Watercress and Health Research Pack
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**Watercress: good for every body.**

**The scientific research so far.**

Watercress has been grown on our farms in the UK since the 1850’s, with many claiming it as the 'original' fast food. However, despite this leafy green’s rich history, many people are unaware of the powerful health benefits watercress possesses, especially concerning its anti-carcinogenic properties and its combative potential against oxidative stress.

A unique defence, which combats DNA damage that leads to the proliferation of cancer, was discovered in 2007, when UK researchers identified the cancer inhibiting properties of watercress.

The research that has attracted so much scientific attention has been focussed upon a unique reaction, which occurs when watercress is chewed or digested. This process brings the enzyme myrosinase into contact with glucosinolates that are found abundantly in watercress- precursors for compounds called isothiocyanates (ITC’s). Much of the published research has investigated a particular type of ITC called phenethyl isothiocynate (PEITC), of which watercress is its highest natural source.

In 2007 The Watercress Alliance, made up of The Watercress Company, Vitacress and Bakkavor, sponsored the first published study on watercress carried out by Professor Ian Rowland Et Al at the University of Ulster. The study included 60 participants made up of 30 smokers and 30 non-smokers. During the treatment stage, participants were required to eat one portion of raw watercress daily (85g) for 8 weeks in addition to their normal diet.

The results demonstrated that watercress supplementation led to a decrease in DNA damage in white blood cells by 22.9%, as well as showing an increase in blood concentration of two key antioxidants: lutein and B-carotene. The enhanced levels of these antioxidants increases the cells capacity to combat the effects of free radicals, offering protection against the changes that occur in human cells’ that may lead to cancerous development.

Professor Rowland comments "Our earlier published research into the effects of watercress extracts on human cells in vitro, had indicated that watercress had the potential to reduce DNA damage. DNA damage can have many adverse consequences in the human body, so it was very exciting and satisfying to show that the damage could be reduced when watercress was consumed by volunteers, in a real life situation."

This discovery kick-started the momentum for research into the health benefits of watercress, opening up a wealth of opportunity for scientific development.

In 2009, Professor Graham Packham, from the University of Southampton, headed a research project looking specifically at the phenethyl isothiocynate (PEITC) contained within watercress.

The research found that the gluconasturtiin within watercress is a precursor for the production of PEITC, which has been proven to interfere with a protein called hypoxia inducible factor (HIF), which plays a vital role in the development of cancerous cells.

As tumours grow they rely upon a constant supply of oxygen necessary for their development. However as they grow larger the centre of the growth becomes distanced from the oxygen supply, subsequently receiving less oxygen and fewer nutrients. To counter this, cancer cells send out signals that are responsible for encouraging the growth of new capillaries into the tumour. PEITC, found within watercress, has been shown to block the signals of this protein, proving vital in the suppression of cancerous cell development and tumour growth.
Professor Packham commented in 2009 saying “The research takes an important step towards understanding the potential health benefits of this crop since it shows that eating watercress may interfere with a pathway that has already been tightly linked to cancer development.”

It wasn’t long before more research was uncovered, with Fogarty Et Al (2012) widening the focus of the powerful properties watercress possesses in preventing DNA damage by applying it to an exercise environment.

The study used a sample of ten 'healthy' males who were required to ingest 85g of watercress daily for 8 weeks. The participants were then asked to engage in high intensity exercise on a treadmill. This was compared to a control group where no watercress was to be consumed with the same program of activity, as well as a further group who consumed watercress acutely 2 hours before exercise.

The results found that exercise increases DNA damage, as well as increasing lipid peroxidation, whilst watercress consumed chronically or acutely attenuated DNA damage as well as lipid peroxidation. This provides support for the powerful protective effects of this leafy green vegetable and highlights its contribution to mitigating exercise induced DNA repair.

Commenting this week on his previous research and future expectations for watercress, Dr. Fogarty said, "We all know that regular exercise is extremely beneficial for our overall health. What we often forget is that each specific workout can actually be quite stressful. The type of stress that exercise causes is actually quite similar to the other types of stresses our bodies are exposed to, such as environmental pollution, cigarette smoke and even sun light. The role of foods such as watercress in our diet is therefore of extreme importance, as we've shown it to help dampen the body's response to stress. So, regardless if you run marathons, swim the channel or simply walk slightly quicker because you're late for the train to work, watercress can help your body deal with the daily stresses it is exposed to."

Fogarty continues, "The next stage of our research is to find out if this interesting food can also help improve exercise performance. There are so many weird and wonderful chemicals found in this plant that we've only touched the tip of the iceberg in regards to what it can do for overall human performance, not just the Chris Froomes of this world."

These new horizons for watercress research are already well underway, with some impressive contributions coming from the South West.

Dr Kyle Stewart, a GP trainee in Torbay, and a collaboration of Torbay Medical Research Fund and The University of Exeter are currently extracting and classifying clinically useful urease inhibitors from watercress.

Dr. Stewart commented “The urease enzyme is implicated in a range of pathological states in humans, and it may very well be that this powerful natural product could be the basis of novel therapeutics both in primary and secondary care. Of particular importance is how this may play a role in overcoming antibiotic multi-resistance in some pathogens. The team has identified clinical and non-clinical markets for watercress and are enjoying the partnership with The Watercress Company in Dorset, who are of paramount importance in helping us understand the botanical aspects of this exciting work.”

It is evident that these health claims aren’t just embellished folklore; they have scientific backing and could provide ground- breaking horizons for households all over the world. So keep an eye on this leafy vegetable and watch it grow. It's no coincidence we've been eating watercress for over 2000 years but we're only just beginning to scratch the surface.
Further research papers and readings that may be of interest.

- **Cancer chemoprevention with dietary isothiocyanates mature for clinical translational research.**
  

- **Development of a Novel Experimental *In Vitro* Model of isothiocyanate-induced Apoptosis in Human Malignant Melanoma Cells**
  

- **7-Methylsulfinylheptyl and 8-methylsulfinyloctyl isothiocyanates from watercress are potent inducers of phase II enzymes.**
  

- **Design, synthesis, and identification of a novel napthalamide-isoselenocyanate compound NISC-6 as a dual Topoisomerase-IIα and Akt pathway inhibitor, and evaluation of its anti-melanoma activity.**
  
• **Molecular targets of isothiocyanates in cancer: Recent advances.**


• **Glucosinolates and isothiocyanates in health and disease**


• **Prostate cancer chemoprevention by dietary isothiocyanates is associated with suppression of lipogenesis**


• **CXCR4 is a novel target of cancer chemopreventative isothiocyanates in prostate cancer cells**


• **Isothiocyanates suppress the invasion and metastasis of tumors by targeting FAK/MMP-9 activity.**

Inhibition of hypoxia inducible factor by phenethyl isothiocyanate

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Phenethyl isothiocyanate (PEITC), a natural dietary isothiocyanate, has anti-cancer activity in various in vitro and in vivo models. PEITC inhibits angiogenesis but the molecular mechanisms that underlie this effect are not known. We have now demonstrated that PEITC is an effective inhibitor of hypoxia inducible factor (HIF), a transcription factor that plays an important role in expression of pro-angiogenic factors. PEITC inhibited the activation of a HIF-dependent reporter construct following incubation of cells in hypoxia, or treatment with the hypoxia mimetic cobalt chloride. PEITC also interfered with the accumulation of HIF1α protein and induction of the endogenous HIF target genes, CAIX, GLUT1, BNIP3 and VEGF-A. The ability of PEITC to inhibit HIF activity was independent of the activity of prolyl hydroxylases, the Von-Hippel–Landau protein and the proteasome, all of which are required for the normal rapid turnover of HIF1α in normoxia. Decreased expression of HIF1α in PEITC treated cells was not associated with changes in the levels of HIF1α RNA suggesting that PEITC may inhibit HIF activity by decreasing translation of the HIF1α RNA. Consistent with this, PEITC decreased phosphorylation of the translation regulator 4E-BP1. Our data demonstrate that PEITC is an effective inhibitor of HIF activity. This may contribute to the anti-angiogenic and anti-cancer effects of PEITC.

1. Introduction

Isothiocyanates (ITCs) are a group of structurally related compounds with potential chemopreventive and anti-cancer activity [1–3]. Natural ITCs derived from cruciferous vegetables have anti-cancer activity in both in vitro and in vivo models, and increased dietary consumption of ITCs has been linked to reduced cancer risk in humans [4]. ITCs are derived by hydrolysis of specific β-thioglucoside N-hydroxysulfate (glucosinolate) precursors by the action of the plant enzyme myrosinase, activated following damage to the leaf (e.g., chopping or chewing). For example, broccoli is a rich source of glucoraphanin, the glucosinolate precursor of sulforaphane (SFN) and watercress is a rich source of gluconasturtiin, the precursor of phenethyl isothiocyanate (PEITC).

The ability of ITCs to inhibit the development of carcinogen-induced cancers is perhaps the best understood activity of ITCs [5–7]. ITCs are thought to inhibit carcinogen-induced carcinogenesis by modulating carcinogen metabolism via (i) inhibiting the activity of phase I cytochrome P450 enzymes and (ii) inducing phase II detoxifying and antioxidant gene expression, including glutathione-S-transferases, NAD[P]H:quinone oxidoreductase, UDP-glucuronosyl transferase and thioredoxin reductase. Induction of phase II gene expression is mediated by the Nrf2 transcription factor [8–10]. In the absence of inducers, Nrf2 is inactivated by association with the cysteine-rich Keap1 protein which inhibits Nrf2, at least in part, by acting as a ubiquitin ligase adaptor protein, targeting Nrf2 for ubiquitination and proteasomal degradation [11–13]. SFN and PEITC are potent activators of Nrf2 and this appears to be dependent on ITC-mediated electrophilic attack and conjugation of several “sensor” cysteines within Keap1 [14–18]. This is thought to release Nrf2 from Keap1-mediated negative regulation. Nrf2 then accumulates in the nucleus where, in concert with the small Maf protein, it binds to antioxidant/electrophile response elements in target genes to activate transcription. Activation of MAP kinase signalling pathways and phosphorylation of Nrf2 may also contribute [17]. The ability of SFN to interfere with benz[a]pyrene-induced gastric tumour formation is significantly reduced in Nrf2 deficient mice, demonstrating the importance of this pathway for ITC-mediated protection from carcinogen-induced carcinogenesis [19].

In addition to their established chemopreventive activity, ITCs also exert activity against established cancer cells [20,21]. For example, PEITC inhibits the growth of PC3 prostate cancer cells, associated with the downregulation of the positive cell cycle regulators, cdk1 and cdc25C, and SFN induces metaphase arrest in pancreatic cancer lines [22,23]. ITCs promote mitochondrial damage and apoptosis in various cells types and PEITC enhances apoptosis in
primary leukaemia cells [24]. ITCs have been demonstrated to slow the growth of various human cancer and oncogene-transformed cell lines when grown as xenografts in immunocompromised animals, and to suppress tumour formation in Apc-deficient immunocompetent animals [22,25–29].

The mechanisms by which ITCs inhibit the growth and survival of established cancer cells are likely to be complex. Similar to effects on Keap1, conjugation of ITCs to key cell regulatory proteins is thought to be important [20]. At early time points following addition to cells, ITCs are bound predominantly to glutathione (GSH), a major cellular antioxidant [30,31]. ITC–GSH conjugates are exported from the cell where hydrolysis of these conjugates leads to regeneration of ITCs. The regenerated ITCs are then taken back up into cells and the net effect of this cycle is a rapid accumulation of ITCs within cells (perhaps up to 100 times over the extracellular concentration) and depletion of intracellular GSH [20]. Once GSH levels are depleted, ITCs conjugate to cellular proteins [20,32]. Presumably conjugation to cell cycle and cell death regulatory proteins leads to altered function, triggering induction of cell cycle arrest and apoptosis. However, the critical targets involved in growth inhibition by ITCs are not known and to our knowledge the only protein other that Keap1 that has been identified as a direct target for ITCs is α-tubulin [33]. SFN has also been demonstrated to inhibit the activity of NF-κB and AP-1 transcription factors [34,35]. Although these transcription factors contain reactive thiol(s), the mechanism of inhibition, and the contribution of these effects to the cell growth inhibitory and apoptosis-promoting effects of ITCs is unclear. In addition to direct conjugation of cellular proteins by ITCs, metabolites of PEITC and SFN may also play an important role, for example, via inhibition of histone deacetylase activity [1]. A further potential mechanism by which ITCs promote cell growth inhibition is via increasing cellular stress. For example, GSH-depletion leads to an increase in intracellular reactive oxygen species (ROS) and in some systems, ITC-induced growth inhibition is suppressed by antioxidants [20,25].

Angiogenesis, the formation of new blood vessels, plays a critical role in tumour development and metastasis and is considered one of the six hallmarks of the cancer cell [36]. Early stage tumours can grow independent of angiogenesis up to a size of 1–2 mm³, however, inadequate supply of oxygen and nutrients and accumulation of toxic metabolites limits growth beyond this size. Thus, the switch to a pro-angiogenic state is a critical step in cancer development [37]. The molecular mechanisms that promote angiogenesis in cancer cells are complex, however, the hypoxia inducible factor (HIF) family of oxygen-sensitive transcription factors play a key role. The best studied member is HIF1α which forms a transcriptionally active heterodimeric complex with HIF1β [38,39]. In normoxic conditions, the expression of HIF1α is maintained at very low levels since it is effectively ubiquitinated and targeted for rapid proteasomal degradation by the Von-Hippel–Landau (VHL) protein, a component of an E3 ubiquitin ligase complex. Recognition of HIF1α by the VHL complex in normoxia is mediated by hydroxylation of specific proline residues (HIF1α Pro402 and Pro564) by oxygen-dependent, Fe-containing prolyl hydroxylases. When oxygen levels are sufficiently reduced prolyl hydroxylase activity is inhibited, and HIF1α is no longer modified and is stabilised. HIF1α then translocates to the nucleus where, as part of a dimeric complex with the constitutively expressed HIF1β protein, it modulates expression of specific target genes involved in the regulation of angiogenesis, e.g., vascular endothelial growth factor-A (VEGF-A), metabolism (glucose transporter type 1 (GLUT1); SLC2A1) and apoptosis/survival (BCL2/adenosine E1B 19kd-interacting protein (BNIP3)) [38,39]. The activity of HIF is also influenced by ROS [40]. Treatment of cells with hydrogen peroxide is sufficient to stabilise HIF1α and induce expression of HIF target genes, even in normoxia [41]. Moreover, HIF activation in normoxia and hypoxia is inhibited by antioxidants such as GSH, its metabolic precursor N-acetylcysteine (NAC), and catalase [42–47]. Both cytosolic and mitochondrial sources of ROS have been implicated in HIF activation [43,48–51].

Several studies have demonstrated that ITCs interfere with angiogenesis pathways. PEITC decreased survival of human umbilical vein endothelial cells (HUVEC) and inhibited the formation of capillary-like tube structures and migration by HUVEC in vitro [52]. PEITC also inhibited ex vivo angiogenesis analysed using the chicken egg chorioallantoic membrane assay. SFN inhibited migration of human dermal microvascular endothelial cells (HMEC1) in an in vitro wound healing assay and inhibited tube formation of HMEC-1 cells on basement membrane [53]. SFN also inhibited the growth of HMEC-1 cells and bovine aortic endothelial cells [33,54].

Angiogenesis plays a critical role early in tumour development, and its inhibition may play a major role in the chemopreventive/anti-cancer effects of ITCs. Since HIF plays a central role in angiogenesis, we have investigated the effects of PEITC on HIF activity.

2. Materials and methods

2.1. Cell culture and chemicals

Human MCF7 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). Human RCC4 renal carcinoma cells were obtained from CR-UK Research Services (London, UK). All cell lines were maintained in Dulbecco’s Modified Eagle’s medium (DMEM; Lonza group Ltd., Basel, Switzerland) supplemented with 10% fetal calf serum (PAA Laboratories, Yeovil, UK), 1 mM L-glutamine and penicillin/streptomycin (Lonza group Ltd.). PEITC, NAC, Catalase, Trolox, cycloheximide, desferrioxamine (DFO) and cobalt chloride (CoCl₂) were from Sigma (Poole, UK). MG132 was from Enzo Life Sciences (UK) Ltd., Exeter, UK. To induce hypoxia, cells were cultured in 5% CO₂, 94.9% Argon, 0.1% O₂ in a sealed chamber.

2.2. Cell growth inhibition and apoptosis assays

MCF7 cells were plated at a density of 1000 cells per well of a 96-well plate in 50 μl complete growth media. RCC4 cells were plated at a density of 5000 cells per well of a 96-well plate in 50 μl complete growth media. The following day cells were treated in triplicate with PEITC or dimethyl sulfoxide (DMSO) as a solvent control, or were left untreated. DMSO was used at a dilution equivalent to the highest concentration of PEITC tested in each assay. After 6 days, relative cell number was determined using the CellTiter 96® AQueous One Solution Reagent (Promega, Southampton, UK) according to the manufacturer’s instructions. Relative cell number was calculated as a percentage of untreated cells. Apoptosis was analysed using the fluorescein isothiocyanate (FITC) annexin V apoptosis detection kit II (BD Pharmingen, Oxford, UK) according to the manufacturer’s instructions.

2.3. Reporter gene assays

MCF7 cells (grown in a 10 cm tissue culture dish) were transfected with 4 µg of the HIF-dependent luciferase reporter construct pGL2-TK-HRE [55] (a kind gift of Giovanni Melillo, Tumor Hypoxia Laboratory, NCI, USA) or pGL3–promoter (Promega) using Transfast (Promega) according to the manufacturer’s instructions. After 24 h, cells were recovered and plated in 96-well plates (2000 cells/well). Cells were incubated for 5 h to allow cells to adhere before being treated to activate HIF in the presence or
absence of PEITC or other agents. In experiments using NAC, Trolox or catalase, cells were pretreated with 10 mM NAC, 100 μM Trolox or 2000 units catalase for 2 h prior to induction of HIF. Luciferase activity was measured after 24 h by addition of 100 μl of BrightGlow (Promega) according to the manufacturer’s instructions.

2.4. Quantitative-reverse transcription-polymerase chain reaction (Q-RT-PCR)

Total RNA was isolated using Trizol (Invitrogen, Paisley, UK) and the quantity and quality of RNA was analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., South Queensferry, UK). cDNA was synthesised using oligo(dT) and MMLV reverse transcriptase (Promega) according to the manufacturer’s instructions. cDNA was synthesised in a 25 μl reaction containing 1 μg of total RNA, oligo(dT) primer and MMLV reverse transcriptase, then diluted to 100 μl using nuclease free water. Q-RT-PCR was performed in 20 μl reactions containing 5 μl cDNA, 10 μl Universal Taqman PCR master mix (Applied Biosystems, Warrington, UK) and 1 μl of the Taqman Gene Expression Assay of interest (Applied Biosystems). Expression assays used for this study were: BNIP3 (Hs00969293_mH), VEGF-A (Hs00173626_m1), CAIX (Hs00154208_m1), GLUT1 (Hs00892681_m1) and β-actin (Hs99999903_m1). All reactions were performed in duplicate using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) according to the following thermal cycle protocol: 94 °C 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Control reactions with no cDNA were run on each plate for each Taqman gene Expression Assay used and no amplification was detected in any control reaction. All expression values were normalised using expression of β-actin as a control.

2.5. Western immunoblotting

ImmunobLOTS were performed as previously described [56] using a mouse monoclonal anti-HIF1α antibody (BD Biosciences, Oxford, UK), a mouse monoclonal anti-HIF2α antibody (Abcam, Cambridge, UK), a rabbit polyclonal anti-4E-BP1 antibody (Cell Signalling Technology, Danvers, MA, USA) and a rabbit anti-β-actin antibody (Sigma). Horseradish peroxidase conjugated secondary antibodies were from GE Healthcare UK (Amersham, UK) and bound immunocomplexes were detected using SuperSignal West Pico Chemiluminescent reagents (Perbio Science UK Ltd., Northumberland, UK).

2.6. Statistics

The statistical significance of any differences was analysed using Student’s t-test with Bonferroni correction to correct for multiple testing.

3. Results

3.1. Growth inhibition and induction of apoptosis by PEITC in MCF7 cells

We selected human MCF7 breast cancer cells to investigate the effects of PEITC on HIF since their response to hypoxia has been well characterised [57,58]. We first characterised the effects of PEITC on the growth and survival of these cells. Consistent with representative experiment is shown. (C) Quantitation of apoptosis in PEITC-treated MCF7 cells (24 h). AV (annexin V) (F1-FL1-H) and propidium iodide (PI; F2-FL2-H) positive cells was determined by flow cytometry. A

Fig. 1. PEITC-induced growth inhibition and apoptosis in MCF7 cells. (A) Representative growth inhibition experiments. MCF7 cells were incubated with the indicated concentrations of PEITC ( ), or DMSO at a dilution equivalent to the highest concentration of PEITC ( ). After 6 days, relative cell numbers were determined using the CellTiter 96® AQueous One Solution reagent. Results are derived from means of triplicate wells (±SD). (B) MCF7 cells were incubated with the indicated concentrations of PEITC for 24 h. The proportion of annexin V (F1-FL1-H) and propidium iodide (PI; F2-FL2-H) positive cells was determined by flow cytometry. A
previous studies demonstrating growth inhibitory effects of PEITC in MCF7 cells [59,60]. PEITC inhibited MCF7 cell growth with an IC50 of 10.8 ± 1.7 μM (mean ± SD) in 6 day assays (Fig. 1A). PEITC also increased the percentage of Annexin V positive cells (a marker of apoptosis), although overall the levels of PEITC-induced apoptosis in MCF7 cells were relatively modest (Fig. 1B and C).

3.2. **PEITC inhibits HIF-dependent transcriptional activity**

To investigate the effects of PEITC on HIF activity, we first analysed the effects of PEITC on activity of a HIF-dependent reporter construct [55]. As a control we also analysed the activity of the SV40-promoter based reporter plasmid pGL3-promoter. Cells were transfected with the HIF or control reporter constructs and cultured under normoxic or hypoxic (0.1% O2) conditions, in the presence of increasing amounts of PEITC or DMSO as a solvent control.

As expected, hypoxia caused a strong induction of the HIF reporter construct, whereas the activity of the control construct was modestly decreased under hypoxic conditions (Fig. 2A). PEITC caused a statistically significant dose dependent decrease in the activity of the HIF reporter in cells under hypoxic conditions with an IC50 of ~3 μM. There was also a trend for PEITC to reduce basal activity of the HIF reporter in cells under normoxic conditions. (Although this is not evident from the data shown in Fig. 2A, due to the strong induction of reporter gene activity by hypoxia, it is clearly shown in subsequent experiments using CoCl2 which gives a lower level of HIF reporter activation, Fig. 2B.) These effects were specific at PEITC concentrations up to 13 μM, since the activity of the control reporter construct was unaltered. However, in some experiments 26 μM PEITC reduced activity of the control reporter under both normoxic and hypoxic conditions (see Fig. 2B). This presumably reflects non-specific inhibition at this higher concentration.

To confirm these findings, we investigated the effects of PEITC on the ability of CoCl2, a well studied hypoxic mimetic, to activate HIF in normoxic conditions. Similar to hypoxia, CoCl2 increased expression of the HIF reporter construct, with little effect on the control reporter construct, and this was inhibited in a dose dependent manner by increasing concentrations of PEITC (Fig. 2B).

3.3. **PEITC inhibits induction of HIF target genes**

Activation of HIF is associated with increased expression of HIF target genes. To determine the effect of PEITC on HIF target genes, MCF7 cells were exposed to hypoxia for 16 h in the presence or absence of PEITC and expression of CAIX, VEGF-A, BNIP3 and GLUT1 were analysed by Q-RT-PCR. CAIX was relatively strongly induced by hypoxia (50-fold) and this was statistically significantly inhibited by PEITC in a dose dependent manner (Fig. 3A). PEITC also caused a statistically significant inhibition of the induction of BNIP3, VEGF-A and GLUT1, although these genes were less strongly induced by hypoxia alone (3–5 fold) and were less sensitive to the inhibitory effects of PEITC. PEITC also inhibited the induction of CAIX expression following treatment of MCF7 cells with CoCl2 under normoxic conditions (Fig. 3B).

3.4. **PEITC inhibits accumulation of HIF1α and HIF2α**

To investigate the mechanism by which PEITC inhibited HIF-dependent transcription, we first analysed the effects of PEITC on
Fig. 3. Regulation of endogenous HIF target genes. (A) MCF7 cells were left untreated (unt) as a control or incubated in hypoxic conditions for 16 h in the presence or absence of the indicated concentrations of PEITC (μM), or DMSO as a control. Expression of (i) CAIX, (ii) VEGF-A, (iii) GLUT1 and (iv) BNIP3 were analysed by Q-RT-PCR. (B) MCF7 cells were left untreated (unt) as a control or treated with CoCl2 (100 μM) for 24 h in the presence or absence of the indicated concentrations of PEITC (μM), or DMSO as a control. Expression of CAIX was analysed by Q-RT-PCR. In both experiments, the amount of DMSO used was equal to that in cells treated with 25 μM PEITC. Data are mean of duplicate determinations, normalised to expression of β-actin. Relative expression in untreated cells was set to 1.0. Statistically significant differences between DMSO and PEITC treated cells are indicated (*p < 0.05; **p < 0.01; ***p < 0.005). All other differences were not statistically significant.
accumulation of HIF1α in cells cultured under hypoxic conditions. MCF7 cells were exposed to hypoxia for 5 h in the presence or absence of PEITC (Fig. 4A). As expected, HIF1α protein levels were barely detectable in cells in normoxia, but were strongly increased in cells exposed to hypoxia, due to inhibition of proteasomal degradation. Consistent with the ability of PEITC to inhibit HIF-dependent transcription, PEITC decreased the accumulation of HIF1α. Similar results were obtained when HIF1α levels were increased in normoxia by treating cells with CoCl2 (Fig. 4B).

In addition to HIF1α and HIF1β, the HIF family of transcription factors also contains HIF2α and HIF3α, which like HIF1α, are also induced by hypoxia and form transcriptionally active heterodimers with HIF1β [61,62]. Although the function of HIF3α has not been well characterised, HIF2α also appears to play a role in angiogenesis and carcinogenesis, and may regulate the expression of distinct, but overlapping, set of target genes compared to HIF1α [61,62]. Like HIF1α, HIF2α is targeted for rapid proteasomal degradation in normoxic conditions via the action of VHL and prolyl hydroxylases [63,64]. To investigate the effects of PEITC on HIF2α, MCF7 cells were exposed to hypoxia for 16 h in the presence or absence of PEITC (Fig. 4C). We selected the 16 h time point because accumulation of HIF2α was relatively slow, but was maintained for a protracted period in MCF7 cells compared to HIF1α. Differences in kinetics of activation of HIF1α and HIF2α have been reported in other cell types [65,66]. Similar to HIF1α, PEITC strongly decreased the accumulation of HIF2α.

3.5. Inhibition of HIF activity by PEITC is independent of prolyl hydroxylases

Ubiquitylation of HIF1α is dependent on the activity of prolyl hydroxylases and inhibition of these enzymes causes accumulation of HIF1α under normoxic conditions. To determine whether inhibition of HIF1α by PEITC required activity of prolyl hydroxylases, we investigated whether PEITC could also inhibit activation of HIF following prolyl hydroxylase inhibition. MCF7 cells were transfected with the HIF and control reporter constructs and treated with PEITC in the presence or absence of the prolyl hydroxylase inhibitor DFO. DFO inhibits prolyl hydroxylases by chelating the Fe atom that is present within the active site of these enzymes. As expected, DFO caused a strong induction in HIF reporter gene activity under normoxic conditions (Fig. 5). Treatment with PEITC caused a statistically significant and dose dependent reduction of HIF-dependent activity. Therefore, inhibition of HIF1α activity by PEITC is not dependent on the activity of prolyl hydroxylases.

3.6. Inhibition of HIF activity by PEITC is independent of VHL and the proteasome

The VHL protein plays a critical role in targeting HIF1α for proteasomal degradation downstream of prolyl hydroxylases. To determine whether the effects of PEITC were dependent on VHL, we analysed the activity of PEITC in VHL-deficient RCC4 renal carcinoma cells that have constitutive HIF1α activity in normoxia [67]. PEITC inhibited the growth of RCC4 cells with an IC50 of 44.0 ± 2.0 (mean ± SD). Therefore these cells are somewhat less sensitive to the growth inhibitory effects of PEITC compared to MCF7 cells. In RCC4 cells, PEITC caused a loss of HIF1α expression (Fig. 6A), decreased activity of the HIF reporter construct (Fig. 6B), and decreased expression of CAIX RNA (Fig. 6C), although somewhat higher concentrations of PEITC were required compared to MCF7 cells.

To determine whether the effects of PEITC were dependent on the proteasome, we analysed whether PEITC interfered with stabilisation of HIF1α by the proteasome inhibitor MG132 under normoxic conditions. Treatment of MCF7 cells with MG132 caused a strong increase in HIF1α expression (Fig. 7). However, this was still effectively reversed by PEITC, as well as the translation inhibitor, cycloheximide. Therefore, the ability of PEITC to decrease HIF1α expression does not require proteasome activity. Interestingly, a modest “recovery” of HIF1α expression was observed in...
inhibit HIF activity whereas pre-treatment with catalase, like NAC, caused a statistically significant ($p < 0.001$) reduction in HIF activity in hypoxic conditions (Fig. 8B and C). However, Trolox did not counter the ability of PEITC to inhibit HIF activity at any concentration, and catalase actually enhanced the ability of a lower concentration of PEITC (3.25 μM) to inhibit HIF activity. Thus, although NAC partially interferes with inhibition of HIF activity by PEITC, this appears to be due to direct conjugation of NAC/GSH to PEITC, rather than an antioxidant activity.

3.8. PEITC does not alter expression of HIF1α RNA

Although stabilisation of HIF1α plays a major role in the induction of HIF1 activity in hypoxic cells, HIF1α RNA transcription and translation are also subject to tight regulation [69]. We therefore analysed the effects of PEITC on the levels of HIF1α RNA (Fig. 9A). The expression of HIF1α RNA was not increased by hypoxia (even following protracted times – 24 h) and PEITC did not alter expression of HIF1α RNA. Similar results were obtained in CoCl2 treated cells. Therefore, transcription of HIF1α RNA is not altered by PEITC.

3.9. PEITC decreases 4E-BP1 phosphorylation in MCF7 and RCC4 cells

Since PEITC did not effect HIF1α degradation or transcription it is likely that PEITC interferes with translation of HIF1α RNA. The 5'-untranslated region (5'-UTR) of the HIF1α RNA is highly structured and, like other RNAs with this feature, its translation is tightly dependent on the activity of the elf4E translation factor [69], elf4E activity is regulated by binding to 4E-BP proteins (of which 4E-BP1 is the most prominent family member) and phosphorylation of 4E-BP1 prevents its interaction with elf4E and therefore allows efficient translation of RNAs with complex 5'-UTRs, such as HIF1α. Therefore, inhibition of 4E-BP1 phosphorylation may contribute to the ability of PEITC to downregulate HIF1α expression. We therefore investigated the effects of PEITC on 4E-BP1 phosphorylation in MCF7 and RCC4 cells (Fig. 9B and C). In control cells, we detected multiple forms of 4E-BP1, consistent with the presence of phosphorylation [70]. When cells were treated with PEITC, the overall levels of 4E-BP1 were not substantially altered, but there was a clear loss of the more slowly migrating, phosphorylated isoforms. Therefore, PEITC treatment causes a loss of 4E-BP1 phosphorylation in MCF7 and RCC4 cells.

4. Discussion

Substantial evidence demonstrates that ITCs, such as PEITC, can exert an anti-cancer activity in diverse in vitro and in vivo models [1–3, 20, 21]. Consistent with this, dietary intake of cruciferous vegetables and urinary excretion of ITC metabolites has been associated with reduced cancer risk in multiple epidemiological studies [4]. Angiogenesis plays a key role in cancer development and is an attractive target for the development of novel anti-cancer therapies. For example, the VEGF neutralising antibody Bevacizumab is approved for treatment of metastatic colorectal cancer. PEITC and SFN reduce angiogenesis [52–54] and this effect may play an important role in the chemopreventive/anti-cancer effects of ITC.

Here, we have demonstrated that PEITC is a potent inhibitor of HIF, a master regulator of hypoxic responses. PEITC acts, at least in part, to prevent the accumulation of HIF1α and HIF2α protein in hypoxic cells. HIF1α is effectively destabilised by the prolyl hydroxylase/VHL/proteasome pathway in normoxia. However, this is not required for PEITC to reduce HIF1α expression since PEITC was effective in VHL-deficient cells, and PEITC could inhibit HIF activity induced directly by inhibition of prolyl hydroxylases or the
proteasome in normoxia. Moreover, PEITC did not alter expression of HIF1α RNA following hypoxia or treatment of cells with CoCl2. Thus, PEITC appears to interfere with translation of HIF1α RNA. Recent data demonstrate that SFN, which also has anti-angiogenic activity, may also inhibit HIF activity by acting on HIF1α RNA translation in human tongue squamous cancer and prostate cancer cell lines [71]. By contrast, a reduction of HIF1α RNA levels by SFN was reported in human microvascular endothelial cells [53].

Although inhibition of HIF1α translation appears to be one mechanism by which PEITC interferes with HIF activity, it is possible that PEITC may act via multiple mechanisms. In some experiments (e.g., see Figs. 4B and 7) lower concentrations of PEITC appeared to be more effective than higher concentrations in decreasing HIF1α expression, although HIF activity remained effectively inhibited. This suggests that there may be complex, concentration-dependent effects of PEITC on HIF and further work is required to determine whether inhibition of HIF1α translation is the only mechanism by which PEITC targets HIF activity. Notably, the C-terminal transactivation domain of HIF1α contains a redox

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Fig. 6. Effect of PEITC on HIF activity and HIF1α expression in VHL-deficient RCC4 cells. (A) RCC4 cells were left untreated as a control or incubated with the indicated concentrations of PEITC (μM) for 24 h. Expression of HIF1α and β-actin were analysed by immunoblotting. (C) RCC4 cells were transfected with (i) pGL2-TK-HRE or (ii) control pGL3-promoter reporter constructs and treated with the indicated concentrations of PEITC (open bars) or equivalent amounts of DMSO as a solvent control (closed bars). Luciferase activity was measured after 24 h. Data shown are means of triplicate determinations (±SD) and are representative of two individual experiments. (D) RCC4 cells were treated with the indicated concentrations of PEITC (μM) or DMSO as a control (equivalent to 40 μM PEITC). After 24 h, expression of CAIX was analysed by Q-RT-PCR. Data are mean of duplicate determinations, normalised to expression of β-actin. Relative expression in untreated cells was set to 1.0. In (C) and (D), statistically significant differences between DMSO and PEITC treated cells are indicated (*) *p < 0.05; **p < 0.01; ***p < 0.005). All other differences were not statistically significant.

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Fig. 7. Effect of PEITC on proteasome inhibitor-induced HIF activity. MCF7 cells were left untreated, or treated with DMSO or the indicated concentrations of PEITC or cycloheximide (10 μg/ml) in the presence of MG132 (25 μM) for 1 h. Expression of HIF1α and β-actin were determined by immunoblotting.
regulated cysteine residue that is essential for HIF activity [72] and this may be targeted directly by PEITC.

Growth inhibitory effects of ITCs may involve direct conjugation and functional modification of key cell regulatory proteins and/or via increased ROS production following depletion of intracellular GSH [25,31,68]. ROS generally promote activation of HIF [41–47] and consistent with this, we demonstrated that NAC and catalase decreased induction of HIF activity in hypoxic conditions. However, only NAC, which contains a free thiol, reversed the ability of PEITC to inhibit HIF activity, whereas the non-thiol based antioxidants catalase and Trolox either had no effect, or enhanced the inhibitory effects of PEITC. These data

![Graphs showing the effect of NAC, Trolox, and catalase on inhibition of HIF activity by PEITC.](image)

Fig. 8. Effect of NAC, Trolox and catalase on inhibition of HIF activity by PEITC. (A) MCF7 cells were transfected with the pGL2-TK-HRE reporter construct. Cells were then pretreated with NAC for 2 h or left untreated before being treated with the indicated concentrations of PEITC or equivalent amounts of DMSO as a solvent control. HIF activity was induced by incubating cells in hypoxic conditions for 24 h prior to analysis of luciferase activity. (B) As in (A), except cells were pretreated with Trolox (100 μM) for 2 h instead of NAC. (C) As in (A), except cells were pretreated with catalase (2000 units) for 2 h instead of NAC. Data shown are means of triplicate determinations (±SD) and are representative of two individual experiments. In all graphs; DMSO (closed bars), PEITC (open bars), antioxidant and DMSO (grey bars), antioxidant and PEITC (hatched bars). Statistically significant differences between PEITC and PEITC + antioxidant treated cells are indicated (*p < 0.05; **p < 0.01; ***p < 0.005). All other comparisons between PEITC and PEITC + antioxidant treated cells were not statistically significantly different.
suggest that inhibition of HIF activity by PEITC does not directly involve modulation of intracellular ROS but rather may be due to conjugation of ITCs to cellular protein(s) involved in controlling HIF activity. Thus, we believe the inhibitory effects of NAC are due to its ability to protect cellular proteins from electrophilic attack, rather than acting to limit ROS per se. NAC may act directly as a "sink" to conjugate PEITC, or as a metabolic precursor to maintain cellular GSH levels.

PEITC also prevented the accumulation of HIF2α. HIF2α is closely related to HIF1α and is also induced by hypoxia [62]. However, there are important differences between HIF1α and HIF2α, both in terms of their regulation by hypoxia and in their function [62]. Knock-out experiments demonstrate that HIF1α and HIF2α are non-redundant, but both play roles in vascularisation in vivo, and HIF1α and HIF2α may target overlapping, but distinct sets of target genes. Interestingly, HIF2α appears to play a dominant role in growth/survival in VHL-defective renal cell carcinoma [73–75] and expression of HIF2α in the absence of HIF1α in VHL-deficient renal cell carcinoma is associated with elevated MYC activity [76].

A key question is how PEITC inhibits translation of HIF1α RNA. The 5'-untranslated region (5'-UTR) of the HIF1α RNA is highly structured and, like other RNAs with this feature, its translation is tightly dependent on the activity of the elf4E translation factor [69]. elf4E is an mRNA cap-binding protein that mediates the binding of the elf4E complex to the 5'-cap structures of mRNA. elf4E activity is regulated at multiple levels including phosphorylation and binding to 4E BP proteins (of which 4E BP1 is the most prominent family member). 4E BP1 is itself regulated by phosphorylation; 4E BP1 phosphorylation prevents its interaction with elf4E and therefore allows efficient translation of RNAs with complex 5'-UTRs, such as HIF1α. PEITC has previously been shown to inhibit 4E BP1 phosphorylation in HCT-116 (colorectal cancer) and PC3 (prostate) cancer cells [70] and we observed similar effects in RCC4 and MCF7 cells. Thus, one potential mechanism by which PEITC may inhibit HIF activity is by reducing 4E BP1 phosphorylation and HIF1α RNA translation.

Interestingly, the mTOR kinase, a major upstream upregulator of 4E BP1 phosphorylation [77], contains several conserved cysteine-residues which have been suggested to play an important role in controlling mTOR activity [78]. Moreover, the mTOR inhibitor rapamycin also causes a decrease in HIF1α translation [79]. We are currently testing whether these cysteine-residues within mTOR are directly modified by PEITC and the role that this might play in inhibition of 4E BP1 phosphorylation and HIF1α RNA translation.

Acknowledgements

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![Graphs](image-url)
cells. This work was supported by the Biotechnology and Biological Sciences Research Council, Cancer Research UK, Vitacress Salad Leaves and the Watercress Alliance. S Syed Alwi is supported by the Government of Malaysia and The University of Putra Malaysia.

References


Watercress supplementation in diet reduces lymphocyte DNA damage and alters blood antioxidant status in healthy adults

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ABSTRACT

Background: Cruciferous vegetable (CV) consumption is associated with a reduced risk of several cancers in epidemiologic studies.

Objective: The aim of this study was to determine the effects of watercress (a CV) supplementation on biomarkers related to cancer risk in healthy adults.

Design: A single-blind, randomized, crossover study was conducted in 30 men and 30 women (30 smokers and 30 nonsmokers) with a mean age of 33 y (range: 19–55 y). The subjects were fed 85 g raw watercress daily for 8 wk in addition to their habitual diet. The effect of supplementation was measured on a range of endpoints, including DNA damage in lymphocytes (with the comet assay), activity of detoxifying enzymes (glutathione peroxidase and superoxide dismutase) in erythrocytes, plasma antioxidants (retinol, ascorbic acid, α-tocopherol, lutein, and β-carotene), plasma total antioxidant status with the use of the ferric reducing ability of plasma assay, and plasma lipid profile.

Results: Watercress supplementation (active compared with control phase) was associated with reductions in basal DNA damage (by 17%; P = 0.03), in basal plus oxidative purine DNA damage (by 23.9%; P = 0.002), and in basal DNA damage in response to ex vivo hydrogen peroxide challenge (by 9.4%; P = 0.07). Beneficial changes seen after watercress intervention were greater and more significant in smokers than in nonsmokers. Plasma lutein and β-carotene increased significantly by 100% and 33% (P < 0.001), respectively, after watercress supplementation.

Conclusion: The results support the theory that consumption of watercress can be linked to a reduced risk of cancer via decreased damage to DNA and possible modulation of antioxidant status by increasing carotenoid concentrations.


KEY WORDS Watercress, cruciferous vegetables, DNA damage, antioxidants, humans, cancer biomarkers

INTRODUCTION

Increased vegetable intake, particularly of cruciferous vegetables (CVs) such as cabbage, cauliflower, broccoli, Brussels sprouts, watercress, and mustard greens, is associated with a decreased risk of several cancers in human population studies (1–9). However, not all associations, which have mostly been obtained from epidemiologic studies, are necessarily causal, and thus, intervention studies with specific dietary factors of interest are crucial.

CVs, especially those of the Brassica variety, have been shown to display several anticarcinogenic properties in vivo, as reviewed by Steinkellner et al (10) with the various underlying mechanisms having been summarized by others (11). Some of these mechanisms include alterations in the activities of metabolic enzymes (12), resulting in reduced carcinogenicity of dietary or environmental carcinogens in vivo (13), reduction of oxidative DNA damage levels in humans after supplementation with Brussels sprouts (14), and reduced DNA damage in human lymphocytes ex vivo in conditions of increased oxidative stress after supplementation with cruciferous and leguminous sprouts (15). Moreover, in vitro studies, extracts of Brussels sprouts have been shown to reduce the genotoxic effects of hydrogen peroxide in human lymphocytes (16), and those of cruciferous and leguminous sprouts reduced genotoxic effects of hydrogen peroxide in human colon cancer (HT-29) cell lines (15). In terms of the active chemical species, CVs are rich sources of glucosinolates, a class of sulfur- and nitrogen-containing glycosides that are hydrolyzed (by plant myrosinase or intestinal microflora) to form isothiocyanates. These isothiocyanates have been shown, in several in vitro and in vivo studies, to display anticarcinogenic properties as reviewed previously (9, 17). Watercress (Rorippa nasturtium-aquaticum) in particular contains one of the highest concentrations of glucosinolates per gram weight of any vegetable (18, 19) as well as containing high concentrations of carotenoids such as lutein and β-carotene (20). These phytochemicals have also been associated with various anticarcinogenic properties, including antioxidant activities. Members of the Cruciferae family have also been shown to contain high amounts of phenolic compounds (21).

Despite the widely documented protective effects of CVs, only a few studies have investigated their effects on cancer risk.
with the use of human intervention trials. These trials have either been of parallel or crossover designs, with subject numbers ranging from 10–43, intervened with either broccoli, Brussels sprouts, or cruciferous and leguminous sprouts for between 3 d and 21 d in healthy subjects, measured improvements in a range of surrogate endpoints relating to cancer risks, including glutathione S-transferase (GST) activity, lymphocyte DNA damage, and urinary metabolite excretion profiles (14, 15, 22, 23). The aim of the present study was to investigate whether a diet supplemented with watercress had any effects on intermediate endpoints (biomarkers) of cancer risk by using blood as a surrogate tissue. We measured the concentrations of various antioxidants, activities of metabolizing enzymes, and DNA damage in lymphocytes.

SUBJECTS AND METHODS

Subjects
The study was conducted in sixty volunteers [mean (±SD) age: 32.98 ± 10.97 y; range: 19–55 y]; 30 were men and 30 were women, 30 were nonsmokers and 30 were smokers (smoked 15–25 cigarettes/d). All subjects were healthy and nonusers of dietary supplements or medications, as determined using a prescreening health and lifestyle questionnaire and standard clinical tests. The study was conducted with the prior approval of the ethics committee of the University of Ulster and with the informed consent of participants.

Study design
The study design was a single blind, randomized crossover trial. The volunteers were randomly assigned to either the treatment (watercress supplemented) or control group during the first phase of the study. It was ensured that each of these groups contained equal numbers of men and women and of smokers and nonsmokers. During the treatment phase, the subjects consumed one portion (85 g) of raw watercress daily for 8 wk in addition to their normal diet. During the control phase (8 wk), the subjects were asked to maintain their habitual diet. The control and the treatment phases were separated by a 7-wk washout phase. All volunteers completed a 7-d food diary during each phase of the trial. The watercress used for this intervention study was a commercially available product produced by Vitacress Ltd (Southampton, United Kingdom). The subjects were supplied with fresh watercress (85 g bag/d) during the supplementation phase, and it was purchased from a local supermarket every 2–3 d. Fasting blood samples were collected before and after each phase (week 0, week 8, week 15, and week 23) by venepuncture into EDTA- or lithium heparin–containing tubes, as required. All blood samples were processed on ice. Lymphocytes were isolated by using Histopaque-1077, according to the manufacturer’s instructions (Sigma Diagnostics, St Louis, MO), and plasma samples were prepared by centrifugation at 1000 × g for 10 min, 4 °C. Red blood cell concentrate (washed twice with phosphate-buffered saline) samples were also collected. Plasma and red blood cells were immediately stored at −80 °C, whereas lymphocytes were stored frozen in liquid nitrogen. All biological measurements were carried out at the end of the intervention in batches containing equal number of active and control phase samples in each batch, and the researchers were blinded to these samples during analyses.

Methods

Watercress component analysis
A random bag of watercress was selected from each weekly supply during the 8-wk period that subjects consumed watercress. These samples were immediately stored at −80 °C (in an unopened form, stored in its original protective atmosphere) until analysis. Originally, sample bags of watercress were collected for both watercress intervention periods to allow comparison of levels throughout the 6-mo study. However, a freezer breakdown resulted in the loss of the material collected during the first watercress consumption period, and, consequently, all data related to samples of supplemented watercress are derived from the second intervention period. Watercress leaves were processed by using methods previously detailed by Mellon et al (24) with the use of liquid chromatography–mass spectroscopy (LC-MS). All glucosinolate standards had been previously purified, and flavonoid standards were either obtained from Extrasynthese (Genay, France) or had been previously purified from broccoli (quercetin-3-O-sophoroside and various hydroxycinnamic acid gentiobiose derivatives) and lettuce (quercetin-3-O-(6′-malonyl-glucoside)). The samples were freeze-dried and milled to a fine powder before extraction. All samples were analyzed in triplicate, by using ion-pair LC with UV-vis and also ion-pair LC/electrospray ionization (ESI) MS (to further confirm identities). The LC gradient for glucosinolate and phenolic analysis is a multipurpose chromatographic method that simultaneously separates glucosinolates and phenolics. The samples (40 mg) were extracted in 1 mL 70% acetonitrile (MeCN) at 70 °C for 20 min before being processed by the method previously detailed, with the use of sinigrin as the extraction standard. An injection volume of 20 μL was used. Glucosinolate and phenolic analyses were performed by using the negative ion electrospray ramped cone voltage method. Hydrolysis product analyses were performed by using the same LC/MS system.

DNA damage in lymphocytes
Peripheral blood lymphocytes, previously isolated and stored in liquid nitrogen, were thawed and screened for single strand breaks (SBs) in DNA by using the single cell gel electrophoresis (Comet) assay (25). Formamidopyrimidine DNA glycosylase (FPG) modification to the method was also used to allow additional oxidative purine damage to be assessed, according to the method of Collins et al (26). In brief, after the lysis stage, a separate slide to assess oxidative damage was washed in FPG reaction buffer (0.02 mmol/L Tris-HCL, 0.4 M NaCL, 1 mmol/L EDTA, and 0.5 mg/mL BSA, pH 7.5) for 3 × 5 min. After this, 40 μL of FPG (16 U/mL) was applied to the cells and incubated at 37 °C for 30 min. All slides were then transferred together to an electrophoresis chamber. In addition, basal DNA damage (SB) was measured in lymphocytes subjected to increased oxidative insult ex vivo by pretreating lymphocytes with 150 μmol H2O2/L for 5 min, 4 °C, before the measurement of SBs. The mean (percentage DNA in tail) was calculated from 50 cells per gel (each sample in duplicate) and the mean of each set of data were used in the statistical analysis.

Biochemical assays with the use of plasma and erythrocytes
The ferric reducing/antioxidant and ascorbic acid concentration (FRASC) assay was used in the simultaneous measurement
of ascorbic acid and the total antioxidant capacity of plasma (ferric reducing ability of plasma, FRAP), as described in the methods of Benzie and Strain (27, 28). Working FRASc reagent was prepared by mixing 25 mL acetate buffer (300 mmol/L \[3.1 \text{ g C}_2\text{H}_4\text{NaO}_4 \text{ g H}_2\text{O}\) (Riedel-de Haen, Seelze, Germany), pH 3.6) and 16 mL C\(_2\text{H}_3\text{O}_2\) [BDH Laboratory Supplies, Poole, UK] per liter of buffer solution, 2.5 mL TPTZ solution [10 mmol/L \[2,4,6\]-tripyridyl-s-triazine (Fluka Chemicals, Buchs, Switzerland) in 40 mmol/L HCl (BDH)] and 2.5 mL FeCl\(_3\) \(6\text{H}_2\text{O}\) solution (20 mmol/L; BDH). The Hitachi 912 autoanalyzer (Roche, Basel, Switzerland) was used to obtain both the FRAP value and the ascorbic acid concentration of the samples. This was done via monitoring the 0–4 min absorbance change of paired aliquots of water (40 \(\mu\)L added to 100 \(\mu\)L sample) and ascorbate oxidase (40 \(\mu\)L of a 4 \(\mu\)L/mL solution added to 100 \(\mu\)L sample)-treated samples run in parallel. The 4 min absorbance change of the aliquot diluted in water is referred to as the FRAP value. From this value, the absorbance change of the aliquot diluted with ascorbate oxidase was subtracted and the difference in the 2 readings was due specifically to the ascorbic acid (vitamin C) in the sample. By using the difference of the 2 readings (with and without ascorbic oxidase) and the stoichiometric factor of the reaction, ascorbic acid concentration of plasma was calculated. Glutathione peroxidase (GPX) in red blood cell concentrate was measured on the Hitachi 912 autoanalyzer (Roche, Basel, Switzerland) using a commercial kit (RANSEL kit; Randox Laboratories Ltd, Crumlin, CO Antrim, United Kingdom) according to manufacturer’s instructions. RCC aliquots were diluted in phosphate buffer (20 \(\mu\)L RCC in 4 mL phosphate buffer). SOD and GPX controls (Randox Laboratories Ltd) were run every 20 tests. The % CVs of the interbatch controls for both the assays were <5%. The GPX and the SOD results were standardized to red blood cell concentrate (RCC) hemoglobin concentration, and the final results were expressed in U/g hemoglobin. Plasma lutein, retinol, \(\alpha\)-tocopherol, and \(\beta\)-carotene were analyzed by simultaneous determination by using the HPLC method described by Thurnham et al (29). Plasma total cholesterol, HDL cholesterol, and triacylglycerols were measured on the Hitachi 912 autoanalyzer by using commercial kits (Roche diagnostics, Lewis, United Kingdom) according to kit manufacturer’s protocols, and plasma LDL cholesterol was calculated from the other 3 lipid profile parameters by using the Friedewald formula (30).

**Statistics**

All values are expressed as mean \(\pm\) SD, unless otherwise specified. The mean values are shown for all subjects \((n = 60)\) during their supplementation (watercress) phase and during their control (no watercress) phase. The differences in the mean were calculated using paired samples \(t\) test for normally distributed data, whereas Mann-Whitney \(U\) test or Wilcoxon’s signed-rank test were used for data that were not normally distributed. For the blood biomarker measurements, the results are presented as treatment effects, this was achieved by calculating individual differences between the values before and after treatment for both the control and the supplementation phases for each subject. The statistical tests were then carried out on the difference (after – before) in values between treatment (watercress) and control phases. All blood biomarkers were measured in duplicates, and the average of the 2 values were taken as the final result. The results were also tested for dietary treatment and smoking interactions by using a univariate general linear model, and where significant interactions with smoking were observed, the data were further analyzed separately for subgroups of smokers and nonsmokers. All statistical analyses were performed by using the SPSS software version 11.0 (SPSS Inc, Chicago, IL).

**RESULTS**

Analysis of watercress leaves was carried out to determine average contents of key phenolic compounds and glucosinolates present in the leaves, as shown in Table 1. In terms of the phenolic components in watercress leaves, several glycodies of quercetin, including rutin, were present, although the major phenolics were derivatives of hydroxycinnamic acid. A number of glucosinolates were also present, with 2-phenylethylglucosinolate predominating.

Mean daily energy, macronutrients, and the relevant micronutrients intake of the subjects \((n = 58)\) during the control phase (no watercress supplementation) and active phase (watercress supplementation) of the crossover trial are shown in Table 2. Two of the sixty subjects failed to return a completed food diary in each phase; body mass index data were available for 54 of the 60 subjects. However, all \((n = 60)\) who started the intervention study finished it to completion, so all blood measurements were carried out with a full set of samples. No statistical differences in body mass index \((n = 54)\), energy intake, and macronutrient intakes were observed between the control phase and the watercress phase of the study. However, the mean intakes of dietary fiber \((P < 0.05)\), vitamin C \((P < 0.001)\), vitamin E \((P < 0.01)\), folate \((P < 0.05)\) and carotene \((P < 0.001)\), were higher during the watercress phase of the study than during the control phase.

The effects of watercress consumption on peripheral blood lymphocyte DNA damage in terms of basal DNA damage (SBs), basal plus oxidative purine DNA damage (basal plus oxidative), and finally the ability of the lymphocytes to resist exogenous (basal) DNA damage [150 \(\mu\)mol/L hydrogen peroxide challenge

### TABLE 1

<table>
<thead>
<tr>
<th>Phenolic or glucosinolate</th>
<th>Fresh weight</th>
<th>Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-3-O-Sophoroside, 7-O-Glucoside</td>
<td>0.09 ± 0.02</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Q-3-O-Glc-(6'-Malonyl-Glc)</td>
<td>0.13 ± 0.029</td>
<td>1.43 ± 0.295</td>
</tr>
<tr>
<td>Q-3-O-Sophoroside</td>
<td>0.05 ± 0.005</td>
<td>0.59 ± 0.05</td>
</tr>
<tr>
<td>Q-3-O-Rutinoside (Rutin)</td>
<td>0.05 ± 0.004</td>
<td>0.6 ± 0.017</td>
</tr>
<tr>
<td>7-Methylsulfinylethyl-GLS</td>
<td>0.1 ± 0.002</td>
<td>1.07 ± 0.025</td>
</tr>
<tr>
<td>8-Methylsulfinylethyl-GLS</td>
<td>0.06 ± 0.07</td>
<td>0.68 ± 0.148</td>
</tr>
<tr>
<td>3-Indolylmethyl-GLS</td>
<td>0.04 ± 0.07</td>
<td>0.43 ± 0.26</td>
</tr>
<tr>
<td>2-Phenylethyl-GLS</td>
<td>1.53 ± 1.41</td>
<td>17.98 ± 4.31</td>
</tr>
<tr>
<td>4-Methoxy-3-indolylmethyl-GLS</td>
<td>0.065 ± 0.017</td>
<td>0.791 ± 0.05</td>
</tr>
<tr>
<td>Total HCA derivatives</td>
<td>9.40 ± 0.832</td>
<td>109.03 ± 4.22</td>
</tr>
</tbody>
</table>

\(^1\) All values are \(\bar{x} \pm SD\). Mean values of 8 bags of watercress, sampled once per week from one watercress intervention period (8 wk).
found that both basal DNA damage and H2O2 challenge had a highly significant increase (P < 0.001) in the H2O2 challenge, Figure 1) were found to have significant interactions. These interactions are shown in Figure 1. The percentage tail DNA values (± SEM) are presented as treatment effects; ie, the group means (active and control) of individual differences between the values after and before treatment, with all statistical analyses being carried out on mean of difference values between the watercress phase and control phase. Significant decreases in DNA damage were observed (basal, and basal + oxidative purine) for the subjects when consuming watercress. In the group as a whole, the mean differences between treatments were 17%, −2.65 ± 1.17 (P = 0.03) for basal DNA damage; 22.9%, −4.53 ± 1.14 (P = 0.002) for basal + oxidative-induced DNA damage; and 9.4%, −3.10 ± 1.66 (P = 0.07) for the H2O2 challenge. The data were further analyzed for smoking interactions by using a univariate general linear model (P < 0.01). We found that both basal DNA damage and H2O2 challenge had significant interactions, and hence we tested the effects of treatment in the 2 subgroups per se. In smokers, the mean difference between treatments for basal DNA damage was 25.6%, −3.98 ± 1.72 (P = 0.03); for H2O2 challenge was 15.5%, −5.05 ± 2.54 (P = 0.056). In the non-smoking group, the mean differences between treatments for basal DNA damage was 8.53%, −1.31 ± 1.58 (P = 0.4); for H2O2 challenge, was 3.4%, −1.14 ± 2.12 (P = 0.6).

The data shown in Table 3 show that watercress supplementation significantly increased the plasma concentration of the carotenoids measured. Plasma β-carotene concentration rose by ≈33% and lutein by 100% after watercress supplementation, mean differences between treatments of 0.10 ± 0.024% and 0.17 ± 0.019 μmol/L (both P < 0.001, by using Wilcoxon’s signed-rank test) were observed, respectively. Of the carotenoids, only β-carotene evidenced a significant smoking interaction, with a highly significant increase (P < 0.001) in β-carotene concentrations observed for nonsmokers and a lower significant increase (P < 0.05) observed for smokers during the watercress phase compared with the control phase. No significant changes due to watercress supplementation were observed for plasma concentrations of α-tocopherol, retinol, or vitamin C or in the total antioxidant potential of plasma (FRAP value). Generally, lipid profiles (LDL, HDL, and total cholesterol) were unaffected by watercress consumption, with the exception of plasma triacylglycerol concentration, which showed a decrease of about 10% (−0.13 ± 0.1) during the active (supplemented) phase compared with the control phase (P = 0.07, Wilcoxon’s signed-rank test). In response to watercress, red blood cell GPX and SOD activity did not change significantly in the total population.

**DISCUSSION**

There is considerable scientific consensus from epidemiologic studies that CVs may reduce risk of cancers, including cancers of the lung (5, 6), prostate (7), colon (31), and of the lymphatic system (8, 32). However, debate on the issue concerns which types of Cruciferae are most important and whether the epidemiologic observations can be confirmed with experimental studies conducted in humans. The present study has focused on watercress, which has a particularly high content of glucosinolates and other potential anticancer phytochemicals including carotenoids. An in vitro study within our laboratory showed that watercress extract can protect cells (HT-29; colon cancer cell line) against DNA damage levels induced by genotoxic agents such as H2O2 and fecal water (33). In the present study, we reported a decrease in all our measures of DNA damage in lymphocytes in response to watercress consumption in humans in vivo. This is consistent with previous studies on vegetable consumption reported by Riso et al (34) and Pool-Zobel et al (35). Two of the 3 measures of DNA damage (basal DNA damage and H2O2 challenge, Figure 1) were found to have significant interactions with smoking, and, on further subgroup analyses, we found the reductions in these variables as a result of watercress supplementation were of greater magnitude and more significance in smokers than in nonsmokers. This may reflect a higher toxin burden present in the smoking group, as has been indicated by a significantly lower total antioxidant status at baseline in smokers than in nonsmokers (P < 0.01, results not shown), possibly suggesting a greater benefit of consumption of watercress for this compromised group. A recent study by Kang et al...
the smokers and nonsmokers were analyzed for treatment effect per se. Univariate general linear model, and, when such an interaction was evident, interactions between smoking and DNA damage measures were tested by a

FIGURE 1. Comparison of the effects of watercress supplementation phase (■) with that of a control phase (□) on lymphocyte DNA damage in the total study population (n = 60), in smokers (n = 30), and in nonsmokers (n = 30) determined by using the Comet assay. Lymphocytes were collected from the subjects before and after watercress supplementation and were untreated (basal) or treated ex vivo with formamidopyrimidine DNA glycosylase (basal + oxidative) to assess oxidized purines or hydrogen peroxide (H₂O₂ challenged) to test for DNA damage resistance. DNA damage was measured as the mean of the individual difference (after – before supplementation) in the active and control phases. *Significantly different from the control phase: *P ≤ 0.05, **P ≤ 0.01 (paired-samples t test). Interactions between smoking and DNA damage measures were tested by a univariate general linear model, and, when such an interaction was evident, the smokers and nonsmokers were analyzed for treatment effect per se.

(36) which used a commercially available green vegetable drink (Angelica keiskei–based juice) given to smokers daily for 8 wk, also reported a significant decrease in lymphocyte DNA damage.

However, in contrast to our data and that of other authors, some recent articles have shown little or no association between vegetable consumption and biomarkers of cancer risk. In a 24 d, parallel design, intervention study performed in 43 healthy male and female subjects (nonsmokers), consumption of fruit and vegetables (600 g) or supplementation with vitamins and minerals compared with placebo had no effect on oxidative DNA damage measured in mononuclear cells or urine (37). A further article on the “6 a day study” reported that the fruit and vegetable diet enhanced resistance of plasma lipoproteins to oxidation and increased erythrocyte glutathione peroxidase activity (38). The reasons for the limited or different effects observed in this study in comparison with our study may be related to the choice of study design (parallel compared with crossover), population size (15 subjects compared with 60) or the difference in exposure period (24 d compared with 56 d). Moller and Dragsted et al (37, 38) did, however, exercise dietary control over their subjects, whereas our study simply supplemented the normal diet of the participants. However, dietary assessment with 7-d food diaries indicated that watercress supplementation did not alter the overall dietary habits of our subjects, as evidenced from similar energy and macronutrient intakes during the supplemented and control phases of the study. The increased intakes of some micronutrients (vitamin C, vitamin E, folate, and carotene) during the active phase were most likely to be attributable to watercress supplementation.

The mechanisms that produced the antigenotoxic effects in this study due to watercress supplementation are unknown, although this may be related to antioxidant status (39). Watercress is known to be a rich source of the carotenoids lutein and β-carotene (20). Therefore, it was not surprising that plasma concentrations of these carotenoids were significantly elevated after watercress intervention. A recent placebo-controlled trial with carotenoid supplements (lutein, β-carotene, and mixed carotenoids) conducted in postmenopausal women for 56 d showed a decrease in endogenous lymphocyte DNA damage as a result of carotenoid supplementation (40). Therefore, increases in the in vivo concentrations of lutein and β-carotene may contribute to the decrease in DNA damage levels in lymphocytes observed in the present study. The greater difference in the concentrations of β-carotene in nonsmokers than in smokers as a result of watercress supplementation may indicate that this carotenoid was used up to a higher extent in smokers, perhaps reflecting a greater requirement of this antioxidant in smokers. In support for this concept, a similar observation was also made in a previous study, in which plasma lutein and β-carotene status increased more markedly in nonsmokers than in smokers as a result of green vegetable supplementation (41). Furthermore, the elevated plasma lutein in particular may provide additional health benefits to watercress consumers in terms of cardiovascular risk (42) and macular degeneration (43). A recent prospective study also reported that high plasma concentrations of carotenoids (including β-carotene) were associated with a reduced risk of mortality from cancers and cardiovascular diseases (44). Finally, because plasma lutein can reflect intake of green leafy vegetables (45) as well as of CVs (46), the increased concentrations of the lutein as a result of watercress supplementation indicated good compliance in the study participants during the
supplement phase of the present study. Watercress contains a high concentration of glucosinolates along with other bioactive phytochemicals (e.g., lutein) that may have also contributed to the antigenotoxicity observed in lymphocytes as a result of supplementation. Note that consumption of watercress had no effect on the activity of antioxidant enzymes such as SOD or GPX. This observation further supports previous observations from our laboratory (15) that CVs do not exert their protective activity via antigenotoxicity observed in lymphocytes as a result of supplementation. However, the effects of watercress on GST activity in the present study and the correlations with GST single nucleotide polymorphisms will be dealt with in a separate article.

In conclusion, data from our randomized, single blind human study and the correlations with GST single nucleotide polymorphisms will be dealt with in a separate article.

We sincerely thank the volunteers who participated in this study. We also thank the technical staffs, Jimmy Coulter, Neil Dennison, and Sheila Dobbin of the School of Biomedical Sciences, University of Ulster, Coleraine, for their technical assistance during the project.

IRR and CIRG were the principal investigators of the study and were responsible for the study design and in charge of the overall operation. SH, LAB, CIRG, and JRP were the intervention study conducts. SH and LAB were responsible for the blood biomarker analyses. JW and MB were responsible for dietary intake assessment and coding of the food diaries. RB was responsible for the watercress component analyses. IB was responsible for the statistical analyses and data interpretation. CIRG, SH, and LAB was responsible for the manuscript preparation, and IR was responsible for critically reviewing the manuscript. None of the authors had any financial or personal conflict of interest.

REFERENCES


Acute and chronic watercress supplementation attenuates exercise-induced peripheral mononuclear cell DNA damage and lipid peroxidation

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Abstract
Pharmacological antioxidant vitamins have previously been investigated for a prophylactic effect against exercise-induced oxidative stress. However, large doses are often required and may lead to a state of pro-oxidation and oxidative damage. Watercress contains an array of nutritional compounds such as β-carotene and α-tocopherol which may increase protection against exercise-induced oxidative stress. The present randomised controlled intervention was designed to test the hypothesis that acute (consumption 2 h before exercise) and chronic (8 weeks consumption) watercress supplementation can attenuate exercise-induced oxidative stress. A total of ten apparently healthy male subjects (age 23 (SD 4) years, stature 179 (SD 10) cm and body mass 74 (SD 15) kg) were recruited to complete the 8-week chronic watercress intervention period (and then 8 weeks of control, with no ingestion) of the experiment before crossing over in order to compete the single-dose acute phase (with control, no ingestion). Blood samples were taken at baseline (pre-supplementation), at rest (pre-exercise) and following exercise. Each subject completed an incremental exercise test to volitional exhaustion following chronic and acute watercress supplementation or control. The main findings show an exercise-induced increase in DNA damage and lipid peroxidation over both acute and chronic control supplementation phases (P<0·05 v. supplementation), while acute and chronic watercress attenuated DNA damage and lipid peroxidation and decreased H2O2 accumulation following exhaustive exercise (P<0·05 v. control). A marked increase in the main lipid-soluble antioxidants (α-tocopherol, γ-tocopherol and xanthophyll) was observed following watercress supplementation (P<0·05 v. control) in both experimental phases. These findings suggest that short- and long-term watercress ingestion has potential antioxidant effects against exercise-induced DNA damage and lipid peroxidation.

Key words: Exhaustive exercise: Watercress prophylaxis: DNA damage: Reactive oxygen species

Free radicals are produced in mammalian cells during a complex array of physiological processes, which, if left uncontrolled, have the potential to cause metabolic damage(1). Free radical activity is moderated by a complex antioxidant network(2), which has the capacity to temper potentially negative cellular effects(3). The human body depends on the intake and recycling of dietary antioxidant vitamins such as vitamins C, E and β-carotene in order to support the enzymatic systems(4); however, a systemic increase in free radical production may overwhelm antioxidant defences leading to a state of oxidative stress(5) in the form of molecular damage to DNA, cellular lipid membranes and protein molecules(6).

It has been established that various modes and intensities of exercise can lead to an exacerbated state of oxidative stress, although the exact mechanism of free radical production remains elusive(7). Despite this, pharmacological interventions have frequently been investigated for a prophylactic effect against oxidative stress induced by exercise(8–11), and although research has shown a prophylactic effect with exogenous antioxidant consumption, in some cases, large doses of antioxidants are required. As it has been shown that an overconsumption of oral antioxidants may lead to a pro-oxidant state, causing a disturbance in redox biochemistry(12), it is therefore imperative that food sources naturally high in antioxidant vitamins are considered, due to their capacity to provide increased systemic and cellular protection without excessively elevating in vivo antioxidant vitamin concentrations(13).

Cruciferous vegetables, in particular cauliflower, cabbage, broccoli and watercress, are known to reduce oxidative DNA damage during in vitro experimentation in human cells(14).

Abbreviations: LOOH, lipid hydroperoxides; PC, protein carbonyls.

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More specifically, Gill et al.\(^\text{15}\) demonstrated that watercress, which contains per gram weight one of the highest concentrations of glucosinolates and carotenoids of any vegetable, protects lymphocyte DNA against damage in smokers, while Boyd et al.\(^\text{16}\) suggested that crude watercress extracts are also known to provide increased protection against DNA damage associated with human colon cancer cells and H\(2\)O\(_2\) exposure.

Given that exhaustive exercise promotes oxidative stress, causing severe perturbations to cell structure and function, there is a need to ascertain an antioxidant vitamin-rich vegetable that has the potential to counteract exercise-induced oxidative stress. Although recent research has indicated that watercress may play a role in lymphocyte cell protection\(^\text{15}\), the efficacy of short- and long-term watercress ingestion on exercise-induced oxidative stress, to the best of our knowledge, is not yet known.

We therefore hypothesise that acute and chronic watercress consumption will provide effective prophylaxis against exercise-induced oxidative stress. Thus, the primary aim of the present investigation is to examine the efficacy of acute and chronic watercress ingestion against exercise-induced oxidative stress. A randomised controlled experimental design incorporating a comprehensive assessment of oxidative stress indices was used to test this hypothesis.

**Methods**

**Human subjects and experimental design**

A total of ten apparently healthy male subjects were recruited to participate in the chronic phase of the experiment first. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the University of Ulster Research Ethics Committee. It is noted that randomised assignment of participants to a supplemented or control group and incorporating a cross-over element is the most robust method of testing food supplements. However, due to the availability of this naturally occurring product and the quantities required to complete the experiment, delivery of product could only be guaranteed during the optimum growing months. For this reason, the participants completed 8 weeks of chronic watercress ingestion before washing out for an additional 8 weeks and then subsequently completing 8 weeks of no ingestion (control). Upon the completion of the chronic phase (lasting in total 24 weeks), eight out of the original ten participants volunteered to take part in the acute phase with an additional two participants recruited. A randomised cross-over design was utilised in the acute phase with participants washing out for a 2-week period between the intervention and the control. Please see Table 1 for participant characteristics. Participants with a family history of any known haematological or cardiovascular-related condition were excluded from the study. Subjects were also excluded if they smoked or were taking any form of antioxidant supplement. All subjects were assigned to complete two phases of experimental testing, of which one was a chronic interventional phase lasting 24 weeks and the other an acute experimental phase, consisting of a randomised cross-over assignment to either watercress supplementation 2 h before exhaustive exercise or the control group. All commercially available watercress was supplied by Vitacress (Vitacress Limited). All participants were instructed to refrain from altering their usual dietary habits throughout the experimental phases of testing. Written informed consent was obtained from all subjects before participation. A schematic overview of each experimental phase is provided in Figs. 1 and 2.

**Chronic interventional phase**

Before chronic supplementation and control, a baseline venous blood sample was collected for quantification of lipid-soluble antioxidants. Participants were then required to consume one 85 g portion\(^\text{15}\) of raw watercress on a daily basis for a total of 8 weeks. Watercress was ingested in a single sitting between the hours of 12.00 and 14.00 daily in combination with other foods. Following 8 weeks of supplementation, all participants completed an exercise test to volitional exhaustion while venous blood was drawn at rest and after exercise to determine lipid-soluble antioxidant status and other indices of oxidative stress. Following an 8-week washout period\(^\text{15}\), participants entered an 8-week control phase with no watercress ingestion and then repeated the exercise test to exhaustion.

**Acute experimental phase**

During the acute experimental phase, participants were randomly assigned using a simple lottery system to either a supplemented or control group. For the supplementation group, participants were asked to consume a single portion of

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**Table 1. Participant characteristics**

(Mean values and standard deviations, \(n\) 10)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Chronic Mean</th>
<th>SD</th>
<th>Acute Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>23</td>
<td>4</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>74</td>
<td>15</td>
<td>80</td>
<td>19</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>179</td>
<td>10</td>
<td>175</td>
<td>12</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>23</td>
<td>3</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>VO(<em>{2})(</em>\text{max}) (ml/kg per min)</td>
<td>58</td>
<td>6</td>
<td>52</td>
<td>6</td>
</tr>
<tr>
<td>Maximum heart rate (bpm)</td>
<td>185</td>
<td>8</td>
<td>182</td>
<td>8</td>
</tr>
</tbody>
</table>

*bpm, Beats per min.*

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\(\text{Fig. 1. Experimental time line for the chronic phase.}\)
85 g of raw watercress and 500 ml of water within 30 min between 10.00 and 10.30 hours. Following ingestion, participants rested for 2 h before completing an exercise test to volitional exhaustion. The control group also completed the exercise test to volitional exhaustion, and did not ingest watercress but did consume 500 ml of water. Following a 2-week washout period\(^ {17}\), all participants crossed over to consume either watercress or nothing and were retested as above.

**Exhaustive acute exercise protocol**

To avoid the potential effects of diurnal variation, each participant was required to consistently attend the laboratory testing sessions at the same time of day (09.00 hours) for all phases. On each arrival of participants at the laboratory, body mass and stature were measured using standard methods (see Table 1). All participants were required to complete a treadmill test to volitional exhaustion in which the protocol was specifically designed to be progressive and incremental in order to elicit VO\(_{2}\text{max}\). Treadmill speed was set at 11 km/h with a 1% gradient rise at each 1 min interval until volitional fatigue. Validation of VO\(_{2}\text{max}\) was confirmed when (1) the RER was above 1.15 arbitrary units, (2) a plateau in the oxygen uptake/exercise intensity relationship (2 ml/kg per min) and (3) a heart rate of within 10 beats per min of age-predicted maximum (220 – age). Oxygen uptake was measured using standard laboratory gas analysis (Cosmed Quark b2), while heart rate was measured via a portable short-angle telemetry device (Polar Sports Tester). All subjects achieved maximum oxygen uptake.

**Supplementation compliance**

Subjects were asked to complete a weekly journal detailing total amounts of watercress ingested over the chronic interventional phase.

**Haematology**

Following a standardised 12 h overnight fast, whole blood was drawn from a prominent forearm ante-cubital vein using the vacutainer method (Becton-Dickinson). Blood was collected at baseline (pre-supplementation), at rest (post-supplementation/pre-exercise) and exercise (immediately post-exhaustive exercise) for the quantification of a range of lipid-soluble antioxidants, and at rest and exercise for various indices of oxidative stress. Blood was drawn into either di-potassium EDTA or serum separation tubes. EDTA and serum separation tubes (once clotted) were centrifuged (MSE Centuar 2; Labcare) at 3000 rpm for 10 min at −4°C. Aliquots of plasma and serum were stored at −80°C for a maximum of 3 months. All samples from the same participant were analysed within the same batch. Hb (g/dl) was measured using a \(\beta\)-Hb photometer (Hemocure Limited), and packed cell volume (%) was measured using the standard microcapillary reader technique to aid in the correction of all biochemical markers for acute exercise-induced plasma volume shifts using the equations of Dill & Costill\(^ {18}\).

**Biochemical analysis**

**DNA.** Lymphocytes were isolated from the whole blood (EDTA) by layering 3 ml onto Histopaque-1077 (3 ml) and centrifuged (MSE Centaur 2; Labcare) at 3500 rpm (relative centrifugal force 1356) for 30 min at room temperature. The opaque mononuclear layer was aspirated and washed three times using 15 ml PBS (one tablet added to 200 ml of double-distilled water, 0.2 M, pH 7.2). The comet assay was subsequently carried out on prepared cells using the protocol of Singh et al.\(^ {19}\). Dakin fully frosted slides (3 inch \(\times\) 1 inch; 1.2 mm thick; Richards Supply Company Limited) were covered with 100 \(\mu\)l of 0.5% normal-melting point agarose and allowed to solidify under a coverslip (22 \(\times\) 40 mm, no. 1 thickness; GBH, Laboratory Supplies). Then, 50 \(\mu\)l of cells (1 \(\times\) 10\(^7\)) were mixed with 50 \(\mu\)l of low-melting point agarose layered on top of the
normal-melting point agarose and allowed to solidify under a coverslip. The coverslip was then removed and the slides were placed in lysis buffer (25 mM-NaCl, 100 mM-Na-EDTA, 10 mM-Tris, 1% Triton X and 10% dimethyl sulf oxide, pH 10) for 1 h at 4°C to break down the cell membranes. The slides were removed and placed in a horizontal electrophoresis unit containing electrophoresis buffer (300 mM-NaOH, 1 mM-EDTA, pH 12.5) for 20 min to allow the DNA double helix to relax and unwind. Electrophoresis (25 V, 300 mA, 0.15 V/cm) was performed for 20 min at room temperature. Following electrophoresis, the slides were rinsed with neutralising solution (0.4M-Tris, pH 7), stained with 50 μl ethidium bromide and a coverslip was applied. A random sample of fifty cells from each slide was analysed using a Hewlett-Packard VGA monitor and Fenestra Comet software program (version 2.22) at magnification of 400 X using an epifluorescent microscope (Olympus BH2). Tail length was selected to report DNA damage, as it acts as a sensitive measure of low levels of DNA damage. This assay was performed on each experimental day using fresh lymphocytes. The intra/inter-assay CV were <9 and <11%, respectively.

**Lipid hydroperoxides.** Serum lipid hydroperoxides (LOOH) were measured spectrophotometrically using the method of Wolff. This ferrous iron/xylene orange assay quantifies the susceptibility to Fe-induced LOOH formation in the blood. The presence of Fe ions in the assay protocol might therefore yield slightly higher LOOH values compared with other methods. Briefly, 90 μl serum were incubated with 10 μl catalase for 30 min at room temperature. To this solution, 900 μl ferrous iron/xylene orange reagent 1 (250 μM-ammonium ferrous sulphate, 100 μM-xylene orange, 100 μM-sorbitol and 25 μM-H2SO4) were added and incubated for a further 30 min at room temperature in the dark. Standard solutions were prepared from H2O2 in the range of 0–50 μmol/l and also incubated for 30 min with the ferrous iron/xylene orange 1 reagent, after which the samples were centrifuged in a Beckman microfuge for 5 min to remove any flocculated material. The absorbance of the supernatant was read spectrophotometrically (U-2001; Hitachi) at 550 nm against the standard curve that was linear in the range of 0–5 μmol/l. The intra/inter-assay CV were <2% and <4%, respectively.

**Protein carbonyls.** Protein carbonyls (PC) are a reliable measure of protein oxidation and were quantified with an assay kit purchased from Cayman Chemical. After thawing, the serum sample was diluted 1:64 by adding 5 μl of serum to 315 μl of the sample diluent. Then, 50 μl of this diluted sample were added to a ninety-six-well plate in triplicate along with an appropriate standard curve (part no. H2163) and blanks in addition to 100 μl of H2O2 colour reagent (part no. H2288). After 30 min of incubation at room temperature, the plate was read on a Dynatech MRX 650 plate reader with Revelations software (American Instrument Exchange) using 550 nm as the primary wavelength. The intra/inter-assay CV were <3 and <6%, respectively.

**Lipid-soluble antioxidants.** Plasma retinol, α-tocopherol, γ-tocopherol, β-carotene, lycopene and xanthophyll were analysed by simultaneous determination using the HPLC method of Thurnham et al. Tocopherol acetate was used as the internal standard during this procedure. Briefly, 200 μl of the internal standard were added to each extraction while recovery was measured by injecting 50 μl tocopherol acetate directly on HPLC with each batch of samples. The internal standard was used to monitor sample recovery and the results were adjusted accordingly. Then, 1000 μg of tocopherol acetate were added to 100 ml of heptane and magnetically stirred. The stock solution (1 ml) was then diluted in 249 ml ethanol (95%) and stored at 4°C until use. Thereafter, 100 μl of 10 ml-SDS were added to 1 ml of the plasma sample and mixed. Then, 200 μl of the internal standard were added to each sample along with two blanks (for recovery). These were then vortexed for 1 min and 1 ml heptane (containing 50 mg butylated hydroxytoluene) was added to each sample and vortexed for 5 min. The samples were centrifuged for 10 min at 1000 g at 10°C. Thereafter, 700 μl of the supernatant were discarded and the samples were left to dry under N2 in a fume cupboard. Each sample was reconstituted with 100 μl of the mobile phase (750 ml acetonitrile, 200 ml methanol, 50 ml dichloromethane, 500 mg butylated hydroxytoluene and sonicated for 30 min to degas) and vortexed. The samples were dispensed into HPLC vials and placed on sampling carousel for analysis. For the analysis, the samples were combined with the mobile phase at a flow rate of 1.5 ml/min at 21°C. The HPLC system comprised a Waters 510, Waters 2996 photodiode array detector, connected to a Waters 717 autosampler and a Waters Sunfire™ C18, 3.5 μ, 4.6 X 100 mm column. The temperature of the column was maintained using a Jones chromatography 7900 series HPLC column oven. Samples were analysed using a range of wavelengths (325–450 nm) to detect retinol, tocopherol and carotenoid peaks. The concentration of retinol, α-tocopherol, γ-tocopherol, β-carotene, lycopene and xanthophyll in each sample was corrected in relation to the percentage of the internal standard recovered for each sample. The intra/inter-assay CV were <6 and <10%, respectively.
**Statistical analysis**

Statistical analysis was performed using the SPSS statistics package (version 15.0, SPSS). A prospective calculation of power was performed using the equations of Altman ([15](#)) and based on experimental DNA data by Gill et al. ([15](#)). Data were analysed using parametric statistics following mathematical confirmation of a normal distribution using Shapiro–Wilk tests. Baseline, pre- and post-intervention data were analysed using a two-way (A × B) mixed ANOVA which incorporated one between-subject (group: watercress v. control) and one within-subject factor (state: rest v. exercise). When a significant interaction effect was detected, within-participant factors were analysed using Bonferroni-corrected paired-sample *t*-tests. Between-participant differences were analysed using a one-way ANOVA with an *a posteriori* Tukey’s honestly significant difference test. The α level was estimated at *P*<0.05 and all values are reported as means and standard deviations, unless otherwise stated.

**Results**

**Compliance**

A total of ten participants (100%) completed the chronic phase of the study, and compliance to supplementation throughout was 98% (548 consumed doses from 560 possible doses). The ten participants also completed the acute phase (100%) and compliance with the supplementation protocol was 100% (ten consumed from ten possible doses).

**Indices of oxidative stress**

DNA damage increased following exercise in both control conditions (chronic-phase increase of 61% from rest, *P*<0.05 v. supplementation; acute-phase increase of 63% from rest, *P*<0.05 v. supplementation; Table 2); however, watercress consumption attenuated DNA integrity following exercise in both control conditions (chronic phase, *P*<0.05 v. control; acute phase, *P*<0.05 v. control; Table 2). Similarly, there was a rise in LOOH from rest to exercise in both control conditions (chronic-phase increase of 15% from rest, *P*<0.05 v. supplementation; acute-phase increase of 16% from rest, *P*<0.05 v. supplementation; Table 2), which was conversely decreased as a function of watercress supplementation (chronic phase, *P*<0.05 v. control; acute phase, *P*<0.05 v. control; Table 2). PC concentration remained unchanged following exhaustive exercise in both chronic and acute supplementation phases (*P* > 0.05; Table 2).

**Reactive oxygen species**

There was a comparatively higher production of H$_2$O$_2$ as a function of exhaustive exercise in both control conditions (chronic phase, *P*<0.05 v. supplementation; acute phase, *P*<0.05 v. supplementation; Fig. 3), while watercress ingestion decreased H$_2$O$_2$ concentration in both experimental groups (chronic phase, *P*<0.05 v. control; acute phase, *P*<0.05 v. control; Fig. 3).

**Lipid-soluble antioxidants**

Table 3 shows the effect of watercress and exercise on plasma antioxidant concentration. There was an increase in α-tocopherol concentration following chronic watercress ingestion (*P*<0.05 v. control), while α-tocopherol, γ-tocopherol and xanthophyll all increased in the acute phase (*P*<0.05 v. control). Following exhaustive exercise, α-tocopherol increased in both acute watercress and control states (*P*<0.05 v. rest); however, exercise decreased α-tocopherol in the chronic watercress group (*P*<0.05 v. control). Exercise also lowered γ-tocopherol concentration across the watercress and control groups for both phases (*P*<0.05 v. rest) and β-carotene decreased in the acute and chronic watercress groups (*P*<0.05 v. control), with no change observed in the control state (*P* > 0.05).

**Discussion**

Strong experimental evidence indicates that exhaustive exercise may activate the production of free radical species and cause damage to important biological molecules such as DNA, lipid

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**Table 2. Indices of oxidative stress at rest and exercise for the watercress-supplemented and control groups for both experimental phases**

<table>
<thead>
<tr>
<th></th>
<th>Chronic phase (n 10)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Acute phase (n 10)</th>
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<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>Exercise</td>
<td>Rest</td>
<td>Exercise</td>
<td></td>
<td>Rest</td>
<td>Exercise</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA damage (tail DNA %)</td>
<td>7·2 0·7</td>
<td>7·4 0·3</td>
<td>2·2</td>
<td></td>
<td></td>
<td>6·8 1·8</td>
<td>7·5 0·3</td>
<td>9·8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid hydroperoxides (mmol/l)</td>
<td>6·0 1 15·4*</td>
<td>4·7 61·0†</td>
<td>5·8 1·3</td>
<td>15·6* 5</td>
<td>62·7†</td>
<td></td>
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</tr>
<tr>
<td>Protein carbonyls (per mg/protein)</td>
<td>1·2 0·2</td>
<td>1·1 0·2</td>
<td>1·2 0·2</td>
<td>0·9 0·4</td>
<td>35·1†</td>
<td></td>
<td></td>
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<tr>
<td>Watercress</td>
<td>8·0 0·1</td>
<td>8·9 0·2</td>
<td>1·1 0·3</td>
<td>0·9 0·3</td>
<td>3·40</td>
<td>8·0 0·2</td>
<td>1·0 0·2</td>
<td>6·2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Δ%: Change from rest.

* Between-group differences at exercise (*P* < 0.05).

† Within-group differences between rest and exercise (*P* < 0.05).
and protein(24). Watercress is a green leafy vegetable that possesses one of the highest concentrations of antioxidant vitamins, and is known to protect cells against oxidative stress, in particular DNA damage(15). The primary purpose of the present study was to ascertain the effects of acute and chronic watercress ingestion against exercise-induced oxidative stress. The study demonstrates that exhaustive aerobic exercise may cause DNA damage and lipid peroxidation; however, these perturbations are attenuated by either short- or long-term watercress supplementation, possibly due to the higher concentration of lipid-soluble antioxidants following watercress ingestion.

**Exercise-induced oxidative stress**

Previous work from our laboratory and, indeed, others demonstrates that intense exhaustive exercise can increase damage to circulating lymphocyte DNA and lipid membranes(10,24). The comet assay is a sensitive, valid and versatile tool for the measurement of single- and double-strand breaks in DNA(20) and has been previously used to detect DNA damage following exhaustive exercise(8,10,25–27). Moreover, many of these reports attribute an increase in free radical production as the main cause of exercise-induced DNA damage; however, the exact mechanism(s) for the production of primary free radical species during exercise remains elusive, although free radicals generated within the skeletal muscle are often proposed as the main protagonist(26). Considering the vast majority of human investigations that have quantified oxidative stress in plasma, serum or leucocytes, it is imperative to attempt to identify the primary source of free radical generation during exercise(20). Evidence from the present investigation portrays a potential primary mechanism of cellular exercise-induced DNA damage which may be mediated by H$_2$O$_2$. The up-regulation of leucocytes and neutrophil enzyme activation is known to cause an increased release of superoxide(30,31), and superoxide has the capacity to directly damage DNA(32) and/or be dismutated to H$_2$O$_2$ by superoxide dismutase(33). H$_2$O$_2$ can readily diffuse across cell membranes(29), and depending on the availability of transition metal ions, hydroxyl radicals may be generated which can cause single- and double-strand breaks in DNA and base-pair modification(34). The change in H$_2$O$_2$ as a function of exercise in both control groups provides a tentative explanation for DNA damage observed. An elevated H$_2$O$_2$ concentration may also be a direct mediator of DNA damage through the disruption of DNA repair pathways(35). Furthermore, H$_2$O$_2$ can be generated from a number of extracellular pathways during exercise and may also be independent of superoxide formation. The auto-oxidation of neurological chemicals such as catecholamine has been shown to increase following exercise resulting in H$_2$O$_2$ formation(36). In addition, the oxidation of dopamine by monoamine oxidase releases H$_2$O$_2$ as a metabolic by-product(37).

Superoxide and hydroxyl radical production may also initiate vascular cell membrane lipid peroxidation, which may generate LOOH and facilitate further DNA damage. PUFA decomposition can produce an array of metabolic by-products including mutagenic compounds and lipid peroxidation intermediates(37), including alkyl free radicals(38) and malondialdehyde(39). Both molecules are potentially capable of directly damaging DNA, while alkyl free radicals, in particular, may perpetuate the process of lipid peroxidation(37). Unsaturated alkenals are also by-products of lipid peroxidation, which can lead to the production of PC(39). PC are normally produced following the oxidation of amino acids and have previously been used as a sensitive measure of protein damage during high-intensity exercise(40). Exhaustive exercise has been found to have no effect on protein oxidation throughout any condition, which perhaps suggests that protein oxidation has a greater affinity with exercise duration rather than intensity(41,42).

**Prophylactic effect of watercress**

Watercress is a cruciferous vegetable of the *Brassica* variety and contains, per gram weight, one of the highest concentrations of glucosinolates and carotenoids of any vegetable(43). Glucosinolates can be hydrolysed by the enzyme myrosinase (released from plant cells during the chewing process) to form isothiocyanates which have been shown to possess anticarcinogenic properties and reduce DNA damage properties in both animal models and humans(44). Cellular exposure to isothiocyanates

![Fig. 3. Hydrogen peroxide concentrations at rest and following exhaustive exercise within both supplemented (n = 10) and non-supplemented experimental phases (n = 10). * Within-group difference (P < 0.05 vs. rest); † between-group difference (P < 0.05 vs. rest).](https://www.cambridge.org/core/core/terms)
Table 3. Plasma lipid-soluble antioxidants at baseline, rest and exercise for the watercress-supplemented and control groups for both experimental phases (Mean values and standard deviations)

<table>
<thead>
<tr>
<th></th>
<th>Chronic phase (n=10)</th>
<th>Acute phase (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (mmol/L)</td>
<td>Rest (mmol/L)</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td><strong>α-Tocopherol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Watercress</td>
<td>118</td>
<td>21</td>
</tr>
<tr>
<td>Control</td>
<td>122</td>
<td>10</td>
</tr>
<tr>
<td><strong>β-Tocopherol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Watercress</td>
<td>1·8</td>
<td>0·7</td>
</tr>
<tr>
<td>Control</td>
<td>1·6*</td>
<td>0·8</td>
</tr>
<tr>
<td><strong>β-Carotene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Watercress</td>
<td>1·1</td>
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</tr>
<tr>
<td>Control</td>
<td>0·7</td>
<td>0·01</td>
</tr>
<tr>
<td><strong>Xanthophyll</strong></td>
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<td></td>
</tr>
<tr>
<td>Watercress</td>
<td>0·2</td>
<td>0·05</td>
</tr>
<tr>
<td>Control</td>
<td>0·1*</td>
<td>0·07</td>
</tr>
<tr>
<td><strong>Retinol</strong></td>
<td></td>
<td></td>
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<tr>
<td>Watercress</td>
<td>2·1</td>
<td>0·4</td>
</tr>
<tr>
<td>Control</td>
<td>1·9</td>
<td>0·2</td>
</tr>
</tbody>
</table>

Between-group differences (P<0·05).
† Within-group difference (P<0·05).
‡ Change from baseline to rest.
§ Change from rest to exercise.

Watercress ingestion attenuates DNA damage.
and antioxidants may represent a possible mechanism for the observed protection of cell DNA following both chronic and acute supplementation phases. LOOH also reduced following exhaustive exercise under both supplemented conditions but increased in the control groups. These data would lend support to the suggestion that watercress may provide effective in vivo protection against H_{2}O_{2} production as a function of exercise. It is also plausible that the elevated lipid-soluble antioxidants (under both supplemented protocols) are directly scavenging superoxide and therefore result in a net decrease in H_{2}O_{2} production \cite{45}. The observed increase in lipid-soluble antioxidants, as that demonstrated following exercise, may also play a key role in the protection against cell membrane lipid peroxidation.

Watercress has been shown to increase important lipid-soluble antioxidants and, in particular, the tocopherols and carotenoids. Gill et al.\cite{15} identified a 33% increase in β-carotene, a 26% increase in α-tocopherol and a 35% increase in ascorbic acid, thus highlighting watercress’s potential to elevate lipid- and aqueous-soluble systemic antioxidants. In contrast, we observed no change in β-carotene or retinol under either experimental condition. However, the lipid-soluble antioxidants α-tocopherol, γ-tocopherol and xanthophyll were all elevated following supplementation with watercress, and, in doing so, indicates the potential for watercress to act as a source of blood-rich antioxidants. Xanthophyll is an effective scavenger of singlet oxygen\cite{46} and has shown antioxidant activity that is particularly effective in protecting DNA\cite{47}. The increased concentration of xanthophyll following the acute dose of watercress may therefore have played a contributory role in the increased protection of lymphocyte DNA in this supplemented group. Moreover, an increase in lipid-soluble antioxidants occurred as a function of exercise and is perhaps as a result of membrane-bound antioxidants being released into the vascular medium during the lipolysis of adipose tissue\cite{48}. The notion that exhaustive exercise may increase systemic levels of lipid-soluble antioxidants comes from a plethora of evidence suggesting that an increased mobilisation of fatty acids may give rise to α-tocopherol\cite{24,49,50} in addition to other fat-soluble antioxidants such as retinal and lycopene\cite{51}. PC are generated following the oxidation of amino acids and have previously been used as a sensitive measure of protein damage during high-intensity exercise\cite{40}. However, we observed no change in PC following exhaustive exercise with or without watercress ingestion, and this is in contrast to the observed DNA damage and lipid peroxidation. As previously reported, protein oxidation may have a greater affinity with exercise duration rather than intensity\cite{41,42}, and it is also conceivable that muscle damage may be a potential mediating factor in protein oxidation following exercise.

**Conclusion**

The present study suggests that dietary watercress intervention provides effective protection against exercise-induced oxidative stress, with no additional benefits gained from long-term watercress consumption. The prophylactic effect of watercress may be due in part to the high concentration of antioxidants or other cytoprotective compounds.

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**References**


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