recovery period, significantly more cells exhibited decreased $\Delta\Psi_m$ was observed in cultures treated with etoposide but no such decrease was apparent in response to kaempferol (Fig. 2).

3.3. Effect of kaempferol exposure on DNA integrity

Cells incubated with 1, 10, 20 or 100 μM kaempferol possessed increasingly elevated levels of DNA strand breaks, as revealed by alkaline hydroxyapatite chromatography and SCGE (Fig. 3A,B). Following recovery in fresh media, the level of strand breakage in kaempferol-treated cultures had declined to levels observed in response to the negative control treatments (Fig. 3C). Menadione and etoposide, used as a positive control for DNA strand breakage, induced strand breakage in the range of 30–70% as determined by alkaline hydroxyapatite chromatography and in excess of that quantifiable by the highly sensitive SCGE assay.

The SCGE assay, modified by inclusion of FpG, was used to assess the level of oxidised DNA purines [30]. For the 90-min incubation, analysis was confined to kaempferol concentrations of 1–20 μM as 100 μM treatment, as described (Fig. 3B), induced maximal strand breaks as measured by SCGE. Thus, further DNA strand breaks introduced by excision of FpG-sensitive sites would not have been detectable at this concentration. However, it was possible to measure oxidised purines in response to 100 μM kaempferol following the recovery period when DNA strand breakage was decreased (Fig. 3C). No increase in oxidised purines was observed after 90-min exposure to 1–20 μM kaempferol (Fig. 3D) or after the recovery period in response to the 1–100 μM kaempferol treatments (Fig. 3D).

3.4. Membrane integrity

DNA damage was not paralleled by changes to membrane integrity. Following 90-min incubation in kaempferol, no change to the extent of LDH leakage was observed (Fig. 4A) and there was no decline in the level of fluorescence within FDA-loaded cells relative to negative control treatments (Fig. 4B). Following the recovery period, membrane integrity, measured by FDA staining or trypan blue uptake, remained unaffected by the kaempferol treatments (Fig. 4C,D).

3.5. Cell proliferation following the post treatment recovery period

A decline in ^{3}H-thymidine incorporation was observed for cells treated with 10, 50, 100 μM kaempferol (Fig. 5A),

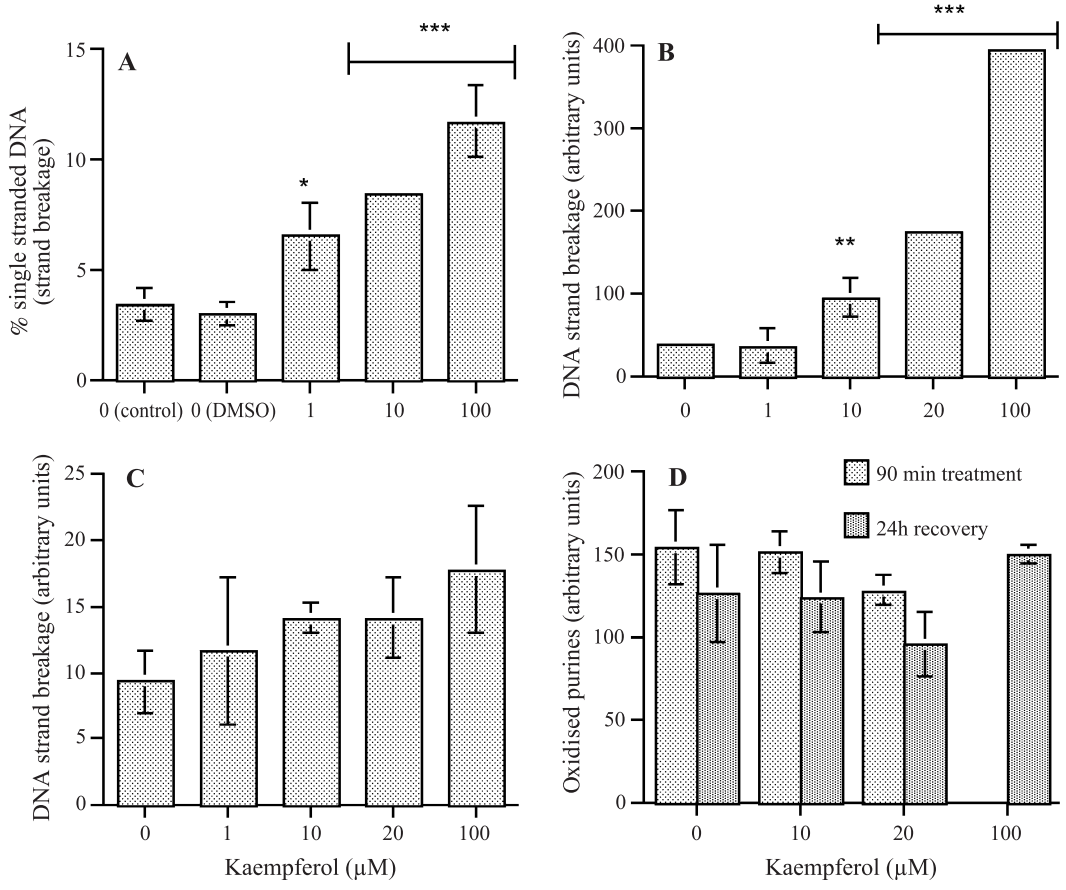

Fig. 3. DNA damage following exposure to kaempferol. The proportion of single-stranded DNA following incubation in alkali and hydroxyapatite-chromatography (A) or measurement of nucleoid tail fluorescence intensity after SCGE (B,C) was used as an indicator of single strand breaks immediately following the 90 min (A, B) treatment or after the recovery period (C). In addition, the level of oxidised purines (D) was estimated via detection of FpG-sensitive sites using SCGE. Data are means±SD (A, n=6, B–D n=4) *P=0.020, **P=0.015, **P<0.001 vs. DMSO control.

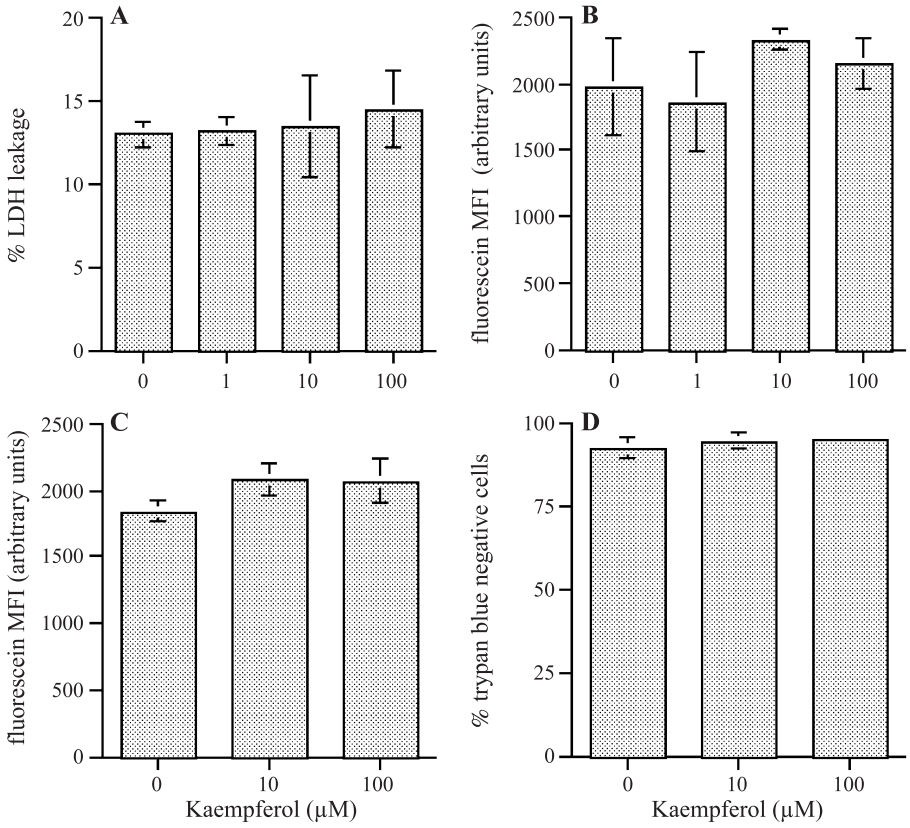

Fig. 4. Membrane damage following kaempferol exposure. Membrane damage was determined via LDH leakage (A), by retention of fluorescein fluorescence within FDA-loaded cells (B,C) and also via resistance to trypan blue staining (D) either immediately following the 90-min exposure to kaempferol (A,B) or after the recovery period in non kaempferol supplemented media (C,D). Data are means±SD (n=3). No significant differences were detected between kaempferol treatments vs. negative controls. Note that post recovery period the media fluorescence intensity (MFI, arbitrary units) of fluorescein within cultures treated with 1 μM etoposide decreased to 1658±68 compared to a DMSO control value of 1841±75% and 81.78±1.18 % of the cell population were resistant to trypan blue staining.

but there was only a trend towards decreased cell number following exposure to 100 μM kaempferol (Fig. 5B).

3.6. Cell cycle distribution following the post treatment recovery period

Cell cycle analysis (Fig. 6) revealed a significant decline in the proportion of cells in G_1 and to a lesser extent S-phase and an increase in cells in G_2-M phase following exposure to 100 μM kaempferol. There was also a significant increase in the proportion of cells with sub-G_1 DNA content. No changes to cell cycle distribution or sub-G_1 DNA content were apparent for cell populations exposed to 1 or 10 μM kaempferol.

3.7. Caspase-3 detection following the post treatment recovery period

An increase in the proportion of cells expressing active-caspase-3 was detected in cultures exposed to 100 μM kaempferol (Fig. 7). In comparsion to the 12.02±1.02% cells expressing active casapse-3 in response to 100 μM kaempferol, 52.47±4.5% of cells treated with camptothecin expressed active-caspase-3.

4. Discussion

Of the myriad of effects attributed to flavonoids, both their potential to exert antiproliferative and cytotoxic activity towards aberrant cells and also their ability to act as antioxidants has attracted attention as possible mechanisms contributing to the pathopreventative effects of fruit- and vegetable-rich diets [8–10].

There is, however, conflicting evidence for the efficacy of kaempferol and other flavonoids as antioxidants. In assessing flavonoid cytotoxicity and lipid peroxidation inhibiting activity, Cos et al. [34] identified kaempferol as possessing the highest antioxidant selectivity index, resulting from the maximal nontoxic dose divided by the IC_{50} value for lipid peroxidation. Conversely, quercetin, a highly abundant flavonol, which is frequently reported to exert protection against oxidative stress (e.g., Refs. [35–38]), exhibited a low antioxidant selectivity index. Kaempferol decreases oxidative DNA damage in isolated human lymphocytes [37] and decreases reactive oxygen levels and increases cell survival in oxidatively stressed HT-22 neuronal cells [38]. However, kaempferol does not protect differentiated Caco-2 colon adenocarcinoma cells, a frequently employed model for normal intestinal enterocytes, from oxidative stress-induced

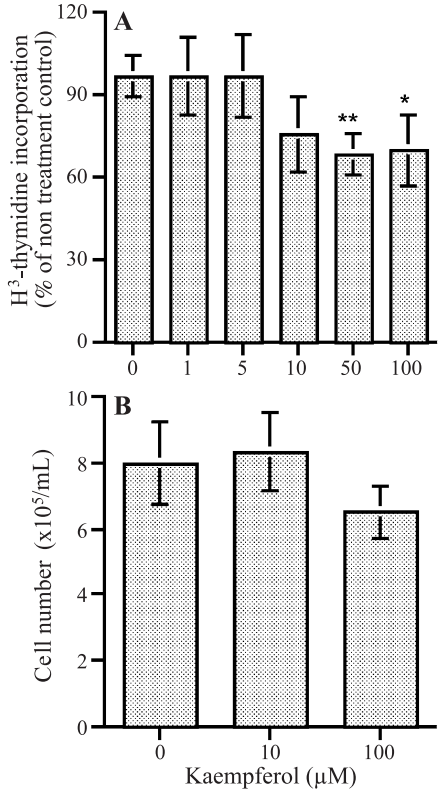

Fig. 5. Cell proliferation. Delayed ^{3}H-thymidine incorporation (A) was used to assess effects of kaempferol on replicative activity. Cells were treated with DMSO or kaempferol and the extent of ^{3}H thymidine incorporation compared to water-treated controls. Cell counts were also obtained (B). Data are means±SD (n=3) *P=0.039 **P=0.009 vs. DMSO control.

DNA damage [36] or H_2O_2–Fe^{2+} induced lipid peroxidation [39]. Kaempferol also exhibits pro-oxidant activity within acellular and cellular in vitro assay systems [40,41].

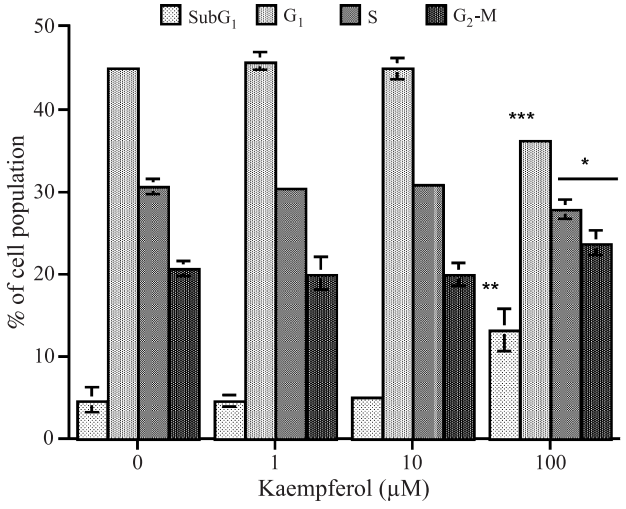

Fig. 6. Cell cycle distribution post recovery period. HL-60 cells were cultured for 90 min in the presence of DMSO or 1, 10 and 100 μM kaempefrol, washed and allowed to culture for a further 24 h in the absence of kaempefrol. Cell cycle distribution was analysed by flow cytometry. Data are means±SD (n=3) from a representative of three repeat experiments. Repeat experiments revealed an identical trend. *P<0.05, **P=0.007, ***P<0.001 vs. DMSO control.

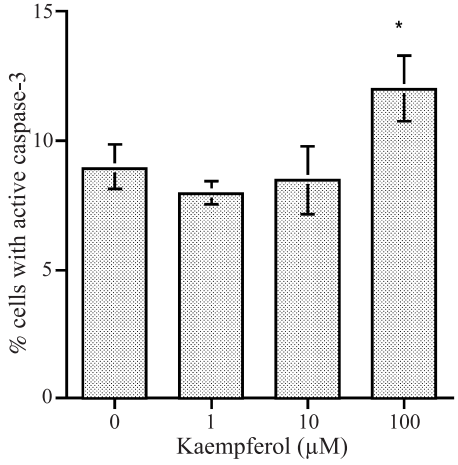

Fig. 7. Detection of active-caspase-3 post recovery period. HL-60 cells were cultured for 90 min in the presence of DMSO or 1, 10 and 100 μM kaempefrol, washed and allowed to culture for a further 24 h in the absence of kaempefrol and labelled with PE-conjugated polyclonal anti-active casapse-3 antibody. 52.47±4.5% cells were labelled following treatment with the apoptosis inducer camptothecin. Data are means±SD (n=3), *P=0.027 vs. DMSO control.

Such apparently contradictory data is mirrored by studies of flavonoid-rich diets. For example, decreased lymphocyte DNA damage is reported for individuals fed kaempferol- and quercetin-enriched diets, whereas elevated DNA damage was associated with phenol-depleted diets [42]. Conversely, Young et al. [43] observed evidence of decreased oxidative stress for individuals fed a diet depleted of flavonoid-rich fruit and vegetables. As stated by Young et al. [43], such results highlight the absence of a clearly identified role for dietary polyphenols in disease prevention.

Here, HL-60 cells loaded with DHR allowed the analysis of ROS levels in relation to changes in DNA and membrane integrity and cellular survival following kaempferol exposure. A previous study of kaempferol and HL-60 cells reported an LC_{50} of 125 μM following a 24-h exposure, which was correlated with pro-oxidant activity leading to lipid peroxidation [18]. Here, no increased membrane damage or heightened levels of ROS were apparent. Measurement of mitochondrial membrane potential indicated that rhodamine fluorescence, indicative of ROS, was unlikely to have been affected by any effect of kaempferol on mitochondrial function.

Thus, it would appear that prolonged incubation with high concentrations of kaempferol is required for the development of an overtly oxidatively stressed environment and for significant loss of membrane integrity within HL-60 cells. However, there was a concentration-dependent increase in DNA strand breakage. Paradoxically, strand breakage was detected even though the incubation in kaempferol (1–100 μM) initially resulted in a decrease to intracellular ROS levels. There was, however, no increase in the level of oxidised DNA purines implicating a highly localised and specific mechanism for kaempferol-induced DNA damage and not a generalised increase in oxidative stress. Similarly,

the flavonoids quercetin, myricetin and silymarin induce DNA strand breaks in a variety of cultured human cells without elevating levels of oxidised pyrimidines [44].

It is possible that DNA damage results from highly localised changes in ROS generation that do not translate into a general increase in oxidative stress. $HO^{\cdot}$ generation close to the DNA molecule may be responsible for strand breakage. Kaempferol (10–100 μM) promotes, in an Fe^{3+}/Cu^{2+} stimulated reaction, DNA damage and lipid peroxidation in isolated rat nuclei [40] but kaempferol antioxidant/pro-oxidant activity may be altered by localised changes to its immediate subcellular microenvironment. As an example, $HO^{\cdot}$ formation in the presence of ferric-chelates may be elevated, unaffected or inhibited by kaempferol depending on the nature of the chelate and/or the presence of ascorbate [45].

Kaempferol-induced DNA damage is not sustained within the cell population but probably accounts for the decline in ^{3}H-thymidine incorporation for cells exposed to >10 μM kaempferol. Cell cycle analysis revealed an increase in the proportion of cells within the G_2-M phase of the cell cycle and a decline in the proportion of G_1 and S-phase cells. Kaempferol induces G_2-M arrest in a number of cell types [9]. This accumulation in G2-M may directly result from the kaempferol-induced DNA damage. However, in OCM-1, human melanoma cells kaempferol induced G_2-M arrest results from impaired dephosphorylation of $p34^{cdc2}$ kinase by inactivation of the phosphatase cdc25c [46].

A variety of outcomes may follow G_2-M arrest. Cells may either re-enter the cell cycle following effective DNA repair, undergo apoptosis or changes to ploidy may develop due to exit from the cell cycle block in the absence of efficient DNA repair (e.g., Ref. [47]). Treatment of HL-60 cells for 12 h with 60 μM of the flavonoids apigenin, myricetin or quercetin induces apoptosis but kaempferol is ineffective within this period [22]. High concentrations of kaempferol are also required to exert antiproliferative activity towards Caco-2 (EC_{50} 163 μM) and HT-29 (EC_{50} 137 μM) colon carcinoma and MCF-7 (EC_{50} 155 μM) breast cancer cells, in each instance with little evidence of cytotoxicity [48].

Here, an increase in the proportion of cells with a sub-G_1 DNA content was observed in response to 100 μM kaempferol treatment. This is commonly, though not exclusively, associated with DNA fragmentation occurring during apoptosis [49]. In addition, an increase in the proportion of cells expressing active caspase-3, a cysteine protease [22], in the absence of detectable membrane damage, as revealed by the trypan blue assay, suggests the induction of an apoptosis-like event within a minor proportion of the cell population. However, a critical event in apoptosis is the sustained opening of the mitochondrial membrane permeability transition (MPT) pore, which results in the dissipation of the mitochondrial membrane potential [50]. Here, only a minor increase in the proportion of cells with decreased mitochondrial membrane potential was observed in response to 100 μM kaempferol after the 90-min exposure. Thus apoptosis is limited or, alternatively, caspase activity and DNA fragmentation may indicate a more general stress-damage status within the cell population.

Thus, despite initially lower intracellular ROS production, kaempferol induces adverse effects on DNA integrity, which might account for a limited antiproliferative effect observed with exposure to high kaempferol concentrations. Natural products, including flavonoids, have been proposed as direct therapeutics or as adjuncts to conventional cytotoxic chemotherapeutics for the treatment of acute myelogenous leukaemia [51]. Induced DNA damage, in an essentially less 'oxidative stressed environment' may be exploitable by rendering aberrant cells more susceptible to co-treatment with therapeutic agents. However, whether kaempferol also exerts such effects in normal non-transformed cells requires examination. Polyphenols may well demonstrate selective antiproliferative or cytotoxic effects against aberrant as opposed to normal cells [52] but Kuo [53] has suggested that intracellular targets of flavonoids, including kaempferol, are common to transformed and nontransformed intestinal epithelial cells.

Whether kaempferol exert such effects in a dietary context is unclear. Physiological flavonoid concentrations probably range from 0.1 to 10 μM L^{-1}, with individual plasma concentrations being generally sub-1 μM L^{-1} [39]. It is possible that individual cells or tissues may accumulate higher concentrations in response to supplementation [6], or that high dosages might more rapidly reflect cellular changes that occur following a prolonged and consistent period of dietary exposure. However, it is perhaps necessary to consider that many non-nutritive phytochemicals in either a dietary or supplemental context are unlikely to exert their activity in isolation from other dietary components. Fruits, vegetables and herbal medicines contain a myriad of other active polyphenols [1,5,7,8], many of which exhibit cell cycle modulatory and apoptosis-inducing activity [5,7,8]. Thus, kaempferol may be a component to such mixtures that ultimately exert bioactivity because of the combination of non-nutritive and micronutrient constituents rather than via one individual compound alone.

Finally, while there is little evidence for deleterious effects of naturally flavonoid rich diets in vivo [8,9,54], given the diversity of flavonoid effects on cellular function [54,55], it may be unwise to propose that enhanced supplemental intakes of flavonoids such as kaempferol will exert a general preventative or therapeutic role against degenerative diseases.

Acknowledgements

This work was funded by the Scottish Executive Environment and Rural Affairs Department (SEERAD). The authors thank Dr. G. Duthie and Prof. J. Arthur for critically reading the manuscript.

References

[1] G. Block, B. Patterson, A. Subar, Fruit, vegetables, and cancer prevention—A review of the epidemiologic evidence, Nutr. Cancer 18 (1992) 1–29.

[2] K.A. Steinmetz, J.D. Potter, Vegetables, fruit, and cancer I: Epidemiology (review), Cancer Cause Control 5 (1991) 325–357.

[3] J.W. Powles, Fruit and vegetables, and cardiovascular disease: a review, Int. J. Epidemiol. 26 (1997) 1–13.

[4] A. Trichopoulou, A. Naska, A. Antoniou, S. Friel, K. Trygg, A. Turrini, Vegetable and fruit: the evidence in their favour and the public health perspective, Int. J. Vitam. Nutr. Res. 73 (2003) 63–69.

[5] N.J. Temple, K.K. Gladwin, Fruit, vegetables, and the prevention of cancer: research challenges, Nutrition 19 (2003) 467–470.

[6] P.C.H. Hollman, M.B. Katan, Health effects and bioavailability of dietary flavonols, Free Radic. Res. 31 (1999) S75–S80.

[7] L. Bravo, Polyphenols: chemistry, dietary sources, metabolism and nutritional significance, Nutr. Rev. 56 (1998) 317–333.

[8] G.G. Duthie, S.J. Duthie, J.A.M. Kyle, Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants, Nutr. Res. Rev. 13 (2000) 79–106.

[9] E. Middleton, C. Kandaswami, T.C. Theoharides, The effect of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer, Pharmacol. Rev. 52 (2000) 673–751.

[10] G. Di Carlo, N. Mascolo, A.A. Izzo, F. Capasso, Flavonoids: old and new aspects of a class of natural therapeutic drugs, Life Sci. 65 (1999) 337–353.

[11] J.A. Knight, Diseases related to oxygen-derived free radicals, Ann. Clin. Lab. Sci. 25 (1995) 111–121.

[12] D. Dreher, A.F. Junod, Role of oxygen free radicals in cancer development, Eur. J. Cancer 32A (1995) 30–38.

[13] H. de Groot, U. Rauen, Tissue injury by reactive oxygen species and the protective effects of flavonoids, Fundam. Clin. Pharmacol. 12 (1998) 249–255.

[14] Y. Chen, R. Zheng, Z. Jia, Y. Ju, Flavonoids as superoxide scavengers and antioxidants, Free Radic. Biol. Med. 9 (1990) 19–21.

[15] J. Torel, J. Cillard, P. Cillard, Antioxidant activity of flavonoids and reactivity with peroxy radical, Phytochemistry 25 (1986) 383–385.

[16] S.R. Husain, J. Cillard, P. Cillard, Hydroxyl radical scavenging activity of flavonoids, Phytochemistry 26 (1987) 2489–2491.

[17] W.F. Hodnick, S. Ahmad, R.S. Pardini, Induction of oxidative stress by redox active flavonoids, in: J.A. Manthey, B.S. Buslig (Eds.), Flavonoids in the Living System, Plenum, New York, 1998, pp. 131–150.

[18] E. Sergediene, K. Jonsson, H. Szymusiak, B. Tyrakowska, I.M.C.M. Rietjens, N. Cenas, Pro-oxidant toxicity of polyphenolic antioxidants to HL-60 cells description of quantitative structure activity relationships, FEBS Lett. 462 (1999) 392–396.

[19] N. Yamashita, S. Kawanishi, Distinct mechanisms of DNA damage in apoptosis induced by quercetin and luteolin, Free Radic. Res. 33 (2000) 623–63320.

[20] I. Duarte Silva, J. Gaspar, A. Rodrigues, G. Gomes da Costa, A. Laires, J. Rueff, Mechanisms of myricetin mutagenicity in V79 cells: involvement of radicular species, Teratog., Carcinog., Mutagen. 16 (1996) 253–268.

[21] N. Yamashita, H. Tanemura, S. Kawanishi, Mechanism of oxidative DNA damage induced by quercetin in the presence of Cu(II), Mutat. Res., Fundam. Mol. Mech. 425 (1999) 107–115.

[22] I.-K. Wang, S.-Y. Lin-Shiau, J.-K. Lin, Induction of apoptosis by apigenin and related flavonoids through cytochrome *c* release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells, Eur. J. Cancer 35 (1999) 1517–1525.

[23] M. Marone, G. D'Andrilli, N. Das, C. Ferlini, S. Chatterjee, G. Scambia, Quercetin abrogates taxol-mediated signalling by inhibiting multiple kinases, Exp. Cell Res. 270 (2001) 1–12.

[24] L.I. Cipak, P. Rauko, E. Miadokova, I. Cipakova, L. Novotny, Effects of flavonoids on cisplatin-induced apoptosis of HL-60 and L1210 leukemia cells, Leuk. Res. 27 (2003) 65–72.

[25] M. Kitamura, Y. Ishikawa, Oxidant induced apoptosis of glomerular cells: intracellular signalling and its intervention by bioflavonoid, Kidney Int. 56 (1999) 1223–1229.

[26] R.P. Haugland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Leiden, The Netherlands, 1996.

[27] C.S. Bestwick, L. Milne, Quercetin modifies reactive oxygen levels but exerts only partial protection against oxidative stress within HL-60 cells, Biochim. Biophys. Acta 1528 (2001) 49–59.

[28] M.J. Burkitt, L. Milne, S.Y. Tsang, S.C. Tam, Calcium indicator dye quin2 inhibits hydrogen peroxide-induced DNA strand break formation via chelation of iron, Arch. Biochem. Biophys. 311 (1994) 321–328.

[29] S.J. Duthie, S. Narayanan, S. Blum, L. Pirie, G.M. Brand, Folate deficiency in vitro induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalised normal human colon cells, Nutr. Cancer 37 (2000) 245–251.

[30] S.J. Duthie, G. Grant, S. Naraynan, Increased uracil misincorporation in lymphocytes from folate-deficient rats, Br. J. Cancer 83 (2000) 1532–1537.

[31] Z. Darzynkiewicz, X. Li, Measurement of cell death by flow cytometry, in: T.G. Cotter, S.J. Martin (Eds.), Techniques in Apoptosis, Portland, London, 1996, pp. 71–106.

[32] C.S. Bestwick, L. Milne, Effects of β-carotene on antioxidant enzyme activity, intracellular reactive oxygen and membrane integrity within post confluent Caco-2 intestinal cells, Biochim. Biophys. Acta 1474 (2000) 47–55.

[33] S.K. Jonas, P.A. Riley, R.L. Willson, Hydrogen peroxide cytotoxicity: low-temperature enhancement by ascorbate or reduced lipoate, Biochem. J. 254 (1989) 651–655.

[34] P. Cos, M. Calomme, J.-B. Sindambiwe, T. De Bruyne, K. Cimanga, L. Pieters, A.J. Vlietinck, D.V. Berghe, Cytotoxicity and lipid peroxidation-inhibiting activity of flavonoids, Planta Med. 67 (2001) 515–519.

[35] S.A. Aherne, N.M. O'Brien, Mechanism of protection by the flavonoids quercetin and rutin against *tert*-butylhydroperxoide- and menadione induced DNA single strand breaks in Caco-2 cells, Free Radic. Biol. Med. 29 (2000) 507–514.

[36] S.J. Duthie, V.L. Dobson, Dietary flavonoids protect human colonocyte DNA from oxidative attack in vitro, Eur. J. Nutr. 38 (1999) 28–34.

[37] M. Noroozi, W.J. Angerson, E.J. Lean, Effect of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes, Am. J. Clin. Nutr. 67 (1998) 1210–1218.

[38] K. Ishige, D. Schubert, Y. Sagara, Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms, Free Radic. Biol. Med. 30 (2001) 433–446.

[39] I.-W. Peng, S.-M. Kuo, Flavonoid structure affects the inhibition of lipid peroxidation in Caco-2 intestinal cells at physiological concentrations, J. Nutr. 133 (2003) 2184–2187.

[40] S.C. Sahu, G.C. Gray, Kaempferol-induced nuclear DNA damage and lipid peroxidation, Cancer Lett. 85 (1994) 159–164.

[41] N. Sugihara, T. Arakawa, M. Ohnishi, K. Furuno, Anti- and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with alpha-linolenic acid, Free Radic. Biol. Med. 27 (1999) 1313–1323.

[42] H.Y. Kim, O.H. Kim, M.K. Sung, Effects of phenol-depleted and phenol-rich diets on blood markers of oxidative stress, and urinary excretion of quercetin and kaempferol in healthy volunteers, J. Am. Coll. Nutr. 22 (2003) 217–223.

[43] J.F. Young, L.O. Dragsted, J. Haraldsdottir, B. Daneshvar, M.A. Kall, S. Loft, L. Nilssson, S.E. Nielson, B. Mayer, L.H. Skibsted, T. Huynh-Ba, A. Hermetter, B. Sandstrom, Green tea extract only affects markers of oxidative status postprandially: lasting antioxidant effect of flavonoid-free diet, Br. J. Nutr. 87 (2002) 343–355.

[44] S.J. Duthie, W. Johnson, V.L. Dobson, The effect of dietary flavonoids on DNA damage (strand breaks and oxidised pyrimidines) and growth in human cells, Mutat. Res. 390 (1997) 141–151.

[45] A. Puppo, Effect of flavonoids on hydroxyl radical formation by Fenton-type reactions; influence of the iron chelator, Phytochemistry 31 (1992) 85–88.
[46] F. Casagrande, J.-M. Darbon, Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1, Biochem. Pharmacol. 61 (2001) 1205–1215.
[47] S. Villamarin, N. Ferrer-Miralles, S. Mansilla, W. Priebe, J. Portugal, Induction of G2/M arrest and inhibition of c-myc and p53 transcription in Jurkat T lymphocytes, Biochem. Pharmacol. 63 (2002) 1251–1258.
[48] S. Kuntz, U. Wenzel, H. Daniel, Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity and apoptosis in human colon cancer cell lines, Eur. J. Nutr. 38 (1999) 133–142.
[49] Z. Darzynkiewicz, X. Li, J. Gong, S. Hara, F. Traganos, Analysis of cell death by flow cytometry, in: G.P. Studzinski (Ed.), Cell Growth and Apoptosis, IRL press, Oxford, 1995, pp. 143–167.
[50] D.R. Green, J.C. Reed, Mitochondria and apoptosis, Science 281 (1998) 1309–1312.
[51] J.L. Liesveld, C.N. Abboud, C. Lu, C. McNair, A. Menon, A. Smith, K. Rosell, A.P. Rapoportc, Flavonoid effects on normal and leukemic cells, Leuk. Res. 27 (2003) 517–527.
[52] Z.P. Chen, J.B. Scell, C.-T. Ho, K.Y. Chen, Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counter parts, Cancer Lett. 129 (1998) 173–179.
[53] S.-M. Kuo, Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells, Cancer Lett. 110 (1996) 41–48.
[54] C.F. Skibola, M.T. Smith, Potential health impacts of excessive flavonoid intake, Free Radic. Biol. Med. 29 (2000) 375–383.
[55] I.D. Silva, J. Gaspar, G. Gomes da Costa, A.S. Rodrigues, A. Laires, J. Rueff, Chemical features of flavonols affecting their genotoxicity. Potential implications in their use as therapeutic agents, Chem.-Biol. Interact. 124 (2000) 29–51.