Impact of oral green tea catechins on UVR-induced skin inflammation

Impact of oral green tea catechins on UVR-induced skin inflammation

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Abstract

Thesis title: Impact of oral green tea catechins on UVR-induced skin inflammation University of Manchester, Gemma Darby, Degree of MPhil 24/02/2014

Acute exposure of human skin to ultraviolet radiation (UVR) results in oxidative stress and an acute inflammatory response (sunburn), manifest clinically as erythema, histologically by a dermal leukocytic infiltrate and biochemically by upregulation of pro-inflammatory mediators. Green tea catechins (GTC) are potent antioxidants and anti-inflammatory agents with potential to offer systemic photoprotection. We hypothesised that oral GTC are bioavailable in human skin and through cyclooxygenase (COX) and lipoxygenase (LOX) inhibition may reduce the production of UVR-induced pro-inflammatory eicosanoids prostaglandin (PG)E_2 and 12-hydroxyeicosatetraeonic acid (12-HETE), and also the UVR-induced erythema response.

UVR spectrum and dosimetry study: This small preparatory study involved 3 subjects (2 female, mean age 36; range 29-50 yr, white Caucasian, phototype II). The aim was to assess the UVR-spectrum and dose to be used in the oral intervention study. UVB and solar simulated radiation (SSR) were compared by assessing the levels of PGE_2 they induced in skin blister fluid. Subjects were given 3x and 4x their sunburn threshold dose (minimal erythema dose, MED) to upper buttock skin using both UVR sources. Suction blisters were raised after 24 hours (hr) and the fluid extracted was assayed for PGE_2 by ELISA. Equivalently elevated levels of PGE_2 were found for both UVR-spectra. Since SSR provides a UVR emission that closely mimics sunlight, this was chosen for the intervention study. PGE_2 levels were very similar for 3x and 4x MED. Less biological variation between subjects was seen with 3x MED and therefore this dose was selected.

Oral GTC intervention study: 16 healthy white Caucasian (male and female) volunteers, mean age 42 (range 29-56 yr), phototype I/II were supplemented with encapsulated GTC (550mg/day) and a small dose of vitamin C (50mg/day) which acted as a stabiliser, for 12 weeks. Pre- and post-supplementation, the volunteer's MED to SSR was assessed visually, the UVR-erythema was quantified with a reflectance instrument, and their UVR-erythema dose response analysed. Buttock skin was irradiated with 3x the MED of SSR and after 24hr, skin punch biopsies and suction blister fluid were taken from un-irradiated and irradiated skin. Urine samples were also collected. Skin, blister fluid and urine samples were analysed for catechin and catechin metabolite content, and blister fluid for PGE2 and 12-HETE levels, using liquid chromatography coupled to electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS).

Following supplementation, no change in MED compared with baseline was seen. However, the UVR-erythema dose response showed a reduction in skin erythema at the highest UVR dose (P=0.006) and there was a reduction in area under the curve (P=0.033). Levels of PGE_2 and 12-HETE were significantly upregulated following UVR, both pre- and post- supplementation. Post-supplementation, a reduction in UVR-induced 12-HETE expression occurred (P=0.015) while PGE_2 was not altered. Intact catechins and catechin metabolites were seen in both skin and skin fluid samples, with more metabolites evident following supplementation although with considerable inter-individual variability. Good compliance with supplementation was evident through urinary catechin analysis.

This pilot study showed that oral GTC are bioavailable in human skin. Evidence of protection against UVR-induced skin inflammation was seen after 12-weeks supplementation with GTC combined with vitamin C, both in the erythemal response, and in production of the pro-inflammatory eicosanoid 12-HETE. Randomised controlled studies are warranted to further explore the use of oral GTC in human photoprotection.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

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Abbreviations

Actinic keratosis.....AK

Activator protein 1 .....AP-1

Activating transcription factor .....ATF

Adenosine.....A

Antigen .....Ag

Antigen presenting cell.....APC

Antioxidant response element .....ARE

Apurinic/apyrimidinic.....AP

Arachionic acid .....AA

Ascorbic acid.....AA

Basal cell carcinoma .....BCC

Base excision repair.....BER

Biologically effective dose .....BED

Catalase .....CAT

Cell mediated immunity .....CMI

Chromophore 6-thioguanine..... 6-TG

c-jun NH-2terminal kinase..... JNK

Commission Internationale de l'Éclairage..... CIE

Contact hypersensitivity.....CHS

Cyclobutane pyrimidine dimer ..... CPD

Cyclooxygenase ..... COX

Cysteine C

Days ..... d

Delayed hypersensitivity ..... DHS

Deoxyribonucleic acid ..... DNA

Dermal-epidermal junction ..... DEJ

Electrophile response element ..... EpRE

Epicatechin ..... EC

Epicatechin-3-gallate ..... ECG

Epigallocatechin ..... EGC

Epigalocatechin-3-gallate ..... EGCG

Erythema index ..... EI

Extracellular matrix ..... ECM

Glucose transporters ..... GLUT

Glutathione perioxidase ..... GPx

Glutathione S-transferase ..... GST

Green tea catechins ..... GTC

Guanine ..... G

Gulonolactone oxidase ..... GULO

γ-glutamylcysteine synthetase ..... γ-GCS

Heme oxygenase 1 ..... HO-1

Horseradish peroxidise ..... HRP

Hour ..... hr

Hydrogen peroxide ..... H_2O_2

Hydroperoxyeicosatetraenoic acid ..... HPETE

Hydroxyeicosatetraeonic acid ..... HETE

Hydroxyl radical ..... OH^-

Interleukin IL

International unit ..... IU

Langerhans cell LC

Leukotriene B_4 ..... LTB_4

lipid hydroperoxides ..... LOOHs

liquid chromatography coupled to electrospray (ESI) ionization tandem mass spectrometry ..... LC/ESI-MS/MS

Minimal erythemal dose ..... MED

Lipoxygenase ..... LOX

Malignant melanoma ..... MM

Medicines and Health Regulatory Agency ..... MHRA

Metalloproteinase ..... MMP

MilliJoules ..... mJ

MilliWatts ..... mW

Minutes ..... mins

Mitogen activated protein kinase ..... MAPK

Musculoaponeurotic fibrosarcome ..... MAF

NAD(P)H dehydrogenase, quinone 1 ..... NQO1

Nanometre ..... nm

Nitric oxide ..... NO

Nitric oxide synthase ..... iNOS

Non melanoma skin cancer ..... NMSC

Normal human epidermal keratinocytes ..... NHEK

Nuclear export signal ..... NES

Nuclear factor erythroid 2-related factor 2 ..... NRF2

Nuclear factor kappa-light-chain enhancer of activated B cells ..... NFκB

Nucleotide excision repair ..... NER

Oxo-7,8-dihydro-2'-deoxyguanosine 8-oxodG

Phospholipase-A_2 ..... PLA_2

Polyunsaturated fatty acid ..... PUFA

Prostaglandin ..... PG

Protein proliferating cell nuclear antigen ..... PCNA

6-4 Pyrimidine pyrimidone photoproducts ..... 6-4 PP

Random controlled trial ..... RCT

Reactive oxygen species ..... ROS

Reference nutrient intake ..... RNI

Ribonucleic acid ..... RNA

Singlet oxygen ..... $^1O_2$

Sodium-coupled transporters ..... SVCT

Sodium dismutase ..... SOD

Sodium hydroxide..... NaOH

Solar simulated radiation ..... SSR

Solid phase extraction ..... SPE

Squamous cell carcinoma ..... SCC

Sun protection factor ..... SPF

Superoxide ..... O^-_2

12-*O*-tetradecanoylphorbitol-13-acetate..... TPA

Thromboxane A_2..... TXA_2

Thymine ..... T

Tumour necrosis factor alpha..... TNFα

Ultraviolet ..... UV

Ultraviolet-A ..... UVA

Ultraviolet-B ..... UVB

Ultraviolet-C ..... UVC

Ultraviolet raiation ..... UVR

CHAPTER 1: Introduction

1.1 Structure and function of the skin

Skin is arguably the largest organ in the human body in terms of weight and surface area (Bickers et al., 1982, Bos and Kapsenberg, 1986). It is a complex and multi-functional organ which is often taken for granted. However, with its ability to offer protection against noxious external stimuli, prevent excessive loss of fluids from the body (Gniadecka et al., 1998) as well as its active involvement in vitamin D synthesis (Holick, 2007), the maintenance of healthy skin is of great importance. The skin is comprised of three main layers: the epidermis, dermis and subcutaneous layer [Figure 1.1].

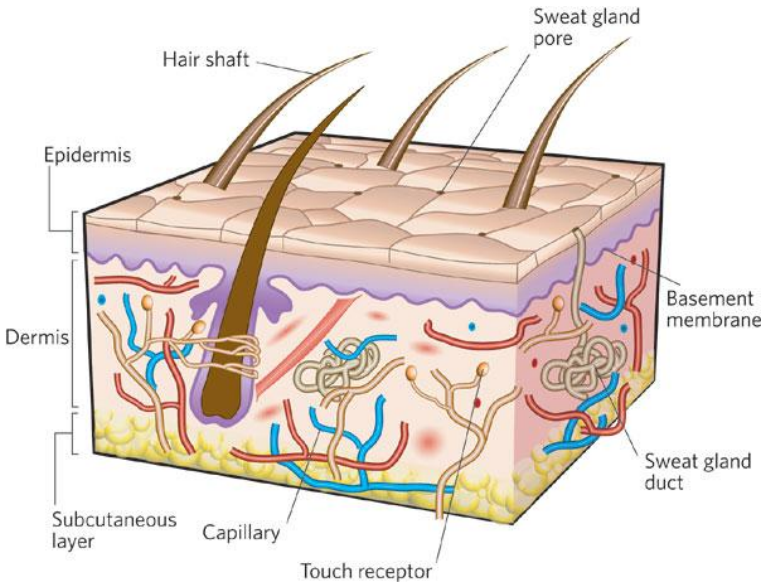


Figure 1.1 Structure of human skin is made up of three main layers: the epidermis (outer layer), dermis and subcutaneous tissue. Image taken from www.deadsea.com.au

1.1.1 Epidermis

The epidermis is the outermost layer of the skin and is approximately 100µm in thickness (Bashkatov, 2005). The five strata that make up the epidermis are: 1) stratum basale (innermost, basal layer); 2) stratum spinosum; 3) stratum granulosum; 4) stratum lucidum (only found in thick skin such as the palms and soles of the feet), and; 5) stratum corneum (the outermost layer). Basal keratinocytes divide to produce two daughter cells, one of which remains in the basal layer for further division, whilst the other migrates upwards towards the stratum corneum. Differentiation and maturation of keratinocytes allows production of the protein keratin. The main transition phase takes place in the stratum

granulosum whereby keratohyaline granules are formed within the cell causing the nucleus to disintegrate. Keratins are surrounded by a thin hydrophobic film creating a semi-permeable barrier containing varying amounts and types of lipids (Nicolaou et al., 2011). This epidermal barrier protects against injury, infection and damage to internal organs as well as preventing the loss of water and proteins (Gniadecka et al., 1998).

Other cells found within the epidermis include melanocytes, Merkel cells and Langerhans cells (LCs). Melanocytes are dendritic cells which account for approximately 5% of all cells in the epidermis. They are interspersed between keratinocytes in the stratum basale and function to synthesise the pigment melanin which is responsible for giving skin its colour. Melanin is also one of the skin's main ultraviolet (UV) absorbing biomolecules offering endogenous protection against ultraviolet-A (UVA). Upon exposure to UVA, melanocytes increase their production of melanin which has the ability to scavenge UVA-induced free radicals. Merkel cells also reside in the stratum basale interspersed between keratinocytes. They are known as mechanoreceptors which are responsible for the sensation of touch. Further up the stratified epithelium, LCs can be found within the stratum spinosum. These antigen presenting cells (APCs) are derived from bone marrow and function as surveillance molecules protecting the epidermis. Their structure allows them to attach themselves to foreign bodies (antigen (Ag)), transporting and presenting them to lymphocytes within the dermis where they undergo neutralisation and elimination.

Directly below the epidermis is the dermal-epidermal junction (DEJ). This basement membrane functions to provide epidermal-dermal adherence, offers mechanical support for the epidermis, and is responsible for the exchange of molecules and for helping the diffusion of nutrients and oxygen from the dermis to lower epidermal layers (Briggaman and Wheeler, 1975).

1.1.2 Dermis

The dermis accounts for approximately 90% of total skin and is 1-4mm in thickness (Bashkatov, 2005). It is positioned directly below the DEJ and divided into two parts: 1) the thinner upper layer (papillary dermis), and; 2) the deeper thicker layer known as the reticular dermis. The papillary dermis is responsible for supplying nutrients to the epidermis via its vascular system and for regulating body temperature. The main dermal

cells are fibroblasts, responsible for the synthesis of extracellular matrix molecules. The reticular dermis provides the strength, structure and elasticity of skin. Elastic fibres which have a central core of amorphous, hydrophobic cross linked elastin are surrounded by fibrillin-rich microfibrils (Watson et al., 1999). Fibrillin-rich microfibrils are the key architectural structures providing a scaffold on which elastin is deposited (Haynes et al., 1997). Their function is to strengthen the elastic fibres enabling transmission of forces between the epidermis and dermis. Bundles of microfibrils are intercalated at the DEJ providing an elastic connection between the elastic fibre network and the epidermis. Thin collagen fibres and small amounts of elastin are identified in the lower papillary dermis whilst, thick collagen and elastin fibres make up the lower reticular dermis which runs parallel to the surface of the skin. Other derivatives found within the dermis include blood vessels, lymph vessels, hair follicles, sweat glands, sebaceous glands and nerve endings.

1.1.3 Subcutaneous layer

The layer positioned directly beneath the dermis is the subcutaneous fat layer. It is approximately 1-6mm in thickness which varies in size depending on body site (Bashkatov, 2005). This layer primarily consists of adipose and connective tissue; however, also residing here are blood and lymphatic vessels (Skobe and Detmar, 2000), nerves and hair follicle roots (Brakebusch et al., 2000). Its main function is to facilitate the regulation of body temperature and protect internal organs (Bashkatov, 2005).

1.2 Ultraviolet radiation (UVR)

The electromagnetic spectrum refers to wavelengths (energy levels) of radiation characteristically separated into x-rays, microwaves, gamma rays, ultraviolet, visible and infrared [Figure 1.2]. The human eye can see ‘visible’ light only (400-700nm). Until recently, visible light was thought to have no adverse effects on the skin; however, a recent study reported visible light has the ability to cause erythema, pigmentation and induce reactive oxygen species (ROS) resulting in indirect damage to macromolecules (Mahmoud et al., 2008). Ultraviolet radiation (UVR) in sunlight is of particular interest to photobiologists because of its high energy and diverse effects on the skin. There are three main types of solar UVR which are UVA, ultraviolet-B (UVB) and ultraviolet-C (UVC). UVC

is completely absorbed by the earth's atmosphere and therefore the skin does not naturally exhibit any adverse effects from it.

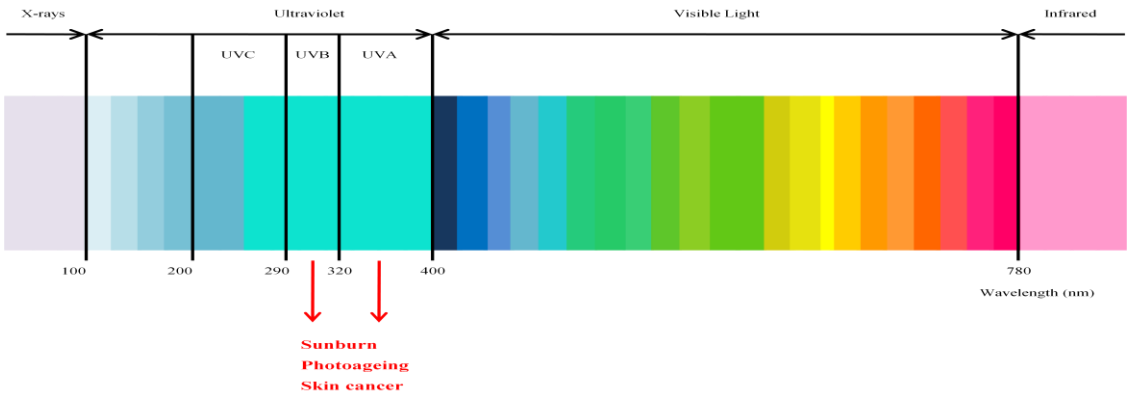


FIGURE 1.2 Electromagnetic spectrum illustrating different types of light including wavelength (nm). Image adapted from www.pro-lite.uk.com

1.2.1 UVB

UVB (290-320nm) is partially absorbed by the ozone layer and is recognised as both a tumour initiator and tumour promoter (Devary et al., 1992, Poon et al., 2005, Streilein et al., 1994). In skin, UVB is largely absorbed in the epidermis, interacting predominantly with epidermal keratinocytes; however, it has the ability to penetrate through epidermal layers into the upper papillary dermis (Dubakiene and Kupriene, 2006). Acute effects of UVB include erythema (sunburn); other effects include photosensitivity disorders and photoimmunosuppression, and in the long term, photoageing and photocarcinogenesis (Shahbakhti et al., 2004). UVB is much more (1000x) effective than UVA in causing skin erythema and several other biological effects; hence despite UVB being present at a much lower percentage in the solar radiation reaching the skin, it is the major contributor to sunburn (UVR-induced erythema).

1.2.2 UVA

Humans are subjected to more UVA (320-400nm) than UVB as it accounts for approximately 90-99% of solar radiation reaching the earth's surface (Pacheco-Palencia et al., 2008, Syed et al., 2006). It has a longer wavelength than UVB allowing penetration deeper into the dermal layers of skin enabling interaction with both epidermal

keratinocytes and dermal fibroblasts (Mahmoud et al., 2008). UVA, like UVB, induces tanning; however, more recently it has been found to contribute to both benign and malignant tumours (Bachelor and Bowden, 2004).

1.2.3 Behavioural changes to UVR exposure

Solar UVR is a key environmental stressor which significantly impacts on skin health. Both social and behavioural changes have resulted in greater exposure to sunlight and subsequently augmented the number of reported cases of skin cancer. Solar UVR is the principal cause of the three main types of skin cancer (basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma (MM)). Climate change has seen a marked increase in ambient UVR levels and it is probable that skin cancer will rise substantially throughout the world in the future. Latest Cancer Research UK figures show there are now >100,000 new cases of skin cancer diagnosed each year with significantly more skin cancers occurring, as only the patient's 1st skin cancer is recorded (CancerResearch-UK, 2013). Therefore, further developments are warranted for more effective strategies of photoprotection.

1.2.4 Chromophores and photosensitisation reactions

When skin is exposed to UVR, three interactions may occur. These include reflection, absorption and scattering (Wondrak et al., 2006). When a photon (packet of light) is absorbed by a chromophore a photochemical reaction is initiated. Chromophores possess a high specificity for a particular wavelength or range of wavelengths. The wavelength of the photon must therefore be within the absorption spectrum of the chromophore for it to be absorbed. Shorter wavelengths are known to exhibit a higher energy and thus cause more damage (Mouret et al., 2006). Upon absorption by the chromophore the photon is said to be converted to a single 'excited state'. This reaction lasts only nanoseconds whereby the energy is transferred from the photon to the chromophore and emitted as fluorescence, heat or forms photoproducts via a photochemical reaction before returning to a 'ground state' (Young, 1997, Hussein, 2005). Another outcome for the 'excited state' molecule involves the momentum spin of electrons in the outer shell, changing to spin in the same direction creating a 'triplet' energy state. This higher energy state is more stable and can last up to 10 seconds before the energy is transferred and emitted as phosphorescence [Figure 1.3]. Scattering may occur within the collagen fibres in the

papillary dermis, where they are have been found to alter the direction of light (wavelength dependent) (Hussein, 2005).

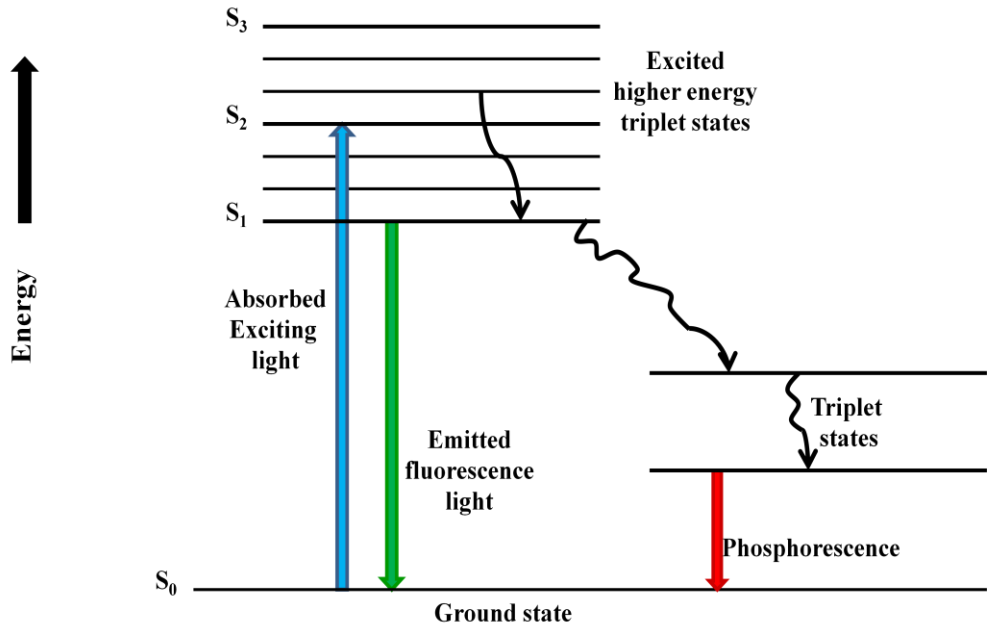


Figure1.3 Jablonski energy diagram adapted from www.files.chem.vt.edu

1.2.5 Action spectrum

An action spectrum is a parameter used to determine the 'relative' effectiveness of different wavelengths of UVR in producing a photobiological effect. In essence, it is used as a 'weighting factor' for the UVR spectrum to discover the biologically effective dose (BED) required for a given effect (McKinlay, 1987). The action spectrum for erythema (sunburn) is a standardised and internationally accepted representation of the effectiveness of wavelengths in the UVR portion of the spectrum to create an erythemal response (Webb et al., 2011) [Figure 1.4]. Research has suggested the action spectra for photocarcinogenesis closely resembles that of UVR-induced erythema indicating a potential inter-relationship between the acute and chronic effects of the sunburn response (de Gruijl and Van der Leun, 1994).

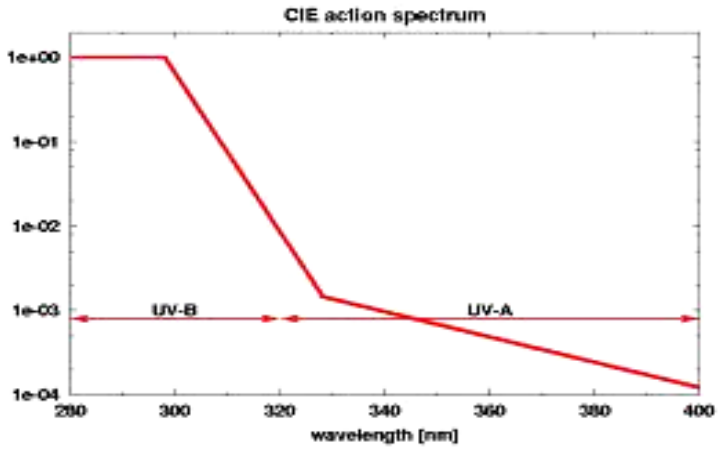


Figure 1.4 Reference action spectrum for UVR-induced erythema in human skin which was proposed in 1987 by McKinlay and Diffey and further adopted as a standard by the Commission Internationale de'l Eclairage in 1998

1.3 Molecular effects of UVR on skin

1.3.1 Direct Effects of UVR

1.3.1.1 DNA Damage

UVB is the most mutagenic form of UVR impacting on human skin with its ability to interact directly with cellular deoxyribonucleic acid (DNA) and thus it is widely accepted that this UVR-induced DNA damage is an underlying factor in the formation of skin cancer. The absorption of UVB by DNA results in dimeric photoproducts which are primarily cyclobutane pyrimidine dimers (CPDs) and to a lesser extent 6-4 pyrimidine pyrimidone photoproducts (6-4 PP) (Mouret et al., 2006) with the most efficient wavelength for their formation around 300nm and 320nm respectively (Freeman et al., 1989). CPD formations occur through absorption of UVB by a double bond in pyrimidine bases within DNA, in particular cysteine (C) and thymine (T). The UVR-modified base forms a stable covalent bond with its neighbouring bases (Goodsell, 2001). CPD mutations are found to typically occur within proliferating epidermal cells residing in the stratum basale (Young et al., 1998) and are predominantly p53 mutations causing TC to TT or CC to TT transitions within the DNA. These particular mutations have been shown to significantly increase the risk of developing skin cancer (Young, 1997, Mouret et al., 2006). Much of this UVR-induced damage is resolved through a DNA repair process known as nucleotide excision repair (NER). This is one of five human DNA repair mechanisms which have evolved. It involves many proteins, transcription factors,

replication factors and DNA polymerases (Friedberg, 2001, Tyson and Mathers, 2007) and works by targeting mutagenic lesions of DNA and eradicating them. There are two pathways by which DNA damage is recognised: (1) global genome repair which is independent of transcription, and; (2) transcription coupled repair which involves actively transcribed genes blocking ribonucleic acid (RNA) polymerase allowing removal and repair of the damaged site (Tyson and Mathers, 2007). Impairment of NER gives rise to the autosomal recessive disease Xeroderma Pigmentosum, increasing a patient's susceptibility to developing skin cancer by x1000 (Tyson and Mathers, 2007, Friedberg, 2001).

1.3.2 Indirect Effects of UVR

1.3.2.1 Reactive oxygen species

ROS are highly reactive molecules formed as a by-product of normal aerobic metabolism and which can also be produced by various stressors. At normal levels they are actively involved in cell signalling and homeostatic regulation. However, following UVR exposure ROS levels increase dramatically causing significant damage to cell structures as well as overwhelming endogenous antioxidant defence mechanisms resulting in oxidative stress (Podda et al., 1998). Examples of ROS include singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$), superoxide ($O^{\cdot}_2$) and hydrogen peroxide (H_2O_2) (Zhang et al., 1997). Increased levels of ROS induce chronic inflammation through the induction of cyclooxygenase-2 (COX-2), inflammatory cytokines tumour necrosis factor alpha (TNFα), interleukin 1 (IL-1), IL-6), chemokines (IL-8, CXCR4) and pro-inflammatory transcription factors; nuclear factor kappa B (NF-κB), c-jun NH-2 terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK).

1.3.2.2 Oxidative DNA damage

It has been extensively documented that oxidation of cellular biomolecules such as DNA occur indirectly through UVR-induced ROS (Zhang et al., 1997, Peak and Peak, 1989). Numerous studies have indicated the ability UVA has to cause indirect damage by $^1O_2$ production (Syed et al., 2006, Montaner et al., 2007, Therrien et al., 1999). In a study by Peak *et al* (1990), $OH^{\cdot}$ was found to induce DNA breakages within the UVA-region (wavelength 364 nm)(Peak and Peak, 1990). Further studies into ROS-generated DNA

damage showed UVR-induced H_2O_2 increases production of 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), an oxidative modified DNA base, in mouse keratinocytes (Beehler et al., 1992) and human skin cells (Orimo et al., 2006). Furthermore, 8-oxodG is now considered as an effective biomarker for assessing oxidative DNA damage in humans (Lam et al., 2012). Damage caused from the generation of ROS is normally repaired by the base excision repair (BER) mechanism (Seeberg et al., 1995). It works by specific DNA glycosylases excising the damaged base at the N-glycosylic bond region of the DNA backbone (between the phosphate base and the sugar) via hydrolysis thus creating an apurinic/apyrimidinic (AP)-site (Seeberg et al., 1995, Friedberg, 2001, Fortini et al., 2003, Tyson and Mathers, 2007). The (AP)-site is removed by an AP-endonuclease or an AP-lyase generating a single strand break which is re-synthesised by DNA polymerase β and re-sealed by DNA ligase (Seeberg et al., 1995).

In addition, a photosensitisation reaction between UVA and its specific chromophore 6-thioguanine (6-TG) is known to induce modifications to the protein proliferating cell nuclear antigen (PCNA). PCNA is actively involved in DNA replication, DNA mismatch repair and excision repair (Montaner et al., 2007, Shivji et al., 1992), thus modifications suggest ineffective functioning of DNA replication and repair mechanisms (Montaner et al., 2007).

1.3.2.3 Lipid peroxidation

Absorption of UVR by cellular membranes may result in significant damage to their lipid-rich structure. Oxidative degradation of lipids is a consequence of ROS, a process known as lipid peroxidation. This is a three stage process of which the stages include initiation, propagation and termination. Polyunsaturated fatty acids within the membrane react with ROS producing an unstable fatty acid radical which in turn reacts with molecular oxygen creating a peroxyl fatty acid radical. Subsequent reactions with free fatty acids result in lipid hydroperoxides (LOOHs), a more stable, propagative molecule which causes damage to cellular membranes during ROS-induced oxidative stress (Girotti, 1998). Interestingly, this more stable molecule has been found to trigger signal transduction pathways through the stimulation of effector molecules and activation of transcription factors and cytokines (Suzuki et al., 1997).

1.3.4 UVR-induced transcriptional activation

1.3.4.1 NFκB

NFκB is a transcription factor belonging to the REL family which plays a major role in inflammatory signalling, activation of inflammatory gene expression (Ahmad et al., 2000) and cellular proliferation (Sarkar and Li, 2003). NFκB is sequestered in the cytoplasm and bound by members of the IκB family of inhibitor proteins which are phosphorylated by IκB kinase complex leading to ubiquitination and degradation. NFκB is translocated into the nucleus activating transcription. Expression of NFκB causes endothelial hyperproliferation and accelerated collagen fibre breakdown through the stimulation of pro-inflammatory cytokines/chemokines (particularly IL-1β and TNFα) in both epidermal keratinocytes and dermal fibroblasts attracting neutrophils containing metalloproteinase (MMP)-8 and MMP-12 (Pacheco-Palencia et al., 2008).

1.3.4.2 AP-1

Nuclear transcription factor activator protein 1 (AP-1) is a heterodimeric transcription factor belonging to FOS, JUN activating transcription factor (ATF) and musculoaponeurotic fibrosarcome (MAF) protein families (Eferl and Wagner, 2003). AP-1 mediates MAPK signalling pathways and thus, is responsible for controlling cellular differentiation, proliferation, apoptosis and for regulating MMPs (Quan et al., 2009). AP-1 has two subunits, *c-fos* and *c-jun* with research suggesting over-expression of *c-jun* in dermal fibroblasts leads to upregulation of MMPs and subsequent degradation of the extracellular matrix (ECM) (Rabe et al., 2006) and furthermore is the most potent transcriptional activator in skin tumours.

1.3.5 UVR-induced Cytokine Signalling

Cytokines are responsible for communicating and transmitting signals between cells. An intricate arrangement of cytokines creates equilibrium between pro- and anti-inflammatory effects. However, upon exposure to UVB, a complex array of cytokines are released by various cells including keratinocytes (Enk et al., 1995) and dermal fibroblasts (de Kossodo, 1995). These pro-inflammatory cytokines are heavily involved in driving the inflammatory response. The major pro-inflammatory cytokines include TNFα and IL-1α, IL-

1β, IL-6, IL-8, IL-10 IL-15 (Strickland et al., 1997). TNFα has been shown to be up-regulated in the human epidermis following exposure to UVB (Oxholm, 1988) and is responsible for triggering apoptotic keratinocytes (sunburn cell formation) post-UVB exposure (Pupe et al., 2003). TNFα and IL-1 are the major players involved at the beginning of the inflammatory response. IL-1 is a recognised leukocyte chemo attractant (Kupper, 1989, Kupper et al., 1987), which has been found to be up-regulated in the epidermis after UVB irradiation (Oxholm et al., 1988). IL-1 also has the ability to increase keratinocyte production of other cytokines such as TNFα, IL-6 and IL-8 (Chung et al., 1996). Furthermore both IL-1 and TNFα are capable of inducing transcriptional activation of a cascade of other molecules in the skin which are involved in the UVR-induced inflammatory response including phospholipase-A_2 (PLA_2), cyclooxygenase (COX)-2 and IL-8 (Storey et al., 2005a). IL-6 is a potent cytokine produced by keratinocytes *in vivo* and *in vitro* following UVR exposure and is thought to function as a mediator of the systemic sunburn reaction (Urbanski et al., 1990). Research has found IL-8 to be up-regulated in the epidermis following irradiation with UVB (Strickland et al., 1997) and is subsequently important in the mediation of UVB-induced inflammation (Storey et al., 2005b). Keratinocyte-derived IL-10 is found to be an important component in UVB-induced immunosuppression (Enk et al., 1995).

1.3.6 UVR-induced eicosanoids

Eicosanoids are biologically active signalling molecules synthesised by the oxidation of 20-carbon essential fatty acids. Many of these organic molecules are associated with inflammation due to their pro-inflammatory properties (Funk, 2001). Arachidonic acid (AA) (20:4n-6) (an omega-6 polyunsaturated fatty acid; PUFA) is found in the phospholipids of cell membranes and is the central precursor for eicosanoid synthesis. The release of AA from cellular membranes is facilitated by the enzyme PLA_2 which cleaves AA by hydrolyzing the fatty acyl group at the sn-2 position of the phospholipids which is rich in AA in the epidermal membrane (Gijon and Leslie, 1999, Gijon et al., 1999). Subsequently AA can also be metabolised via the COX and lipoxygenase (LOX) pathways to produce lipid derived mediators (Funk, 2001, Ziboh, 1992) [Figure 1.5**Error! Reference ource not found.**]. Transformation of AA by COX enzymatic pathway catalyses the formation of the prostanoid family which includes prostaglandins, prostacyclins and thromboxanes. Alternatively leukotrienes are formed via the LOX pathway which

catalyses the insertion of molecular oxygen into AA at the 5, 12 or 15 position producing hydroperoxyeicosatetraenoic acid (HPETE) which can then be reduced to the corresponding hydroxyeicosatetraeonic acid (HETE) (Smith, 1989). Prostaglandin (PG)E_2, thromboxane A_2 (TXA_2), leukotriene (LT)B_4 and 12-HETE are of particular interest in the sunburn response. Human epidermal keratinocytes regularly synthesis PG and it has been suggested PGE_2 production at low concentrations maintains the regulation of epidermal cell proliferation *in vitro* (Buckman et al., 1998). However, post-UVB exposure, COX-2 is up-regulated in keratinocytes resulting in a significant rise in lipid mediators including PGE_2. This therefore, indicates PGs may be important mediators of UVR-induced erythema (Rhodes et al., 2009). Several eicosanoids including PGE_2 and 12-HETE also have properties suggesting their activity in tumour promotion as well as inflammation. *In vitro* studies have demonstrated human skin irradiated with UVB increased concentration of AA, PGE_2, $PGF_{2\alpha}$ and PGD_2 in suction blister fluid 24 hours (hr) post irradiation, correlating with erythema intensity (Black et al., 1980, Black et al., 1978). UV-irradiated human skin cells including endothelial cells and keratinocytes release nitric oxide (NO) in association with PGE_2 causing vasodilatation of the dermal vasculature (Strickland et al., 1997, Deliconstantinos et al., 1995, Rhodes et al., 2001a). Rhodes *et al* (2001) demonstrated NO (known to enhance COX-2 activity) production requires a lower dose of UVB for its induction when compared to levels required to induce PGE_2 (Rhodes et al., 2001a). Other work has shown cells over-expressing COX-2 may inhibit apoptosis as well as increase the risk of neoangiogenesis (Tsujii and DuBois, 1995). The involvement of 12-HETE in the inflammatory response is as a potent keratinocyte derived neutrophilic (Dowd et al., 1985) and lymphocytic (Bacon et al., 1988) chemoattractant *in vitro*. A more recent human study, showed 12-HETE to be elevated in the later phases of the sunburn response suggesting it may play a role in the lymphocytic infiltration (Rhodes et al., 2009).

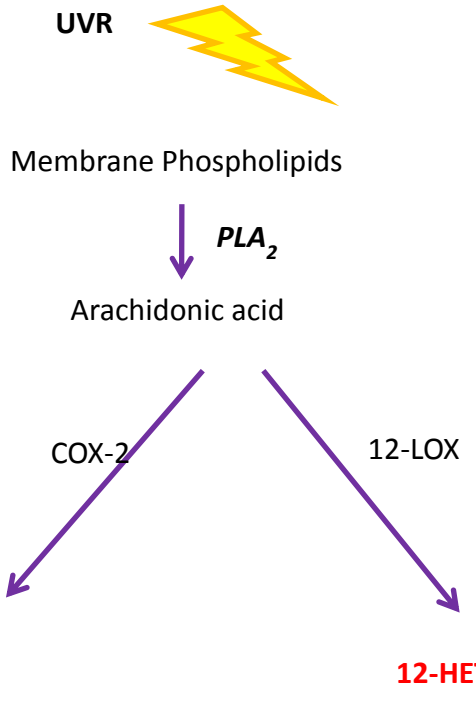


Figure 1.5 UVR-induced release of arachidonic acid (AA) from epidermal membrane phospholipids facilitated by the enzyme PLA_2. Subsequently AA is metabolised by COX and LOX enzymes into lipid derived mediators

1.4 Cutaneous effects of UVR on the skin

1.4.1 The sunburn response

Sunburn is an acute cutaneous inflammatory response which is self resolving. It is identified clinically by erythema and sometimes oedema, histologically by the thickening of the stratum corneum, formation of sunburn cells (apoptotic keratinocytes) and a dermal neutrophilic lymphocytic and monocytic infiltrate (Nicolaou et al., 2011). Biochemically there is increased production of pro-inflammatory mediators including eicosanoids, NO and cytokines. UVR-induced stimulation of cell surface receptors trigger the activation of the MAPK family of proteins and thus may alter transcription and up-regulate expression of pro-inflammatory genes.

The Fitzpatrick classification scale was developed in 1975 by Thomas Fitzpatrick (Fitzpatrick, 1988) and is a well established, though imperfect, tool used worldwide. It determines an individual's sensitivity to sunlight. It is based on a person's history of their skin response to solar UVR by also taking their physical characteristics into consideration [Table 1.1]. The skin type on the scale ranges from numbers I-VI. This classification indicates that people with a low phototype have a higher risk factor for skin cancer

because of their skin's response to UVR when compared to a person with a higher phototype. Therefore, a person with a low phototype is more likely to burn on exposure to the sun and thus are more susceptible to developing both melanoma and non-melanoma skin cancers. At the opposite end of the scale, people with phototype number VI have black skin that rarely burns upon exposure to sunlight.

Table 1.1 Skin phototypes according to the modified Fitzpatrick classification, adapted from (Fitzpatrick, 1988)

Skin Type	Skin Colour	Characteristics
I	Ivory white	Burns easily, never tans
II	White	Usually burns, tans minimally with difficulty
III	White	Burns/tans moderately and uniformly
IV	Beige-Olive	Rarely burns, tans moderately and easily
V	Moderate brown	Rarely burns, tans profusely
VI	Dark brown, Black	Never burns, tans profusely

1.4.1.1 Clinical features of the Sunburn Response

Vasodilatation of the dermal vasculature is one of the initial characteristics of skin following UVR exposure. UVB causes erythema (redness of the skin) and oedema (swelling). Exposure to UVA may also cause erythema but only at very high doses compared with those of UVB (Mahmoud et al., 2008). Erythema is evident as early as 3-4hr post-exposure (Andersen et al., 1991), maximal between 16-24hr (Deliconstantinos et al., 1995), remains elevated at 72hr (Rhodes et al., 2009) and is still evident at 96hr (Strickland et al., 1997). A time course for UVB-induced erythema indicated that both pro- and anti-inflammatory eicosanoids are involved in mediating the induction and resolution of the sunburn response, with COX metabolites mediating vasodilatation in the earlier phases (Rhodes et al., 2009). A previous study showed erythema is mediated at lower doses by NO and at higher UVR doses by the pro-inflammatory eicosanoid PGE_2 (Rhodes et al., 2001a).

1.4.1.2 Histological changes of the Sunburn Response

Following exposure to UVR, the stratum corneum (outermost layer of the epidermis) increases in thickness offering protection and thus helping prevent further damage (Rabe et al., 2006). Approximately 10-15% of UVB has been found to penetrate the upper papillary layers of the dermis (Everett et al., 1966). A dermal leukocytic infiltrate comprising of neutrophils and lymphocytes occurs within hours and is found to be maximal at 24h, with the neutrophil infiltration starting and resolving earlier than the lymphocyte infiltration (Strickland et al., 1997). LOX metabolites are thought to mediate later stages of the sunburn response due to their potent neutrophil and lymphocyte chemoattractant properties (Rhodes et al., 2009, Masoodi et al., 2008).

1.4.1.3 Neutrophilic and mononuclear infiltrate

Cells and tissues recruit neutrophils in response to substances at the site of inflammation. Neutrophils accumulate in the dermis and exert pro-inflammatory activities subsequently releasing further mediators with chemo attractant properties (Rhodes et al., 2009). IL-8 and TNF-α are significant chemoattractants involved in the sunburn response (Strickland et al., 1997). A time course for this neutrophilic infiltrate indicates it occurs 4hr post-UVB irradiation and is still evident at 24hr (Strickland et al., 1997). Research has shown the keratinocytes-derived metabolite 12-HETE generated a mixed neutrophilic and mononuclear infiltrate following an intradermal injection in human skin (Dowd et al., 1985). Black and colleagues (1985) showed 12-HETE to be elevated 12hr post-UVB irradiation in suction blister fluid (Black et al., 1985); however, a more recent study reported sustained elevation of 12-HETE at 72hr, indicative of its potential involvement in the later phase of the sunburn response (Rhodes et al., 2009).

1.4.2 Photosensitivity

In some individuals, exposure to low doses of UVA, and/ or UVB, and/ or visible light elicits atypical responses in the skin. These photosensitivity disorders may be caused by immune malfunction, biochemical disorders, damage to DNA repair mechanisms or by secondary phototoxic and/ or photoallergic reactions caused through exogenous agents such as chemicals, drugs, or plants (Murphy, 2001). Clinical features of the photodermatoses may include pruritis, pain, a wide range of rashes, scarring and, rarely,

photocarcinogenesis (Rizwan et al., 2012). Although treatment depends upon the disease, it often includes the use of phototherapy, broadband sunscreens and reduction in provocation or exacerbation of symptoms through avoidance of sunlight (including protective clothing). Application of antioxidants may also be beneficial through their anti-inflammatory and ROS-scavenging properties, if these contribute to the specific disorder (Lehmann and Schwarz, 2011).

1.4.3 Photoimmunosuppression

The concept of UVR as an immunosuppressor is relatively new. In 1974 Kripke *et al* demonstrated highly antigenic UVR-induced tumours grew only when transplanted into immunosuppressed mice and failed to grow in 'normal' mice due to immunological rejection (Kripke, 1974). Research has found that UVR impairs cell mediated immune (CMI) responses and thus many studies use models of CMI to demonstrate and investigate this. The contact or delayed hypersensitivity (CHS or DHS) model of CMI is widely used within photoimmunology whereby Ag' is applied to the skin, APCs engulf Ag', process it and present it to T lymphocytes, instigating the immune response (Amerio, 2009). UVB was found to induce both local (hapten application corresponds to site of UV-irradiation) and systemic immunosuppression (hapten application away from the UV-irradiation site) by suppressing these reactions when allergens were topically applied to skin (Amerio, 2009). Further studies have shown local immunosuppression to be mediated by alteration in APC function whilst systemic immunosuppression is mediated by modulation of receptors for interleukins (Grewe et al., 1996), cytokines, transcription factors, PGs (Rundhaug et al., 2007) and alteration of signal transducing pathways. Upon absorption of UVB by DNA, CPDs are formed. Research suggests these are a key molecular trigger for immunosuppression in humans and mice (Kuchel, 2005). Further evidence regarding the role of DNA damage in immunosuppression found UVB-induced CPD to cause morphological alterations to LCs as well as impairment of the immune response (Alcalay et al., 1990) and moreover Eggset *et al* (1983) reported LCs within in the epidermis disappeared following exposure to UVB (Eggset et al., 1983). Furthermore, Wang *et al* (2001) showed inhibition of antigen presenting capacity of LCs by the pro-inflammatory cytokine IL-10 (Wang et al., 2001). Aubin *et al* (2003) demonstrated suppressor T cells have the ability to induce apoptosis of APC (Aubin, 2003) with another group reporting solar simulated UVR decreased intra-epidermal T cells

(immunosurveillance) 1 and 2 days (d) post-irradiation thus increasing skin tumour and skin infection development (Di Nuzzo et al., 1998).

The chromophores involved in photoimmunosuppression are not well established however; recent work has focused on urocanic acid, in addition to DNA. Urocanic acid is an epidermal chromophore formed by the deamination of the amino acid histidine mediated by histidine ammonia lyase (Young, 1997). It is found at increased levels within the stratum corneum located in the outer layers of the epidermis. Much of the work on this subject has focused on the UVB region however to a lesser extent UVA also has the ability to induce immunosuppression. A study in human skin cells suggested the involvement of UVA in the induction of melanoma (Mouret et al., 2006).

1.4.4 Photocarcinogenesis

Photocarcinogenesis is a complex process involving three main stages, initiation, promotion and progression (Sander et al., 2004, Khan et al., 2006, Gruber et al., 2007). UVR is directly and/or indirectly involved in all three stages however, the peak wavelength for induction within the UVB range is 293nm (Nishigori, 2006). The most cytotoxic and mutagenic photoproducts are CPD and 6-4 PP formed primarily by UVB (and to a lesser extent UVA) interacting with DNA (Nishigori, 2006). UVA is involved mostly indirectly through generation of excessive amounts of ROS resulting in oxidative stress to macromolecules. Genetic alterations in p53, a tumour suppressor gene involved in cell cycle regulation have been found to be involved in >50% of malignancies including melanoma and non melanoma skin cancers (NMSC). It is thought that p53 aids DNA repair, helping eliminate cells with excessive DNA damage and thus preventing cancer. Unrepaired photoproducts in p53 lead to mutations which initiate photocarcinogenesis. There are three main types of skin cancer which will now be discussed.

1.4.4.1 Basal cell carcinoma

BCC is the most prevalent type of skin cancer accounting for more than 75% of all skin cancers in the UK. Although it may cause substantial morbidity it has little ability to metastasise and thus mortality rates are very low (Sander et al., 2004). BCC occurs primarily in people with sun reactive skin type I or II whom have been subjected to

sunburn as a child or have a strong family history of skin cancer (Wong et al., 2003, Gruber et al., 2007). It originates in epidermal keratinocytes (Young, 1997) thus UVB-induced alterations in DNA, typically GC to AT transitions form mutations in the p53 tumour suppressor gene (Nishigori, 2006). Increased susceptibility for developing BCC has been reported in immunosuppressed patients as well as patients with Xeroderma Pigmentosum and albinism (Wong et al., 2003).

1.4.4.2 Squamous cell carcinoma

SCC is the second most common form of skin cancer originating from keratinocytes in the basal layer of the epidermis (Young, 1997). The causal agent for SCC is UVB-induced CPD and 6-4 PP photoproducts (Brash et al., 1991, Marks, 1996) with C to T and CC to TT transitions identified in p53 (Brash et al., 1991). Further evidence showed a strong relationship between UVR-induced mutations on p53 and the development of SCC (Ziegler et al., 1994). Increased COX-2 expression has also been reported in patients with SCC and furthermore, actinic keratosis (AK), precancerous lesions are thought to transform into SCC (Hussein, 2005). Gruber and colleagues reported that lifelong cumulative sun exposure was a major contributory factor in the development of SCC (Gruber et al., 2007).

1.4.4.3 Malignant melanoma

MM is less common than BCC or SCC; however, it has the highest mortality rate, with 25% of sufferers dying from the disease. MM originates in the melanocytes within the epidermis. Upon UVR-exposure melanocytes synthesise the pigment melanin, an endogenous chromophore for UVB, UVA and visible light which has free radical scavenging properties (Therrien et al., 1999). Disruption of melanocyte homeostasis results in rapid cell proliferation. E-cadherin, a cell adhesion molecule is expressed normally in keratinocytes, melanocytes and LCs and during the induction of melanoma, E-cadherin switches to N-cadherin (expressed normally in dermal fibroblasts and endothelial cells) which results in melanocytes escaping the homeostatic control of keratinocytes (Santiago-Walker et al., 2009). Formation of CPDs by UVA-induced photosensitisation reactions are also thought to be involved in the development of

melanoma. Further reports have indicated intermittent exposure to intense sunlight particularly in childhood is a significant risk factor for the induction of MM (Lautenschlager et al., 2007, Gruber et al., 2007).

1.5 Photoprotection

Overexposure to UVR results in a marked increase in adverse effects on human skin. In addition to the use of conventional sunscreens it is vital to utilise other methods of sun protection such as clothing and restricting UVR-exposure time, particularly between the hours of 10am and 2pm in summer when there is high ambient UVB (Verschooten et al., 2006).

1.5.1 Topical sunscreens

If applied correctly conventional sunscreens are valuable in the prevention of sunburn, cutaneous DNA damage, photoageing and photoimmunosuppression (Lautenschlager et al., 2007). Topical sunscreens, when adequately applied, have the potential to reduce the level of UVR penetration into the skin; however, this is dependent on both the absorption and scattering of UVR photons (section 1.2.4) (Verschooten et al., 2006). There are several compounds used in topical sunscreens which have the ability to absorb photons of UVA and/or UVB (benzophenones, dibenoylmethanes) or block penetration of photons (zinc oxide, titanium dioxide) (Gupta and Mukhtar, 2002). Sunscreens are classified mainly by a sun protection factor (SPF) which is calculated by the minimal erythemal dose (MED) of un-irradiated skin divided by MED of irradiated skin (Verschooten et al., 2006). This refers primarily to UVB protection, while UVA protection may be indicated through a star grading. Limitations of using topical sunscreens include inadequate application (quantity and frequency), the use of too low an SPF and 'substantivity' (relative to water resistance and perspiration) (Lautenschlager et al., 2007). More recent developments in topical sunscreens are those containing antioxidants. A drawback of these was explained in a review article by Richelle *et al* (2006) who described the potential benefit of these is when antioxidants are in their active form and thus are stable both in the preparation and during application allowing penetration of the skin (Richelle et al., 2006).

1.5.2 Systemic photoprotection

Limitations of topically applied sunscreens have given rise to a new concept of sun protection. Endogenous or systemic photoprotection may provide a constant low dose of protection to the entire body surface. In terms of skin, the bioavailability of dietary bioactives is defined as the amount of active compound available (to the skin) after the compound(s) have crossed the intestinal barrier into the bloodstream and thus distributed to all tissues including the skin (Kroon et al., 2004, Richelle et al., 2006, de Mejia et al., 2009). It is possible that topical preparations may be more valuable in protecting the epidermis whilst dietary bioactives may be more beneficial in protecting deeper dermal layers. A further consideration concerning systemic photoprotection is that of competition and interaction from other ingested bioactives such that the physiological effects *in vivo* may vary (Richelle et al., 2006).

1.5.3 Photoprotection by antioxidants

ROS can be produced by mitochondria and membrane structures in cells (Murphy, 2009, Scherz-Shouval and Elazar, 2007) (see section 1.3.2.1). Normally, they are balanced by the continuous production of endogenous antioxidant enzymes (sodium dismutase (SOD), catalase (CAT) and glutathione perioxidase (GPx)) and non-enzymatic antioxidants (β-carotene, glutathione, vitamins E and C). Upon stress from external stimuli, such as UVR, overwhelming levels of ROS are generated causing rapid depletion of endogenous antioxidant defence systems. This disequilibrium may lead to damage of cellular and non-cellular components, in turn oxidative stress and subsequently photoimmunosuppression, photoageing and photocarcinogenesis. In an animal study, mice irradiated with solar simulated radiation (SSR) (25 J/cm^2) showed depletion of endogenous antioxidants in both the epidermis and dermis although the reduction was most severe within the epidermal layers (Shindo et al., 1993) Furthermore, antioxidant depletion in both the epidermis and dermis showed a UVR dose-response. A range of nutrient antioxidants have been utilised to reduce the impact of UVR-induced ROS and free radicals on skin, including punicalagin found in pomegranate (Pacheco-Palencia et al., 2008), proanthocyanidins present in grapes (Mantena and Katiyar, 2006) and quercetin found in apples (Erden Inal and Kahraman, 2000). Many polyphenols are known to scavenge ROS and thus have potential to reduce oxidative injury to biological macromolecules such as

lipids, proteins and DNA. Their notable ability to prevent oxidative damage to DNA (Weisel et al., 2006, Schaefer et al., 2006) suggests their potential as chemopreventive agents.

An area gaining considerable interest is that of the 'antioxidant response element' (ARE) (also known as the electrophile response element; EpRE). Mammalian cells have evolved the ARE to help ameliorate oxidative stress caused to biological macromolecules such as proteins, lipids and DNA. It functions to neutralise ROS and electrophile intermediates and maintain cellular redox homeostasis. AREs are found in the promoter region on phase II drug metabolising and antioxidant genes (Jeyapaul and Jaiswal, 2000, Issa et al., 2006) and are regulated by the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). The pathway is activated upon generation of chemical signals from oxidative stress causing disequilibrium in cellular redox homeostasis. Inhibition of keap1-Cul3-Rbx1-E3 ubiquitin ligase causes increased levels of Nrf2 which subsequently activates its downstream target genes, i.e. glutathione S-transferase (GST), NAD(P)H dehydrogenase, quinone 1(NQO1), heme oxygenase 1 (HO-1), γ-glutamylcysteine synthetase (γ-GCS) via the ARE. Keap1 translocates to the nucleus removing Nrf2 by nuclear export signal (NES). The Nrf2 pathway can be seen in figure 1.6 where it illustrates how antioxidants may modulate cellular events through the activation of NRF2, a recognised molecular target for chemoprevention.

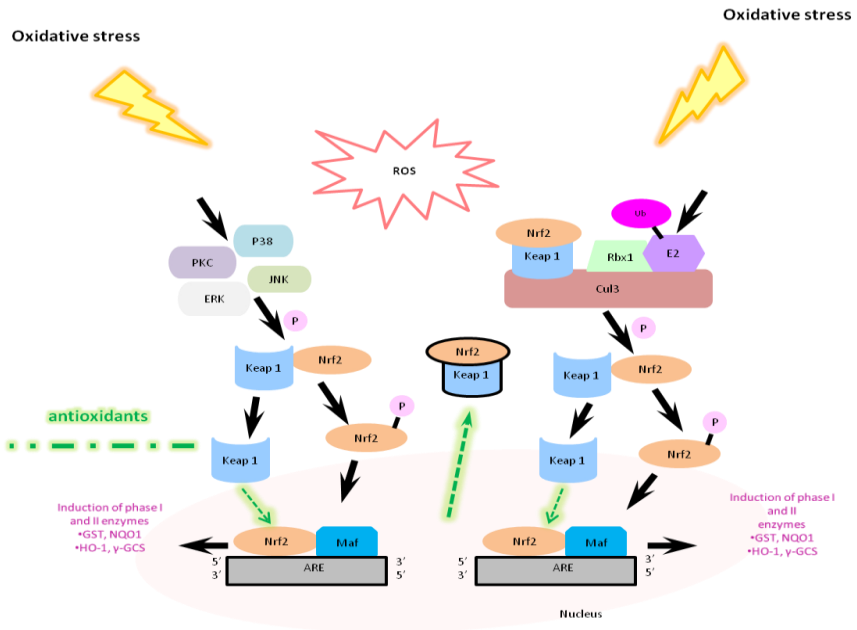


Figure 1.6 Nrf2 signalling pathway acts to ameliorate oxidative damage caused to biological macromolecules. Dietary bioactives such as polyphenols ha have the ability to modulate NRF2-mediated cellular events.

1.6 Green tea catechins

1.6.1 Source and processing

Tea is the second most consumed beverage in the world and is second only in popularity to water. Most of the tea consumed worldwide is black tea but around 20% is consumed as green tea. Tea originates from the plant *Camellia sinensis* and while both black and green tea contain similar amount of flavonoids, they differ in their chemical structure due to the post-harvest processing. During green tea manufacturing the leaves of the plant are extensively steamed and dried at high temperatures, inactivating polyphenol oxidase and thus preventing the oxidation and polymerisation of polyphenolic compounds (Vayalil et al., 2004, Mukhtar and Ahmad, 2000, de Mejia et al., 2009). Polyphenol oxidase is the enzyme responsible for converting monomeric catechins (main polyphenols found in green tea) into theaflavins and thearubigins, the catechin polymers present in black tea (Cabrera et al., 2006).

1.6.2 Classification

Polyphenols are complex molecules with an array of chemical structures subsequently affecting their biological properties, stability, bioavailability (Scalbert and Williamson, 2000) and antioxidant activity (Scalbert and Williamson, 2000, de Mejia et al., 2009, Rice-Evans et al., 1996). Polyphenols have long been reported to offer health benefits but it has only been in recent years that the mechanisms involved have been studied in depth. Polyphenols are broadly divided into tannins, lignins and flavonoids and are synthesised via three main metabolic pathways; terpenes, carotenoids and saponis by the isoprenoid pathway; flavonoids, isoflavonoids, coumarins and lignins via the cinnamic pathway and phenolic compounds, particularly tannins via the shikimik acid pathway (Issa et al., 2006).

Catechins belong to the flavanol subgroup of polyphenols and consist of four main compounds found in green tea are: (-) – epicatechin (EC), (-) – epigallocatechin (EGC), (-) – epicatechin-3-gallate (ECG) and (-) – epigalocatechin-3-gallate (EGCG) [Figure 1.7]. EGCG is the key active compound present in green tea with its high antioxidant capacity and biological activity owing to the hydroxyl groups at the 3’ and 5’ position on the B ring and gallic acid moiety on the C ring (Khan et al., 2006, Henning et al., 2008, Minoda et al., 2010). EGCG is indeed found to be highly bioavailable with peak plasma concentrations

being achieved around 1.5 hours post ingestion (Manach et al., 2005, Lee et al., 2002, Malhomme de la Roche et al., 2010).

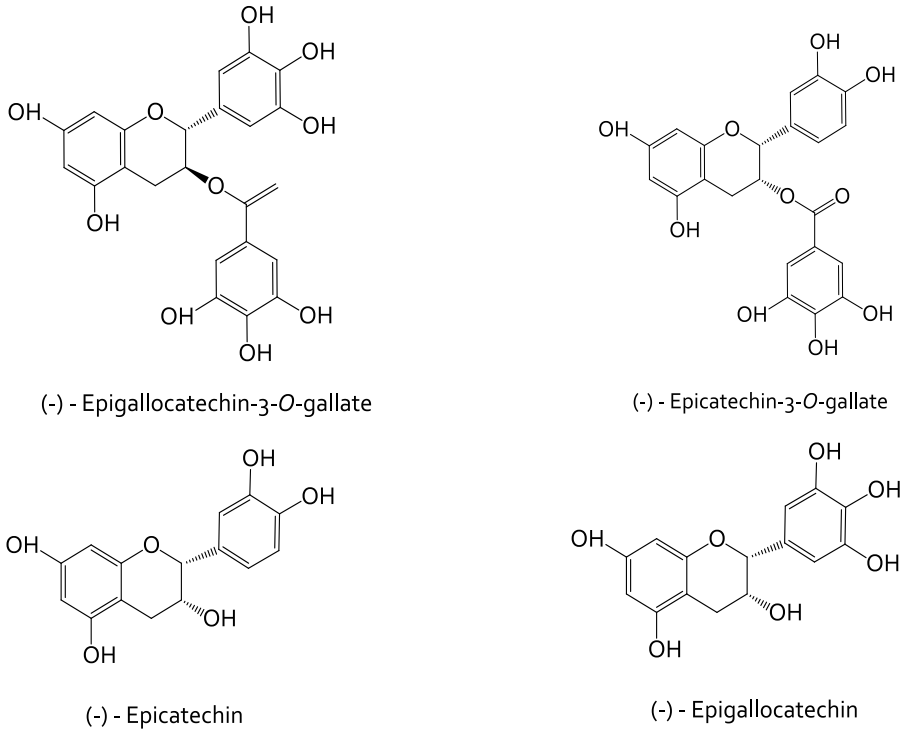


Figure 1.7 Chemical structures of the major catechins found in green tea

1.6.3 Effects on skin

Green tea in itself has been the subject of many epidemiological studies and much of the research in humans around its photoprotective properties has focused on topical application rather than oral ingestion (Malhomme de la Roche et al., 2010). However; it is widely accepted as having both anti-inflammatory and cancer-preventative actions (Katiyar et al., 2000a) and it is hoped these properties could be utilised to treat a variety of skin disorders. Figure 1.8 pictorially describes how they may exert their action via both their antioxidant and anti-inflammatory properties and these are also discussed in more detail below.

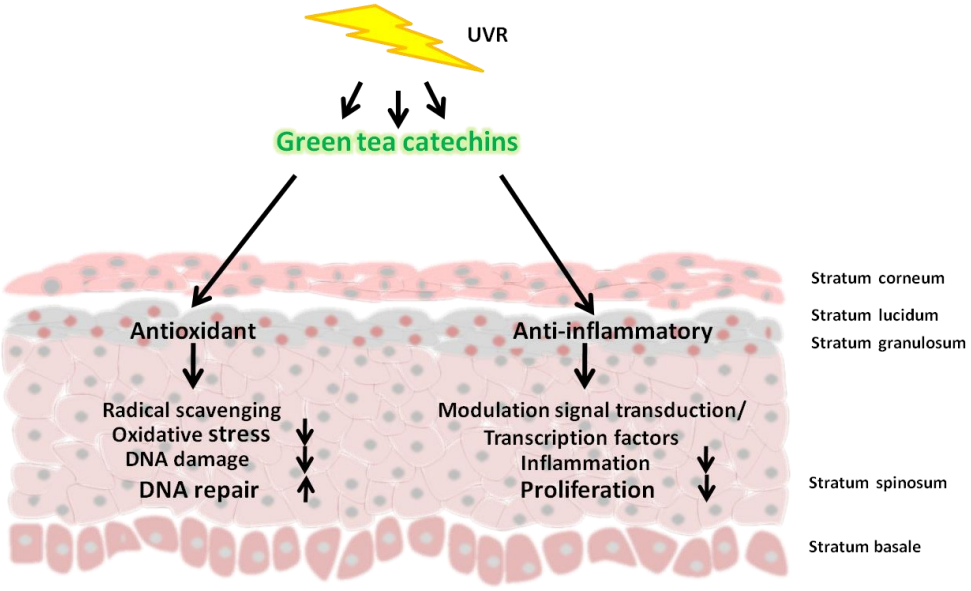


Figure 1.8 Effect of green tea catechins on UVR-modulated molecular pathways in skin

1.6.3.1 Antioxidant effects

Skin cancer is primarily initiated through the UVR-induced formation of CPD. Continuous DNA damage caused either directly from UVB or indirectly from UVA (generation of ROS) may subsequently result in genetic mutations. This is typically evident in the p53 tumour suppressor gene and thus is recognised as a cancer initiator (Tyson and Mathers, 2007). Mice that were pre-treated with topical EGCG, the major catechin in green tea, did not develop UVB-induced photocarcinogenesis (Wang et al., 1992, Mantena et al., 2005). Further work established that EGCG prevented UVB-induced immunosuppression via the induction of IL-12 (Katiyar et al., 1999a), and moreover, found *XPA*-deficient human fibroblast cells treated with EGCG promoted UVB-induced CPD repair, implying NER was the mechanism involved and thus, EGCG can prevent photocarcinogenesis through an EGCG-induced IL-12-dependent DNA repair mechanism (Meeran et al., 2006). A further study reported topically applied green tea catechins (GTC) protected against tumour initiation in the epidermis by inhibiting 12-*O*-tetradecanoylphorbitol-13-acetate (TPA) (involved in tumour promotion) indicating therefore GTC are capable of inducing phase II detoxifying enzymes (Ramos, 2008, Hsu et al., 2003). In human subjects, topically applied GTC reduced UVR-induced DNA damage, erythema and leukocyte infiltration (Katiyar et al., 1999b, Katiyar et al., 2000b). A recent human study assessed 10 volunteers before and after drinking 540ml of green tea. The results showed EGCG afforded concentration-dependent photoprotection to cultured human cells. Samples of peripheral blood cells

taken after green tea consumption showed lower levels of DNA damage than those taken prior to ingestion when exposed to 12 mins of UVA radiation (Morley et al., 2005). A dietary intervention study by Heinrich *et al* (2011) demonstrated women who consumed a green tea beverage (1402mg total catechins) for 12 weeks reduced the skin's sensitivity to UVR-induced erythema and furthermore increased microcirculation and skin structure (Heinrich et al., 2011).

1.6.3.2 Anti-inflammatory effects

It is now well established that green tea has: 1) the ability to modulate signalling pathways [Figure 1.9] including MAPK and transcription factors, particularly, NFκB and AP-1, and; 2) the ability to inhibit pro-inflammatory mediators such as COX (Khan et al., 2006). EGCG has been studied extensively and studies claim its overwhelming anti-inflammatory properties are owing to the galloyl and hydroxyl groups at the 3' position of the compound (Khan et al., 2006). At a cellular level EGCG has been shown to decrease the number of sunburn cells and increase the rate of apoptosis in DNA damaged keratinocytes following UVR. EGCG therefore appears to have a differential effect on benign versus premalignant cells. A study by Vayalil *et al* (2003) found mouse skin pre-treated with EGCG inhibited single UVB-induced phosphorylation of JNK (16-95%) and p38 (100%) in a time-dependent manner (Vayalil et al., 2003). Research has shown EGCG to be an effective inhibitor to UVB-induced activation of NFκB (a transcription factor involved in inflammation) thus sequentially blocking inducible nitric oxide synthase (iNOS) (preventing NO production) (Mukhtar and Ahmad, 2000, de Mejia et al., 2009). Normal human epidermal keratinocytes (NHEK) pre-treated with EGCG were shown to modulate UVR-mediated NFκB activation through the inhibition of IKKα expression (Afaq et al., 2003a). A study by Khan *et al* (2006) reported EGCG is able to inhibit COX-2 expression successfully without affecting COX-1 albeit the results were dose-dependent with optimum results at 10 -100 µmol/L (Khan et al., 2006). One animal study showed that dietary supplementation with GTC inhibited COX-2 expression in mouse skin that had been pre-treated with a tumour-promoter (Kundu et al., 2003). SKH-1 hairless mice given oral GTC showed an inhibitory effect on COX activity (Agarwal et al., 1993) and EGCG was shown to inhibit IL-1B-induced production of PGE_2 and NO in human chondrocytes (Ahmed et al., 2002).

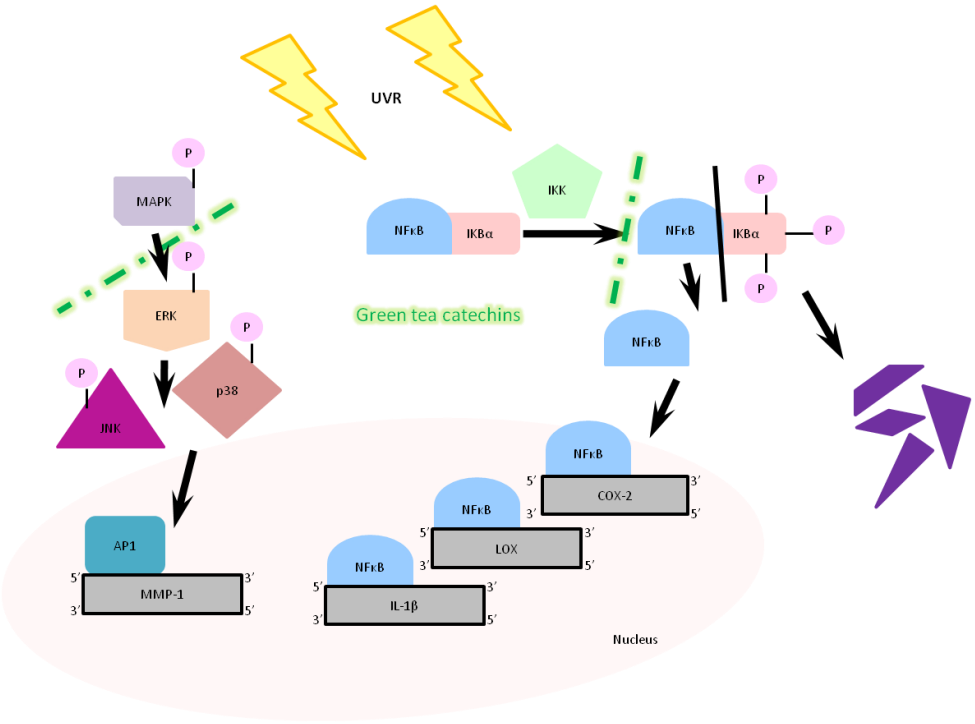


Figure 1.9 MAPK and NFκB pathways are activated in response to stimuli such as UVR and are responsible for a number of inflammatory responses, cellular differentiation, proliferation and apoptosis. GTC inhibit phosphorylation of JNK/p38 and prevent downstream activation of NFκB signalling pathway through inhibition of IKKα, impeding translocation of transcription factors to the nucleus and subsequent induction of gene expression.

1.6.4 Bioavailability and metabolism

The bloodstream delivers nutrients and bioactive compounds to tissues and hence diet is of great importance in the maintenance of skin health (Franzoni et al., 2004). Catechin metabolites found within the blood are known to differ greatly from the native compounds found in pre-ingested food.

Catechins are still intact when they leave the oesophagus (Tsuchiya et al., 1997); however, upon absorption *in vivo* they are observed in a complex array of forms, a subsequent result of three major biotransformation pathways known as methylation, glucuronidation and sulfation [Figure 1.10]. Much of the catechin metabolism occurs within the small intestine, colon and liver. Catechins absorbed within the human intestinal tract are largely metabolized and distributed as conjugated derivatives in blood and these forms are excreted in urine. Catechins are known to have poor stability in the small intestine due to pH, and ROS-induced auto-oxidative reactions and thus hydroxyphenyl-valerlolactones (unique catechin metabolites) are produced by colonic

ring fission. Benzoic acid is one of the final products formed from the bacterial decomposition of catechins. Benzoic acid in the liver is conjugated with a glycine molecule giving rise to hippuric acid, a major catechin excretory compound used as a biomarker of catechin consumption.

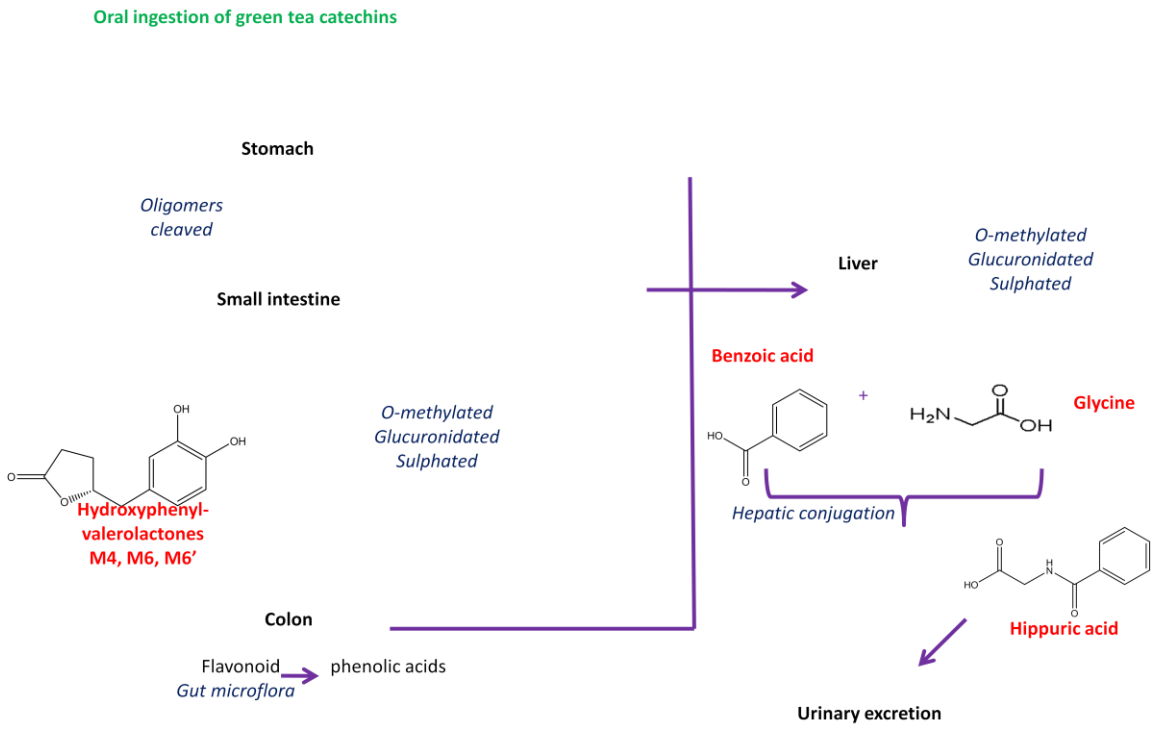


Figure 1.10 Proposed catechin metabolism in humans *in vivo*

1.6.5 Stabilisation by vitamin C

There is evidence to suggest that vitamin C (also known as ascorbic acid) has the ability to stabilise GTC as well as increasing its antioxidant potential. A study by Peters *et al* (2010) established the presence of vitamin C improves catechin bioavailability through enhancing accessability and intestinal uptake from tea through both pre-absorptive and absorptive mechanisms (Peters et al., 2010). Moreover, catechol structures are known to oxidize in a neutral or alkaline environment therefore a further experimental study suggested vitamin C stabilised GTC in an oxygen free environment and, although this may not be directly transferrable *in vivo*, it has been suggested that the presence of vitamin C may increase stabilisation of GTC in the intestines by reducing the pH (Zhen-Yu Chen et al., 1998) subsequently increasing bioavailability.

1.6.6 Safety

Studies have shown high doses of green tea polyphenols (particularly EGCG) pose no toxicity risks after dietary supplementation of 800 mg/d for a period of 4 weeks and there are no pro-oxidant adverse effects *in vivo* (Chow et al., 2003).

1.7 Vitamin C

1.7.1 Sources of vitamin C

Vitamin C is an essential nutrient required for the maintenance of healthy connective tissue providing structure and support to other tissues and organs in the body. Over time humans have lost the ability to synthesise vitamin C through the loss of gulonolactone oxidase (GULO), an enzyme required for the final step of the synthesis via its precursor 2-keto-1-gulonolactone (Padayatty et al., 2003, Rivas et al., 2008). Therefore, vitamin C must be taken orally (diet or supplementation) to maintain efficient vitamin C stores. It is regarded as a potent antioxidant which is found in abundance in a variety of fruit and vegetables with particularly good sources including: oranges and orange juice, red and green peppers, berries, broccoli and spinach.

1.7.2 Vitamin C isomers

There are two main forms of vitamin C: 1) ascorbic acid (reduced form), and; 2) dehydroascorbic acid (oxidised form). Ascorbic acid sequentially releases two electrons. The release of the first electron gives rise to semi-dehydroascorbic acid which is partially oxidised and thus can either accept or donate an electron with the latter generating dehydroascorbic acid. Both forms of vitamin C require transporters to allow absorption in to the body.

1.7.3 Vitamin C transporters

Vitamin C is absorbed in the body by both active transport and simple diffusion. The transporter system used is specifically dependent on the type of vitamin C being absorbed. The two membrane transporters required for vitamin C absorption are: 1) sodium-coupled transporters (SVCTs), and; 2) glucose transporters (GLUTs). SVCTs of

which there are two isoforms (SVCT1 and SVCT2) transport the reduced form (ascorbic acid) across the plasma membrane by active transport whereas the GLUT family of transporters GLUT1, GLUT3 and GLUT4 transfer dehydroascorbic acid by the simple diffusion method. Most cells within the body express both transporter systems however, each acquire absolute specificity for either the reduced or oxidised form (Rivas et al., 2008).

1.7.4 Vitamin C in skin

Vitamin C is a low molecular weight antioxidant which resides in both the epidermal and dermal layers of skin. It is a non-enzymatic hydrophilic antioxidant which is absorbed rapidly (Richelle et al., 2006). Epidermal keratinocytes accumulate high levels of vitamin C facilitating part of the endogenous antioxidant defence system. Lower concentrations are found within the dermis whereby vitamin C is a co-factor for collagen synthesis. These low levels have been found to further deplete with age (Catani et al., 2005). Vitamin C also plays an important role in the protection of fat soluble antioxidants (vitamins A and E) from oxidation.

1.7.5 Reference nutrient intake and toxicity

Current UK dietary reference values for vitamin C intake is 40 mg/d for both men and women which is based on reference nutrient intake (RNI). Richelle *et al* (2006) reported plasma levels of vitamin C remain constant when ingestion is above 400 mg/d due to higher renal and faecal excretion (Richelle et al., 2006). Toxicity is thought to be unlikely as the body does not have the ability to store vitamin C. However, Padayatty *et al* (2003) suggested toxicity of this vitamin warranted further study as to whether it may create a pro-oxidant effect *in vivo* (Padayatty et al., 2003). It is therefore recommended that nutritionally relevant amounts are used within human studies.

1.7.6 Vitamin C and UVR-induced inflammation

Vitamin C alone was found to offer no protection against UVA-irradiated human skin fibroblasts (Offord et al., 2002). Furthermore, a clinical trial confirmed volunteers supplemented with high dose ascorbic acid (3g) for a period greater than 50 days

provided no protection from SSR-induced skin erythema (Fuchs and Kern, 1998). Moreover, other human studies are in agreement that monotherapy with vitamin C does not impact on UVR-induced erythema response in skin (McArdle et al., 2002, Mireles-Rocha et al., 2002, Fuchs and Kern, 1998). However, studies have shown it to have synergistic effect when working in combination with vitamin E. A short (1 week) single-blind human trial of supplementation with α-tocopherol 1200 international units (i.u.) and 2g ascorbic acid daily increased subjects' MED from a median of 50mJ/cm^2 at baseline to a median of 70mJ/cm^2 following supplementation (Mireles-Rocha et al., 2002) and in a further double-blind placebo-controlled human study supplementation with 2g vitamin C and 1000i.u. vitamin E to volunteers for 8 days showed a significant increase in their threshold to UVB-induced erythema (Eberlein-Konig et al., 1998).

1.8 Hypothesis and aims of the thesis

The overall aim of my thesis is to determine the effect of a combination of oral GTC and vitamin C administered at nutritionally relevant amounts on the bioavailability and cutaneous uptake of GTC in human skin. Furthermore, I aim to evaluate the impact of the oral intervention on the sunburn response (UVR-induced inflammation) and examine the modulation of UVR-induced pro-inflammatory eicosanoids PGE_2 and 12-HETE.

I hypothesise that oral GTC are bioavailable in human skin and through COX and LOX inhibition may reduce the production of UVR-induced pro-inflammatory eicosanoids PGE_2 and 12-HETE, which in turn may reduce the UVR-induced erythemal response.

Specific objectives are:

1) To assess the uptake and bioavailability of GTC in human skin;

2) To make preliminary assessment of the impact of GTC and vitamin C on UVR-induced inflammation (erythema);

3) To make a preliminary assessment of the impact of GTC and vitamin C on UVR-induced eicosanoid expression.

CHAPTER 2: Materials and Methodology

A total of 19 subjects were recruited for two studies. The initial study was a UVR-dosimetry study (n=3) to determine the UVR lamp source and dose to be used. The second study was an open oral GTC intervention study (n=16) to determine the effect of a combination of GTC and vitamin C on UVR-induced skin damage.

2.1 Regulatory approvals

Ethical approval was obtained from the North Manchester Research Ethics Committee (ref: 08/H1006/79) and regulatory approval was gained from Salford Royal NHS Foundation Trust R&D department. The Medicines and Health Regulatory Agency (MHRA) did not require an application for this supplement study. The trial was conducted according to the Declaration of Helsinki (Seoul, Republic of Korea, 2008) and all volunteers gave written informed consent before study commencement.

2.2 Volunteers

All subjects (n=19) confirmed their eligibility by answering inclusion and exclusion criteria screening questions [Table 2.1]. Compliance with supplement intake was assessed by urine samples at baseline, day 1, week 6 and week 12 post supplementation.

Table 2.1 Inclusion and exclusion criteria

Inclusion	Exclusion
Healthy white Caucasian	History of skin cancer
Skin phototype I/ II	Taking photosensitising or anti-inflammatory medication
Age 18-65 years	Pregnancy/ breastfeeding
	Taking nutritional supplements
	Ingesting ≥ 2 cups of tea per day
	Sunbed use or sunbathing in last 3 months

2.3 UVR spectrum and dosimetry study.

Subjects (n=3) attended the Photobiology unit, Salford Royal NHS Foundation Trust on 3 consecutive days. On day one: upper buttock skin was irradiated with both TL-12 (section 2.3.1.1) and SSR (section 2.3.1.2) UVR sources. On day two: the participant's erythemal response to each UVR source was assessed both visually (MED) (section 2.3.2.1) and by reflectance spectrophotometry (section 2.3.2.2) after 24hr. The time for each source to provide the subject with 3x their MED was calculated and previously un-irradiated buttock skin was irradiated. On day three: At 24hr post irradiation, suction blisters were raised and fluid extracted (section 2.4.5.2). Blister fluid samples were snap frozen in liquid nitrogen and were assayed for PGE_2 by ELISA (section 2.3.3.1).

2.3.1 Irradiation equipment

2.3.1.1 TL-12 UVR source

For the UVR dosimetry study (n=3), one of the UVR sources used was a Phillips TL-12 20 watt (W) (TL 20W/12 RS/SLV) fluorescent UVR lamp (Philips GmbH, Hamburg, Germany) [Fig 2.1]. The lamp was housed in a black plastic tube containing five apertures on the side, each measuring 10mm in diameter.

Figure 2.1 TL20W/12 UVR source. Hand held broadband UVB light source with five graduated filter apertures allowing a series of UVR doses to be administered simultaneously.

This fluorescent broadband lamp emits a spectrum of radiation between 270-400nm, peaking in the UVB range at 313nm and in the UVA at 365nm [Fig 2.2]. The relative spectral emission of UVB, UVA and UVC is ~44%, 56%, and 1% respectively. The lamp has 5 apertures, such that an approximate geometric series of 10 UVR doses were administered to each volunteer by applying the lamp to the skin for two separate timed applications.

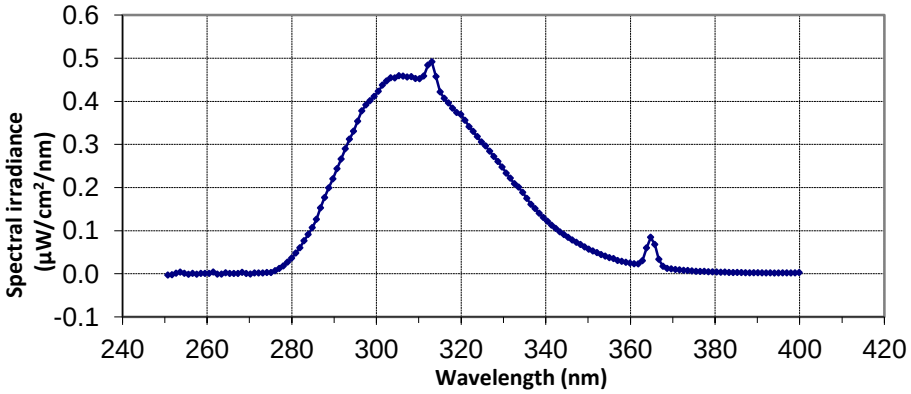


Figure 2.2 Emission spectrum of 20W/12 (provided by Dr D. Allan, Medical Physics Department, Christie Hospital, Manchester, UK)

The series of geometric UVR doses given were as follows:

6.7, 8.1, 11.5, 13.4, 19.7, 24.0, 29.0, 41.2, 47.9, 70.3 mJ/cm^2

Prior to use, the lamp was allowed to warm up for a period of 5 mins and its irradiance was measured to confirm the consistency of output of the lamp. This was recorded using a calibrated UVR radiometer designed by Professor Brian Diffey (serial number MJL59, Medical Physics laboratory, Durham hospital, UK) [Figure 2.3].

Figure 2.3 Diffey UVR Meter for measuring irradiance output of TL 20W/12

2.3.1.2 Solar simulator

The solar simulator (Newport, Spectra-Physics Ltd, model No. 91293) was used in both the dosimetry and open intervention studies [Figure 2.4]. It is often used for experimental

studies in photobiology as it provides a simulation of the UVR components of sunlight that reach the Earth's surface.

Figure 2.4 Solar simulator providing the UVR components of sunlight.

The illuminator housing contains a 1000W xenon arc lamp, an arc lamp ignitor, collimating optics, a light shutter and light shutter power supply. The collimated output beam is 6 x 6 inches emitting radiation in the combined UVB and UVA spectral range (290-400nm) consisting of approximately 3.35% UVB and 96.68% UVA respectively [Figure 2.5].

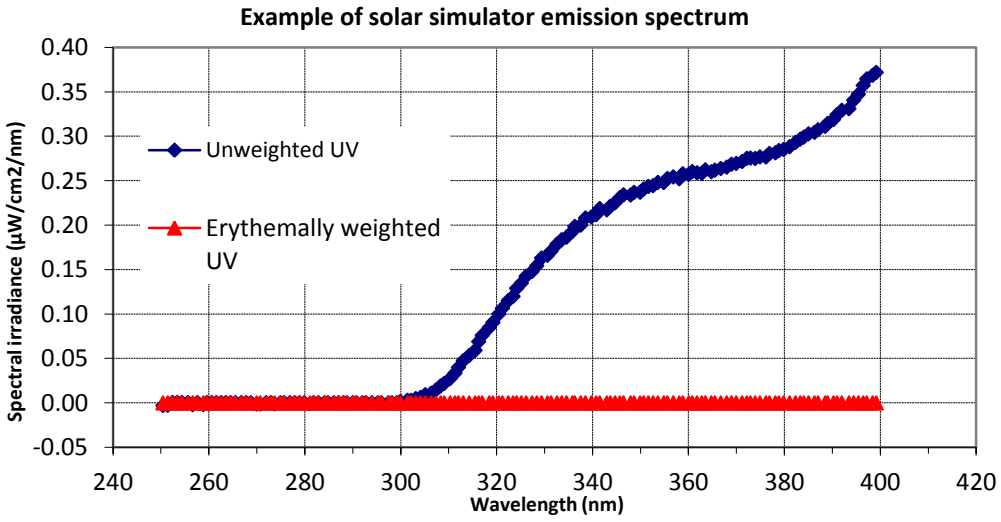


Figure 2.5 Emission spectrum of the solar simulator (provided by Dr D. Allan, Medical Physics Department, Christie Hospital, Manchester, UK.

The solar simulator also contains a dichroic mirror which helps to reduce both infrared and visible light. This enables delivery of UVR eliciting an inflammatory response (erythema) in a realistic time frame.

The dose series used were as follows:

6.6, 8.2, 11, 14, 18, 27, 35, 43, 54, 68 mJ/cm^2

Following a warm up period of 15 mins the consistency of the lamp was checked by measuring its UVR irradiance. This was done using two meters: (1) IL-1400A meter (International Light Inc. Newburyport, USA), and; (2) Waldmann UVR-meter (Herbert Waldmann, GmbH & Co., Villingen Schwenningen, Germany, serial number 10267). Both meters are positioned in the centre of the beam 10cm below the lens, and once they had stabilised the measurements were recorded.

2.3.1.3 Phototesting template

The phototesting template [Figure 2.6] was used with the solar simulator in order to deliver a UVR dose series. The template was made from thick black rubber material which comprised of 10cm x 1cm diameter apertures (Medical Physics department, Salford Royal NHS Foundation Trust, Manchester, UK). The template was placed onto photoprotected upper buttock skin and secured with Micropore™ (3M Healthcare Ltd, UK) hypoallergenic tape. The series of UVR doses given were determined upon the irradiance output of the solar simulator with the apertures covered manually in a timed fashion.

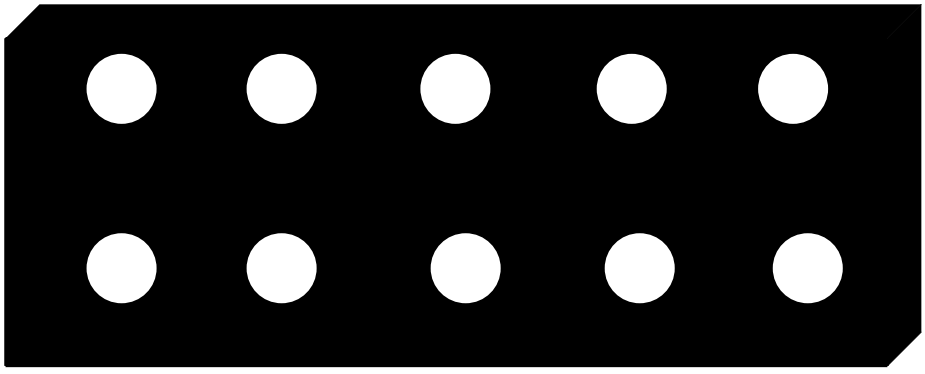


Figure 2.6 Illustration of phototesting template used to give a series of 10 UVR-doses using the solar simulator

2.3.1.4 UVR dosimetry

UVR dose is a measure of UVR irradiance in milliWatts (mW) over a given time (seconds) and is expressed in milliJoules per cm^2 (mJ/cm^2). The UVR dose is calculated as follows:

Dose (mJ/cm^2) = irradiance (mW/cm^2) x time (seconds)

The doses are erythemally weighted to allow comparison of different emission spectrum lamps in terms of their ability to produce erythema. The standard spectrum for erythema is called the *Commission Internationale de l'Éclairage* (CIE) erythema action spectrum (McKinlay, 1987). These 'standardised' graphs are produced by multiplying the lamp irradiance values by the corresponding CIE erythema action spectrum value.

2.3.2 Assessment of erythema

2.3.2.1 Visual assessment

Erythema was assessed visually at 24hr after phototesting to determine the MED. This is a subjective measure of an individual's sunburn threshold and is the smallest dose (mJ/cm^2) of UVR required to cause just perceptible erythema (Dornelles et al., 2004). Studies have indicated a correlation between MED and phototype as used with the Fitzpatrick classification scale (Fitzpatrick, 1988). A study of 193 individuals showed a strong correlation between skin type and MED (Dornelles et al., 2004); however, an earlier study (n=36) found no relationship between the slope of the dose response and sun-reactive skin type (Rhodes and Friedmann, 1992).

2.3.2.2 Reflectance spectrophotometry

The Dia-Stron erythema meter (Dia-Stron Ltd, Andover, UK) was the reflectance instrument used to objectively measure erythema intensity. Erythema was measured 24hr following UVR-exposure [Figure 2.7]. A fibre- optic probe illuminates the skin by shining a white light on to both un-irradiated and UV-irradiated areas. Subsequently, reflected red and green light from the skin is measured using narrow pass interference filters at 546nm and 671nm. Erythema occurs as a result of the vasodilatation of blood vessels in the superficial dermis and the measurement of erythema index (EI) uses haemoglobin (Hb; a protein found in red blood cells) as a cutaneous chromophore of

green light. As the amount of Hb increases the amount of green light absorbed also increases whilst the red light shows little change. EI is calculated by:

EI = $\log_{10}$ = reflected red light / reflected green light

2. 7 Reflectance instrument used to measure erythema index

Prior to measuring erythema, all subjects were rested prone in a temperature controlled room (~23°C) for 15 mins allowing skin and its vasculature to acclimatise to surrounding conditions. Readings were recorded in triplicate from each of the 10 UVR dose sites as well as two sites adjacent to these which acted as controls. The mean control reading was subtracted from each of the mean EI readings giving a change in erythema (ΔE).

2.3.3 Laboratory Methodology

2.3.3.1 Determination of PGE_2 production in blister fluid samples by ELISA

In this small preparatory study (n=3), blister fluid samples assayed for PGE_2 by ELISA as liquid chromatography coupled to electrospray (ESI) ionization tandem mass spectrometry (LC-ESI-MS/MS) has to be run for larger sample batches. The PGE_2 assay was obtained from R&D Systems Ltd (Abingdon, UK). Blister fluid samples were taken for the quantitative analysis of PGE_2 to determine optimal UVR source and dosimetry for subsequent human studies. The principle of the assay involved PGE_2 in blister fluid samples competing against horseradish peroxidase (HRP)-labelled PGE_2 conjugate for the binding sites on a mouse monoclonal antibody. Following a wash to remove all unbound materials, a substrate was added to determine bound enzyme activity. The colour intensity is inversely proportional to the concentration of PGE_2 in the blister fluid.

2.3.3.2 Preparation of reagents for PGE2 assay

2.3.3.2.1 Wash Buffer

20ml wash buffer concentrate was diluted into a volumetric flask using distilled water (dH_2O) to prepare 500ml wash buffer.

2.3.3.2.2 Substrate solution

15 mins prior to use, equal volumes of colour reagent A (hydrogen peroxide, H_2O_2) were mixed with colour reagent B (chromagen) (200µl mixture per well) and stored in a dark place.

2.3.3.2.3 PGE_2 standard

The standard was reconstituted with 1.0 ml dH_2O to produce a stock solution 25,000pg/ml. The solution is prepared and allowed to sit for a minimum of 15 mins prior to use.

2.3.3.2.4 Stock solution dilution series

900µl calibrator diluent RD5-56 was transferred into 2500pg/ml stock solution using a pipette. A further 500µl of calibrator diluent RD5-56 was added to the remaining tubes using the dilution series in figure 2.8.

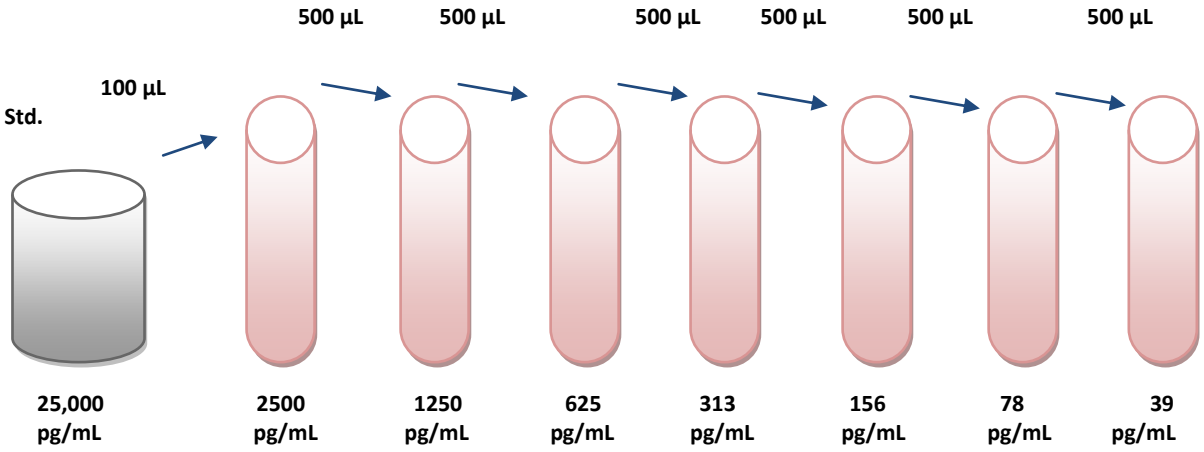


Figure 2.8 Schematic of PGE_2 stock solution dilution series

2.3.3.3 PGE_2 assay methodology

Blister fluid samples were diluted with calibrator diluent RD5-56 using a dilution factor of 8.75 and analysed in duplicate. A further 200µl of calibrator diluent RD5-56 was added to the non-specific binding (NSB) wells in the microplate and 150µL of calibrator diluent RD5-56 added to B_0 wells and used as the zero standard. A final volume of 150µL of standard, control or blister fluid sample was added to the remaining wells; 50µL of primary antibody solution was added to each well excluding the NSB wells. The microplate was covered with the plate sealer and left at room temperature for an hour on a horizontal orbital plate shaker at 500±50 rpm. The HRP-labelled PGE_2 conjugate (50µl) was added to each well excluding NSB wells and the microplate re-covered and incubated at room temperature for two hours on the orbital shaker. Each well was aspirated and washed with 400µl wash buffer for a total of four times to ensure complete removal of all unbound material. Substrate solution (200µl) was added to each well and incubated away from light at room temperature for 30 mins. Sulphuric acid (100µl) was used to stop the reaction. Readings are taken using a microplate reader at 450nm and 540nm to correct for optical imperfections in the plate.

2.4 Open oral GTC intervention study

2.4.1 Supplements

Subjects were supplemented with green tea (540 mg GTC/d) capsules and vitamin C capsules (50 mg/d) [Figure 2.9] for 12 weeks. Supplements were provided by Nestlé Ltd (Lausanne, Switzerland) and packaged by Laboratoire LPH (St Bonnet de Rochefort, France). In total, volunteers took five capsules/d, comprising of three capsules; each containing 450mg green tea extract (total 1350mg tea, 540mg GTC) and two capsules each containing 25g vitamin C. The breakdown of green tea capsules as evaluated by the collaborating Leeds laboratory can be seen in table 2.2.

Figure 2.9 Green tea and vitamin c capsules provided by Nestle Ltd, Lausanne, Switzerland

Table 2.2 Catechin and gallic acid content in green tea capsules (mean values and standard deviations). Contents of three capsules were homogenised and extracted in triplicate

	Content (mg/450 mg capsule	
Green tea catechins (GTC)	**Mean**	**SD**
Gallic acid	0.4	0.0
Catechin	2.1	0.0
Epicatechin	12.5	0.2
Gallocatechin	12.4	0.6
Epigallocatechin	49.3	3.9
Catechingallate	0.3	0.0
Epicatechingallate	26.0	0.2
Gallocatechingallate	4.5	0.4
Epigallocatechingallate	72.6	3.1
Total	180.0	8.3

2.4.2 Study design

For this open intervention study, subjects attended the photobiology unit for a total of nine visits. The length of the visit depended on the procedures being done. The study design was as shown in figure 2.10.

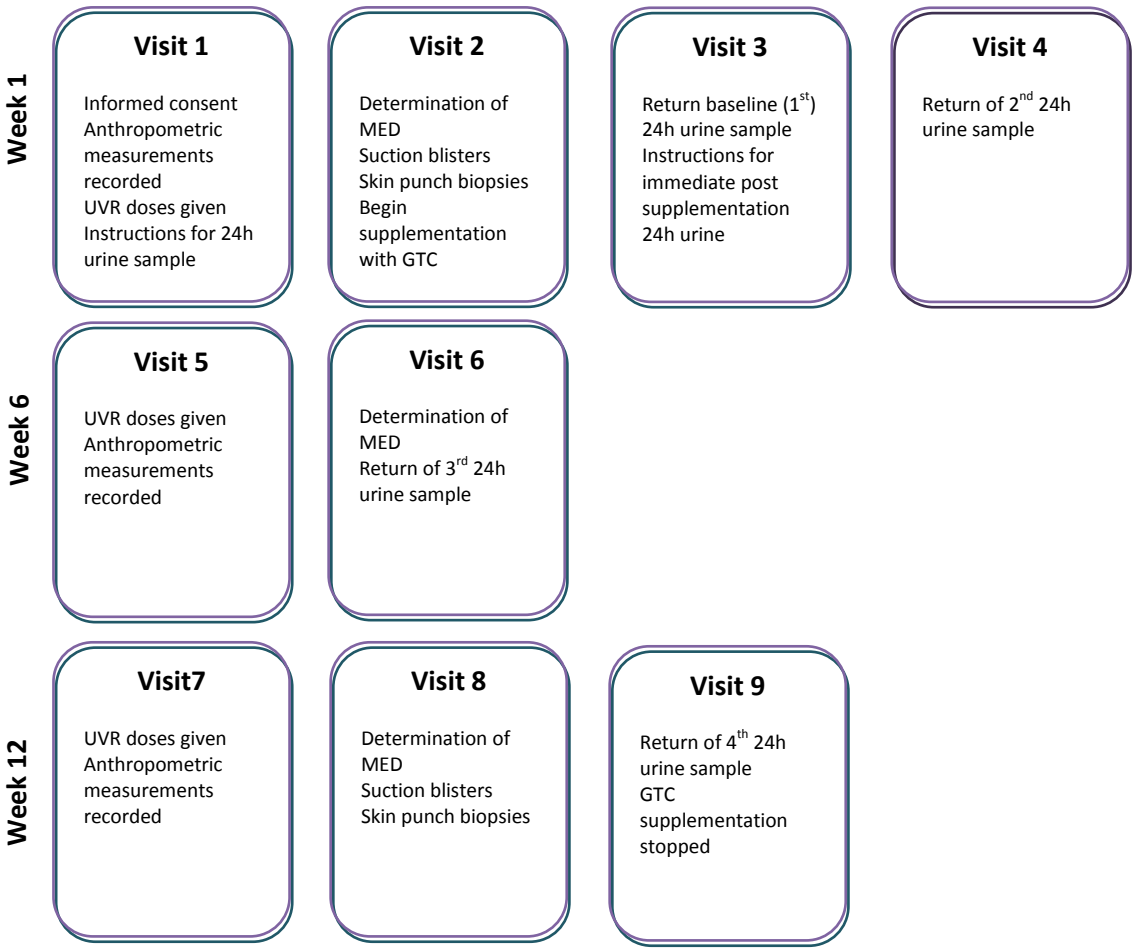


Figure 2.10 Open oral GTC intervention study design

2.4.3 Irradiation equipment

Subjects were irradiated using SSR (section 2.3.1.2). The phototesting template (section 2.3.1.3) [Figure 2.6] was placed on upper buttock in order to deliver the UVR dose series as described in section 2.3.1.4.

2.4.4 Assessment of UVR-induced erythema

Erythema was assessed both visually (section 2.3.2.1) and using a reflectance instrument (2.3.2.2).

2.4.4.1 UVR-erythema dose response modelling

UVR-erythema dose response modelling software (Copyright © Regional Medical Physics Department Gatehead and South Tyneside Health Authority, 1999) was used to produce dose response curves for individual subjects. Changes in EI (ΔE) values were plotted

against UVR dose on a logarithmic scale to produce UVR-erythema dose response curves at 0, 6 and 12 weeks in the supplementation studies. $D_{0.03}$ is referred to as the threshold dose and is an objective measure which approximates a person's MED, the latter being a visual assessment made on an interval scale. The $D_{0.05}$ and $D_{0.1}$ were also used as reference points along the linear portion of the sigmoidal curve. These points allow us to assess how effective a treatment is against UVR-induced erythema [Figure 2.11].

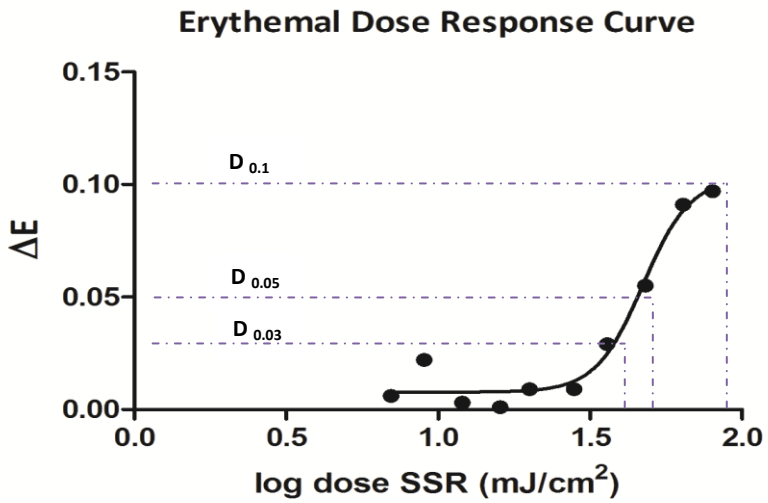
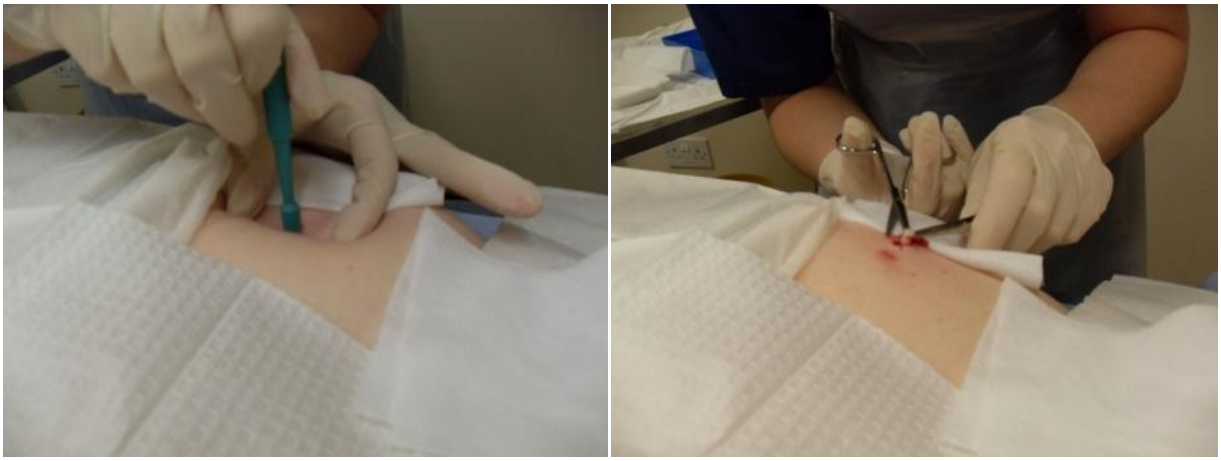


Figure 2.11 Example of UVR-erythema dose response curve in one individual, illustrating change in erythema against log dose of SSR (mJ/cm^2)

2.4.5 Biological Sampling

2.4.5.1 Skin biopsies

In the intervention study, each subject had two 5mm skin punch biopsies removed pre- and post-GTC supplementation (4 samples in total). These were taken from un-irradiated skin on the upper buttock using Militex biopsy punches (Militex Inc., York, Pensylvania, USA) following an injection of local anaesthetic (1% lignocaine; Antigen Pharmaceuticals Ltd; Tipperary, Ireland). The biopsies were placed in 1.8ml cryotubes (Thermo Fisher Scientific; Roskilde, Denmark), and immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis. Wounds were sutured and dressed with sterile Primapore dressings (Smith and Nephew Healthcare; Hull, UK) with sutures being removed 7-10d following procedure [Figure 2.12].

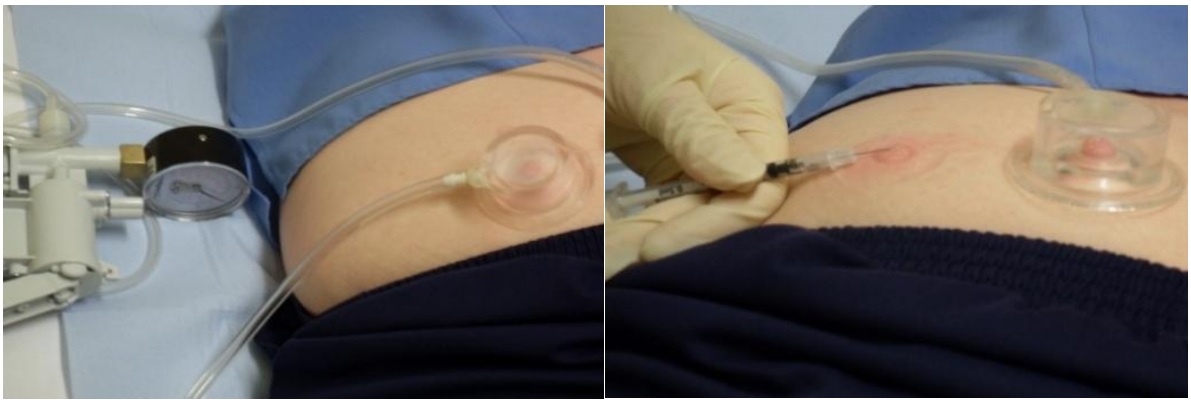


(a) (b)

Figure 2.12 Skin punch biopsies were taken from photoprotected upper buttock skin (a) Skin was sampled under local anaesthetic using a 5mm punch biopsy needle (b) Biopsy was cut from subcutaneous tissue before being snap-frozen in liquid nitrogen

2.4.5.2 Suction blister sampling

Suction blistering is a common procedure for sampling intercellular fluid in human skin studies (Rhodes et al., 1995, Rhodes et al., 2009) [Figure 2.13]. Four suction blister cups, each with a 1cm diameter central aperture (Medical Physics Department; Salford Royal NHS Foundation Trust, Manchester, UK), were applied to upper buttock skin. Two of these were placed on un-irradiated skin and a further two were placed onto skin exposed to 3x the individual's MED 24hr earlier (MED determined by visual assessment as discussed in 2.6.1). This procedure was carried out at week 0 (baseline) and week 12 following GTC supplementation. The suction cups were connected to handheld vacuum pumps (Mityvac; St. Louis, Missouri, USA) using flexible tubing. A suction pressure of 250mmHg was applied and blisters were formed after approximately 90 mins. Blister fluid was aspirated using 0.5ml U-100 insulin syringes (BD Medical; Madrid, Spain) and transferred to a 1.8ml cryotube (Thermo Fisher Scientific; Roskilde, Denmark) containing 25µl blister storage solution (pH 3.6) (see 2.5.7.1 for blister storage solution). Samples were snap frozen in liquid nitrogen and stored at -80°C until analysis.



(a) (b)

Figure 2.13 Suction blister procedure (a) Suction blister formed on photoprotected buttock skin and skin irradiated with 3x MED using suction blister cups with vacuum applied of 250mmHg (b) aspiration of suction blister fluid

2.4.5.3 Urine samples

The volunteers' urine was analysed as a measure of compliance. Urine samples were collected for 24hr using 3L Urisafe polypropylene collection containers (VWR International Ltd; town, UK) containing 3g acidified ascorbate (Sigma; Poole, Dorset, UK) at day 0, day 1, week 6 and week 12, i.e. there were a total of 4 x 24hr urine samples from each subject. Once the urine volumes were recorded, 4x 40ml aliquots per volunteer containing 0.1% of a 10% sodium azide solution were stored at -80°C until analysis.

2.4.6 Laboratory methods

2.4.6.1 Blister storage solution (prior to polyphenol analysis)

Blister storage solution was added to suction blister fluid to minimise the degradation of any GTC uptake in skin. Firstly, 4.8g sodium dihydrogen phosphate (Anala R Normapur®, VWR International Ltd, Leicestershire, UK) and 20g ascorbic acid (Sigma-Aldrich, Poole, Dorset, UK) were mixed together using 10ml distilled water (dH_2O) to wash any surplus reagent from weighing boat. Ethylenediaminetetracacetic acid (EDTA) 0.1g (Sigma-Aldrich, Poole, Dorset, UK) was then added to the mixture which was dissolved in 60ml dH_2O using a stir. The solution was transferred into a 100ml volumetric flask and inverted for approximately 1 min. Distilled H_2O was added using a Pasteur pipette until solution reached 100ml. A pH machine altered the pH to 3.6, providing stabilisation of the catechins in blister fluid samples. Blister storage solution was made fresh at the beginning

of each week to prevent degradation of ascorbic acid. Each vial containing the blister fluid samples also contained 25µl of blister storage solution. Samples were then mixed using a vortex machine, snap frozen in liquid nitrogen and stored at -80°C until analysis.

2.4.6.2 Urine storage solution

Sodium azide solution (10%) was added to urine samples as a biocide and to prevent degradation of catechins. The solution was prepared using 9ml dH_2O and 0.9g sodium azide. After recording the volume of each 24hr urine sample 40ml of each urine sample was transferred into a vial containing 40µl of sodium azide solution. This was mixed thoroughly using a vortex machine prior to be being stored at -80°C until analysis. The sodium azide solution was freshly prepared weekly.

2.4.6.3 ESI LC-MS/MS for Eicosanoid analysis of blister fluid

2.4.6.3.1 Sample preparation

Two pivotal eicosanoids which are up-regulated during the sunburn response are PGE_2 and 12-HETE. These pro-inflammatory mediators were measured in skin blister fluid samples using LC/ESI-MS/MS. Eicosanoid analysis was performed by Dr Karen Massey in the team of our co-investigator Professor Anna Nicolaou, at the University of Bradford.

Skin fluid samples (typically 50-200µl) were diluted with methanol-water (15% w/w) up to 3ml. Subsequently, internal standards (40ng PGB_2-*d*4 and 80ng 12-HETE-*d*8) (Cayman Chemicals) were added and the solution acidified to pH 3.0. Following an incubation period on ice for 30 mins the samples were centrifuged at 3000rpm for 5 mins before being acidified with 0.1M hydrochloric acid (HCL) to pH 3.0 and immediately applied to solid phase extraction (SPE) cartridges (C18-E; Phenomenex).

2.4.6.3.2 LC/ESI-MS/MS analysis

Analysis of skin fluid samples was performed using a Waters Alliance 2695 HPLC pump coupled toan electrospray triple quadrupole Quattro Ultima mass spectrometer (Waters, Elstree, Hertsfordshire, UK). Instrument control and data acquisition were performed using the MassLynx™ V4.0 software. Eicosanoids were analysed on a C18 column (Luna, 5 µm; Phenomenex, Macclesfield, UK). Sample injections (5µL) were performed with a

Waters 2690 autosampler (sample chamber temp 8°C) at a flow rate of 0.2mL/min. An acetonitrile-based gradient system was installed mixing two solvents: (Solvent A) was acetonitrile:water:glacial acetic acid, 45:55:0.02 (v/v/v,) (Solvent B) was acetonitrile:water:glacial acetic acid, 90:10:0.02 (v/v/v). The analytes were separated using the following gradient: 0.0-8.0 min, 0% solvent B; 8.0-8.1 min, 0 to 50% solvent B; 8.0-12.0 min 50% solvent B; 12.0-12.1 min, 50 to 70% solvent B; 12.1-20.0 min 70% solvent B; 20.0-20.1 min, 70 to 0% solvent B; 20.1-30.0 min 0% solvent B.

2.4.6.4 LC-MS/MS for Polyphenol analysis of skin tissue, blister fluid and urine

Polyphenol analysis was completed by Dr Tristan Dew and Miss Kayleigh Clarke, in the team of co-investigator Professor Gary Williamson, at the University of Leeds.

2.4.6.4.1 Urine and skin blister fluid sample preparation

Urine and blister fluid samples were thawed at 5°C with urine samples being adjusted to pH 5.0 with sodium hydroxide (NaOH)(0.1mol/L). A 40µL aliquot of urine or blister fluid was combined with 4µL monosodium phosphate (NaH_2PO_4) solution (0.4mol/L, pH 5.0) containing 200g/L ascorbic acid and 1g/L EDTA, 20µL sodium acetate buffer (0.2mol/L, pH 5.0) containing 0.012µg taxifolin internal standard (Extrasynthese) and 5U sulfatase (Type VIII, Sigma-Aldrich, Poole, Dorset, UK). The next step involved 100U and 200U β-glucuronidase (Type X, Sigma-Aldrich, Poole, Dorset, UK) in NaH_2PO_4 (75mmol/L, pH 6.8) were added to blister and urine samples followed by an incubation period at 37°C for 45 and 60 mins respectively. Subsequently, samples were extracted with 3x 250µL ethyl acetate and the combined extracts dried under nitrogen and frozen at -80°C. Dried samples were reconstituted with 12µL 20% (v/v) acetonitrile containing 1g/L ascorbic acid, and sealed in a microwell plate before analysis.

2.4.6.4.2 Skin biopsy preparation

Skin biopsies were thawed thoroughly at room temperature immediately before extraction and kept on ice throughout. Blood residue was removed by washing the biopsies in hexane. The dermis was separated from the dermis using a scalpel. To the skin, 250µL nitrogen-flushed chloroform containing 0.1g/L butylatedhydroxytoluene, and

250µL sodium dithionite (0.3mol/L) in sodium acetate buffer (0.2mol/L, pH 5.0) were added. Samples were homogenized (Turrax micro homogenizer, IKA), and the samples returned to ice at regular intervals. Subsequently, samples were thoroughly combined using a vortex machine and later separated by centrifugation. The aqueous layer was removed and a second 250µL aliquot of sodium dithionate in sodium acetate buffer added for a repeat extraction. Excess chloroform was removed via nitrogen drying, and the combined extracts mixed with 50µL sodium acetate buffer (0.2mol/L, pH 5.0) containing 0.012µg taxifolin internal standard, 10U sulfatase and 200U β-glucuronidase. After 60 mins incubation at 37^{o}C the extraction proceeded as for blisters/urine, using 3 x 400µL ethyl acetate.

2.4.6.4.3 LC-MS/MS analysis

All samples were analysed using an Agilent 1200 SL HPLC system, which comprised a binary pump, degasser, well plate autosampler (5^{o}C), and column oven (35^{o}C) connected to a 6410 triple quadrupole LC-MS/MS. Samples (5µL) were injected onto a Kinetex C18 microbore column (2.6µm, 150 x 2.1mm; Phenomenex). A binary gradient was installed consisting of LC-MS grade water (Millipore, Darmstadt, Germany) vs. acetonitrile (Fisher Scientific; Loughborough, UK) both with 0.2% (v/v) formic acid, at 0.3mL/min. The gradient started at 5% acetonitrile for the first 5.8 mins, rising to 30% over 29.2 mins and increasing to 95% acetonitrile over 2.4 mins. This remained for a further 3.6 mins to wash the column returning to 5% acetonitrile over 3.6 min and re-equilibrating over a further 10.9 mins. The flow rate was 11L/min and gas temperature 350^{o}C.

Analytes were detected in negative mode, using Dynamic MRM acquisition. Standards used were obtained from the following manufacturing companies. The 3' and 4' mono-methylated forms of EC and EGC were obtained from Nacalai Tesque (Singapore), benzoic acid, 3-hydoxy benzoic acid, hippuric acid, 3,4-dihydroxyphenylacetic acid, and 3-(2,4-dihydroxyphenyl)propionic acid from Fluka Analytical (Poole, UK) and 4-hydroxy benzoic acid from (Sigma-Aldrich, Poole, Dorset, UK). Vanillic acid, 3,5 dihydroxy benzoic acid, gallic acid, syringic acid, 3- and 4- hydroxyphenyl acetic acids and 3-(3hydroxyphenyl)-propionic acid were obtained from Alfa Aesar (Heysham, Lancashire, UK). 3- and 4-methyl gallic acids were obtained from Apin Chemicals (Abingdon, UK) and 2,4-dihydroxy

benzoic acid, 2,4,6-trihydroxy benzoic acid, 2-hydroxyphenyl acetic acid, and 2-hydroxy hippuric acid from Acros Organics (New Jersey, US).

2.5 Statistical analysis

Normality of data was analysed using the Shapiro-Wilk test. Parametric data were tested using paired t-test and non-parametric data used Wilcoxon signed rank tests. Analyses were performed using GraphPad Prism (v5, GraphPad Software, Inc). All data expressed as mean ± SE unless otherwise specified. Statistical significance was accepted at the $P<0.05$ level.

CHAPTER 3: Results

3.1 Introduction to data chapter

The skin is the body's principal interface with external stimuli such as UVR. Exposure to UVR results in both acute and chronic adverse effects on human skin. The acute effects include sunburn (an inflammatory response), immune suppression and photosensitivity while more chronic effects are photoageing and photocarcinogenesis (Swindells and Rhodes, 2004). A notable increase in UVR-mediated skin damage, including skin cancers - BCC increased by 36% (men) and 32% (women) and SCC by 34% (men) and 39% (women) - has occurred between 2000-2002 and 2008-2010 (CancerResearch-UK, 2013). This is believed to be caused through behavioural changes leading to greater exposure to sunlight which, in the future, is predicted to be further exacerbated through more time spent outdoors as a result of climate change. Therefore, adequate sun protection is of extreme importance. The use of dietary bioactives as systemic photoprotective agents, particularly those with combined antioxidant and anti-inflammatory properties, may complement topical sunscreens, thus in combination providing more effective protection against UVR-induced skin damage.

Sunburn is clinically manifest as erythema, with underlying dermal vasodilatation peaking at 24hr (Rhodes et al., 2001b), and histologically shows a mixed dermal neutrophilic and lymphocytic infiltrate peaking at 24-48hr (Strickland et al., 1997, Hawk et al., 1988). A key part of the UVR-induced inflammatory response is the activation of PLA_2 which releases esterified fatty acids including AA from cellular membranes (Gijon and Leslie, 1999, Gijon et al., 1999). Subsequently, AA is metabolized via the COX and LOX pathways to produce the lipid-derived mediators known as eicosanoids (Funk, 2001, Ziboh, 1992). PGE_2 and 12-HETE are two of the most abundant pro-inflammatory eicosanoids identified as being up-regulated during the sunburn response, with up-regulation of PGE_2 known to partly mediate vasodilatation (erythema) (Rhodes et al., 2001b, Rhodes et al., 2009), while 12-HETE, a potent neutrophil and lymphocyte chemoattractant is anticipated to be involved in the white cell recruitment (Rhodes et al., 2009).

Polyphenols are complex molecules with an array of chemical structures which subsequently affect their biological properties, stability, bioavailability (Scalbert and Williamson, 2000) and antioxidant activity (Scalbert and Williamson, 2000, de Mejia et al.,

2009, Rice-Evans et al., 1996). Polyphenols have long been reported to offer health benefits but it has only been in recent years that the mechanisms involved have been studied in depth. There have been many studies performed largely in experimental models which have suggested polyphenols from various sources may help protect against UVR-induced effects on skin (Section 1.5.3). Green tea is one of the most widely studied sources of flavonoids. It is produced from the extensive steaming and drying of fresh leaves and buds of the plant *Camellia sinensis,* preventing the oxidation and polymerisation of its polyphenolic compounds including catechins (de Mejia et al., 2009). Photoprotective effects of GTC have been well documented particularly *in vitro* and in animal models, while studies in humans *in vivo* are scarce. In human subjects, topically applied GTC was found to reduce UVR-induced DNA damage, erythema and leukocyte infiltration (Katiyar et al., 1999b, Katiyar et al., 2000b). A further dietary intervention study demonstrated that in women who consumed a green tea beverage (1402mg total catechins) for 12 weeks, the skin's sensitivity to UVR-induced erythema was reduced while the microcirculation improved (Heinrich et al., 2011). Some of these effects may be in part mediated via the modulation of COX and LOX-produced mediators of UVR-inflammation by EGCG, EGC, ECG and EC, the four main catechins found in green tea (Section 1.6.2). Experimental studies have suggested a decrease in production of PGE_2 and/ or 12-HETE (Hong et al., 2001b, Schewe et al., 2001, Singh and Katiyar, 2011) and oral GTC reduced UVR-induced COX-2 expression and thus PGE_2 production in mouse epidermis (Meeran et al., 2009). However, it is yet to be elucidated whether such findings may be relevant to human skin.

3.2 UVR spectrum and dosimetry study

The aim was to determine the UVR spectrum and dose to use in the human studies by examining UVR impact on cutaneous PGE_2 production.

3.2.1 Subjects and protocol

There were three subjects (2 female, mean age 36; range 29-50 yr, white Caucasian with skin phototype II). The upper buttock skin was utilised. Blister fluid samples were collected from un-irradiated skin and skin irradiated with 3x and 4x the MED of both

broadband UVB (Phillips TL-12; ~56% UVB) and a spectrum of UVA and UVB similar to sunlight (solar simulator; 290-400 nm, 5% UVB, 95% UVA) i.e. SSR, at 24hr prior to sampling (section 2.7.2). The blister fluid was assayed for PGE_2 by ELISA (section 2.3.3.1) in this small preparatory study, as the LC-MS/MS has to be run for larger sample batches.

3.2.2 Results

Equivalently elevated levels of PGE_2 were found for both UVR-spectra (i.e. SSR versus principally UVB). The PGE_2 levels were very similar for 3x and 4x MED (Figure 3.1). Less biological variance between subjects was seen when skin was irradiated with 3x MED. Consequently, 3x MED of SSR was used in the intervention study, the SSR providing a more realistic UV emission, i.e. closely mimicking natural sunlight.

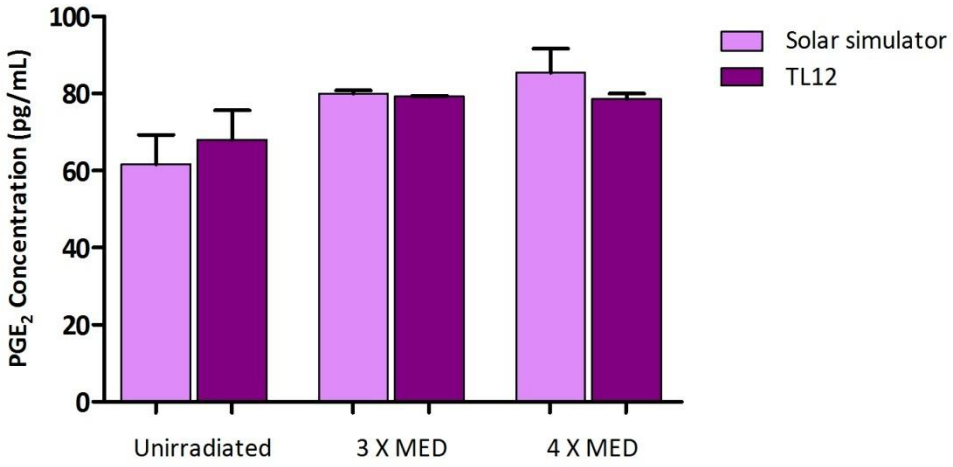


Figure 3.1 UVR-induced PGE_2 production in skin blister fluid following irradiation with TL-12 and SSR sources (n=3). Results are presented as mean ± SEM.

3.3 Oral GTC intervention study

The aim of the main study was to determine whether GTC were bioavailable in human skin and assess the impact of GTC on UVR-induced inflammation and UVR-induced eicosanoid expression.

3.3.1 Subjects

16 healthy white Caucasian (male and female) volunteers of mean age 42 (range 29-56 yr) with skin phototype I/II were recruited. Exclusion criteria were: photosensitivity; history of skin cancers; taking photosensitizing or anti-inflammatory medication; pregnancy; taking nutritional supplements; ≥2 cups of tea per day; and sun bed use or sunbathing in the 3 months prior or during the study. Regulatory approvals were gained as previously discussed (Section 2.1). All volunteers gave written informed consent.

3.3.2 Study design and supplements

The study design was a pilot oral intervention study. Subjects were supplemented with green tea capsules (540mg GTC/d) and vitamin C capsules (50mg/d) for 12 weeks. Each individual's MED and UVR-erythema response was assessed at baseline, 6 and 12 weeks post supplementation. Skin tissue and blister fluid samples were taken from non-UVR exposed skin and skin exposed to 3x MED of SSR at baseline and 12 weeks post supplementation. 24hr urine samples were collected to test for compliance.

3.3.3 UV-irradiation

Erythema was induced using the above SSR source (section 2.3.1.2). MED testing was performed on previously photoprotected upper buttock skin by applying a series of 10 erythemally-weighted UVR doses from 6.6-68mJ/cm^2. MED was assessed at 24hr and defined as the lowest UVR dose to cause just perceptible erythema (Section 2.3.2.1). Subjects were irradiated with 3x the baseline MED prior to tissue sampling.

3.3.4 Eicosanoids

PGE_2 and 12-HETE concentrations (pg/μL) were measured in skin blister fluid samples using LC/ESI-MS/MS (Section 2.4.6.3)

3.3.5 Polyphenol bioavailability

Polyphenol content of urine, blister fluid and skin tissue samples were analysed as discussed (Section 2.4.6.4).

3.4 Statistical Analysis

Statistical analysis of data was performed as described in section 2.5.

3.5 Results

3.5.1 Subjects and compliance with oral GTC

16 subjects were initially recruited to the study. 1 subject withdrew before completion for reasons unconnected to the trial. Of the 15 subjects remaining 4 reported mild nausea following ingestion of GTC supplements however, they continued in the study. Compliance (urinary) analysis showed 1 subject was non-compliant with the remaining 14 participants showing all 4 major catechins and their metabolites in urine at day 1, week 6 and week 12 (Table 3.1). Therefore, 14 subjects (12 female) with a median age of 42.5 yr were included in the study analyses unless otherwise specified.

3.5.2 Skin uptake

3.5.2.1 Skin blister fluid

Blister fluid samples were obtained from a subgroup of participants (n=10) at baseline and following 12-week supplementation with GTC. A range of catechins and their metabolites were detected at both time-points, with a change from the average baseline value calculated (Table 3.1). Hippuric acid, benzoic acid and 4-hydroxybenzoic acid were present in the skin blister fluid samples of all 10 subjects at baseline; levels of all of which appeared to increase following supplementation with GTC. However, a statistically significant change from baseline to week 12 was only observed for benzoic acid (P=0.03). Methylated gallic acid (4-*O*-methyl gallic acid) was found to be present in half of the participants but there was no significant change from baseline. The intact catechin EGC and the unique catechin metabolites M4 and M6 hydroxyohenyl-valerolactones (section 1.6.4) were observed in blister fluid samples of two subjects following supplementation.

3.5.2.2 Skin biopsies

Skin biopsies were obtained from a subgroup of subjects (n=10) at baseline and following 12-week supplementation with GTC. The dermal part of the skin punch biopsy was available for the analyses. Benzoic acid and its 4-hydroxylated form were present in the skin biopsies of all subjects at baseline and week 12, although there was no significant difference in the change from the average baseline value (Table 3.1). The terminal catechin metabolite, hippuric acid, was observed in 6 of the 10 participants post supplementation; however, this change again did not reach significance. Interestingly, a number of intact catechins were seen in a few subjects following supplementation which were not present at baseline, i.e. EC (n=2), EGC (n=1) and its methylated form 4'-*O*-methylated EGC (n=4) and EGCG (n=1). These results indicated that GTC is bioavailable in skin albeit with considerable inter-individual variability.

Table 3.1 Presence of green tea catechins and their metabolites in skin blister fluid and skin tissue samples post-supplementation (week 12; n=10)

	Skin blister fluid		Skin biopsy	
Compound	Change from average baseline value	Detected in n participants	Change from average baseline value	Detected in n participants
Benzoic acid	+36%*	10	ND	10
4-OH-benzoic acid	ND	10	ND	10
Hippuric acid	ND	10	ND	6
4-*O*-Me-gallic acid	ND	5	ND	2
EC	-	-	PPS	2
EGC	PPS	2	PPS	1
EGC-4-Me	-	-	PPS	4
EGCG	-	-	PPS	1
M4 valerolactone	PPS	2	-	-
M6 valerlactone	PPS	2	-	-

Paired t-test performed only for compounds which were present in all 10 subjects. EC, epicatechin; ECG, epicatechin-3-*O*-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-*O*-gallate; ND, no significant difference; PPS, only present post-supplementation. *P = 0.03 (2-tailed paired t-test) compared with baseline.

3.5.3 Urinary metabolites

Urine samples were analysed as a compliance measure. A total of 35 tea phenols and metabolites were investigated of which 20 catechins and/or their metabolites were found to be significantly higher in week 12 urine samples compared to baseline levels (P <0.05; Table 3.2). The urinary results are based on n=13 due to an absent record of one urine

sample. Overall results indicated good compliance with GTC supplementation with all four major catechins observed throughout the 12 week supplementation period, and with a statistically significant difference in levels from day 1 compared to baseline calculated for: EC (P<0.05); ECG (P<0.05); EGC (P<0.05) and; EGCG (P<0.05). Unique catechin metabolites M4 valerolactone, M6 valerolactone and M6' valerolactone were also significantly increased from day one of supplementation throughout the 12 week period. There was variability between subjects regarding the amount and type of compounds present. However, in 100% of the subjects, increased excretion of six compounds (EC, 3'-*O*-methyl EC, 4'-*O*-methyl EC, EGC, 3'-*O*-methyl EGC, 4'-*O*-methyl EGC and M4 valerolactone) was seen in week 12 samples compared to baseline. When based on a daily intake of 129.2µmol of EC and 482.9µmol of EGC the mean excretion of intact EC and EGC (including their methylated forms) at week 12 represented 6.1% and 7.1% respectively. General polyphenol breakdown products hippuric acid, benzoic acid and syringic acid were only elevated from baseline at 12 weeks (P<0.001).

Table 3.2 Green tea catechins and their metabolites in urine at baseline and following 12 weeks supplementation with GTC (data presented as mean values ± SD, n=13)

	Amount excreted in urine (µmol)							
	Baseline		Day 1		Week 6		Week 12	
Compound	Mean	SD	Mean	SD	Mean	SD	Mean	SD
EC †	0.3	0.4	7*	4	5*	4	7*	5
3'-*O*-methyl-EC †	0.06	0.08	0.6*	0.3	0.5*	0.4	0.6*	0.3
4'-*O*-methyl-EC †	0.04	0.05	0.2**	0.2	0.2**	0.2	0.3*	0.2
ECG	0.000	0.002	0.01*	0.01	0.02*	0.01	0.01**	0.01
EGC †	0.2	0.4	22*	13	20*	16	25*	20
3'-*O*-methyl-EGC †	0.01	0.04	0.2*	0.1	0.2*	0.2	0.2*	0.2
4'-*O*-methyl-EGC †	0	0	8**	8	8**	9	9**	8
EGCG	0.00	0.02	0.06*	0.05	0.06*	0.04	0.08**	0.09
Catechin	0.01	0.02	0.2*	0.1	0.1*	0.1	0.2*	0.2
Gallocatechin	0	0	0.4**	0.5	0.3***	0.5	0.6**	0.6
Gallocatechin gallate	0	0	0.003	0.009	0	0	0.01*	0.02
Gallic acid	0.6	0.7	1	1	0.7***	0.5	1**	1
3'-*O*-methyl gallic acid	0.6	0.6	1	1	0.9	0.8	1***	1
3-Hydroxybenzoic acid	1	1	2	2	2	3	4***	4
M4 valerolactone †‡	0.3	0.4	30**	27	18***	25	21**	21
M6' valerolactone‡	0.5	0.7	18**	16	12**	13	15**	15
M6 valerolactone	10	12	33**	25	27***	28	31***	24
Syringic acid	2	1	4	5	3	2	4***	4
Benzoic acid	81	83	95	60	101	132	140***	120
Hippuric acid	4000	2200	5100	2500	4300	1900	5300***	1700

EC, epicatechin; ECG, epicatechin-3-*O*-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-*O*-gallate; M4, 5-(3',4',5'-trihydroxyphenyl)-γ-valeroloactone; M6', 5'-(3',5'-dihyroxyphenyl)-valerolactone; M6, 5-(3',4'-dihydroxyphenyl)-valerolactone.

Mean values significantly different from baseline *P<0.05, **P<0.01, ***P<0.001 (two-tailed paired t test).

† Increased excretion of metabolite from baseline to week 12 in 100% of subjects ‡ M4 and M6' hydroxyphenyl-valerolactone calculated as M6 equivalent.

3.5.4 UVR-erythema dose response analyses

The erythemal response to the UVR dose series applied to the skin was measured using the reflectance instrument at 24hr post UV-irradiation. A UVR-erythema dose response curve was created to assess the difference in erythema following supplementation with GTC. The $D_{0.03}$ was calculated as an objective measure approximating the MED (i.e. the erythema threshold dose). Furthermore, the $D_{0.05}$ and the erythema at the highest dose (68mJ/cm^2) were also calculated as reference points along the linear portion of the sigmoidal curve, as well as the whole erythema response indicated by the area under the curve (AUC). 10 subjects were exposed to the SSR dose range as described in section 2.5.2, therefore all erythema data is based on n=10.

3.5.4.1 MED

The MED was assessed visually at 24hr following UV-irradiation. The median MED at baseline was 35, range 27-43mJ/cm^2 and remained unchanged at 35, range 27-43mJ/cm^2 (*P* = 0.68) following 12 weeks supplementation with GTC [Figure 3.2]. Visual identification of the MED can be seen in figure 3.3 which shows an individual's erythemal responses to the UVR dose-series at 24hr post-SSR. In this instance, their MED remained unchanged (35 mJ/cm^2) following GTC supplementation.

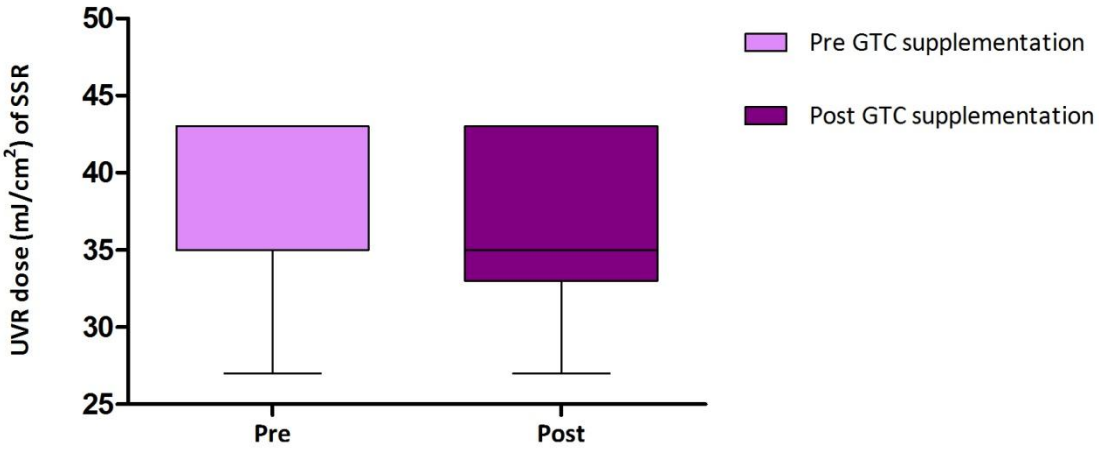


Figure 3.2 MED pre and post supplementation with GTC (n=10). Data presented as a box-and-whisker plot showing median and interquartile range, *P*=0.68

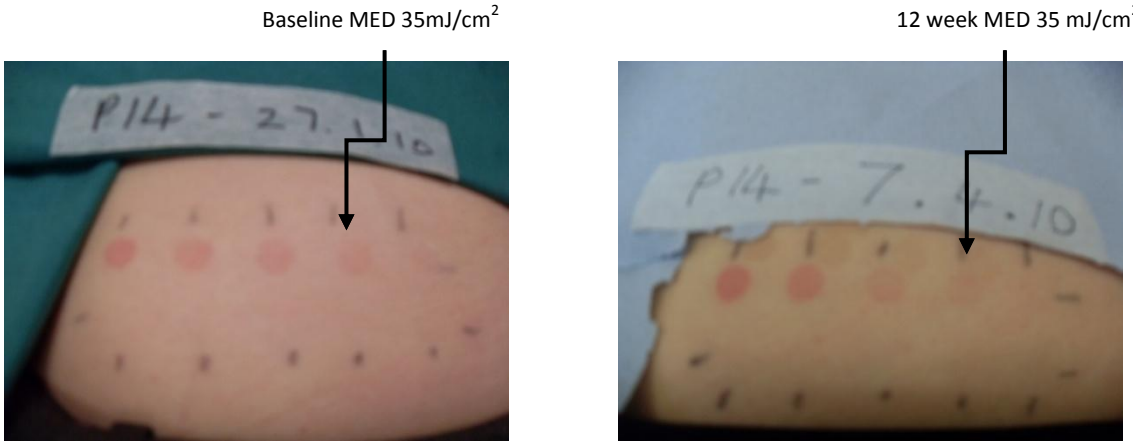


Figure 3.3 Images show an individual's erythemal responses to the UVR dose-series at 24hr post-SSR at baseline (a) and following 12 weeks supplementation with GTC (b). The MED for this volunteer was unchanged from baseline, remaining at 35mJ/cm^2.

3.5.4.2 $D_{0.03}$

The threshold dose ($D_{0.03}$) was determined from the UVR-erythema modelling analysis. A small but non-statistically significant increase was seen in the $D_{0.03}$ following supplementation with GTC, from a mean of 28.1 ± SEM 2.4mJ/cm^2 at baseline to a mean of 32.9 ± SEM 3.5mJ/cm^2 post-supplementation (*P* = 0.177) [Figure 3.4].

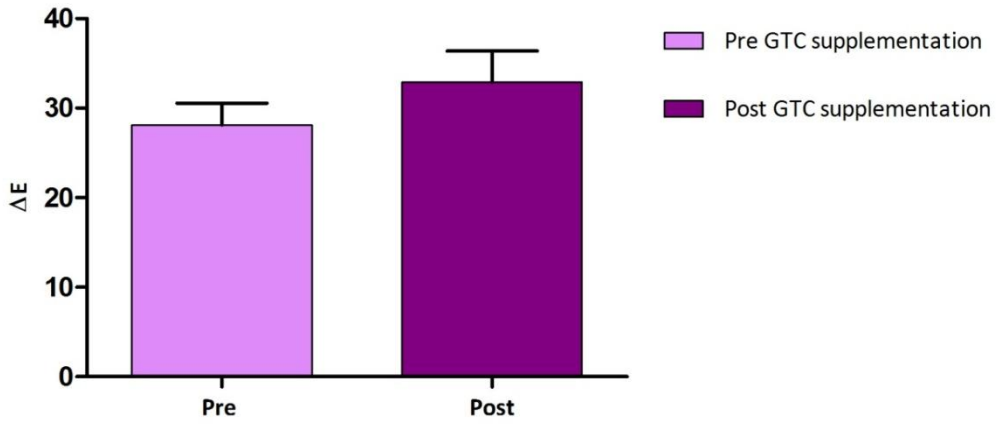


Figure 3.4 $D_{0.03}$ erythema threshold dose pre and post supplementation with GTC (n=10). Data presented as mean ±SEM, *P*=0.177

3.5.4.3 Maximum UVR dose (68mJ/cm^2)

The maximum UVR dose given was 68mJ/cm^2 (erythemally weighted UVR) of SSR. Supplementation with GTC for 12 weeks resulted in a significant decrease in EI at 68mJ/cm^2 from mean 100.2 ± SEM 6.76 at baseline to mean 81.2 ± SEM 7.35 post supplementation, ***P* = 0.006 [Figure 3.5].

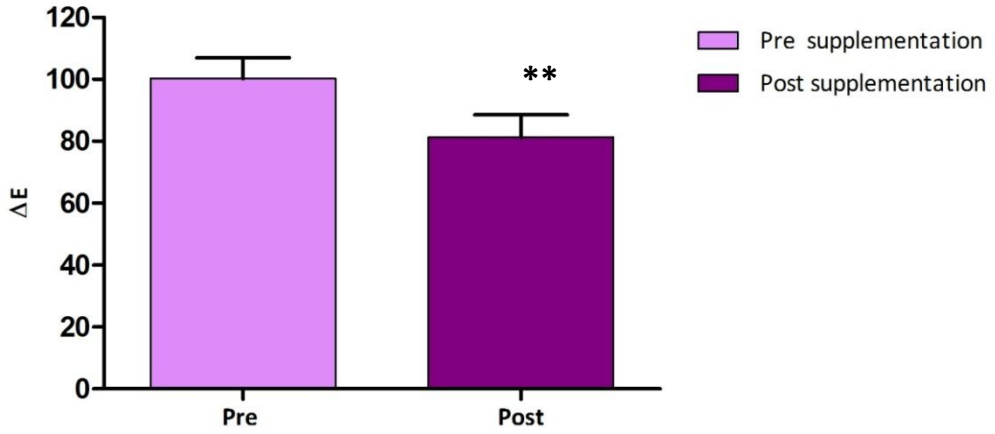


Figure 3.5 Erythema response to SSR at the highest UVR dose (68mJ/cm^2) pre and post GTC supplementation. Data presented as mean ± SEM (n=10) **P = 0.006

3.5.4.4 Area under the curve

The impact of GTC on the UVR-erythema series as a whole was assessed through AUC analysis. This involves using the trapezoid rule to connect a straight line between each set of points defining the curve and calculating the area beneath the curve pre- and post-GTC supplementation. The difference in the AUC pre- and post-supplementation was analysed by 2-way repeated measure ANOVA. This showed a showed a significant reduction in AUC, from a mean of 2888.6 ± SEM 279.5 to 2334.7 ± SEM 340.7, indicative of a reduction in UVR-erythema response, following supplementation (P = 0.033) [Figure 3.6].

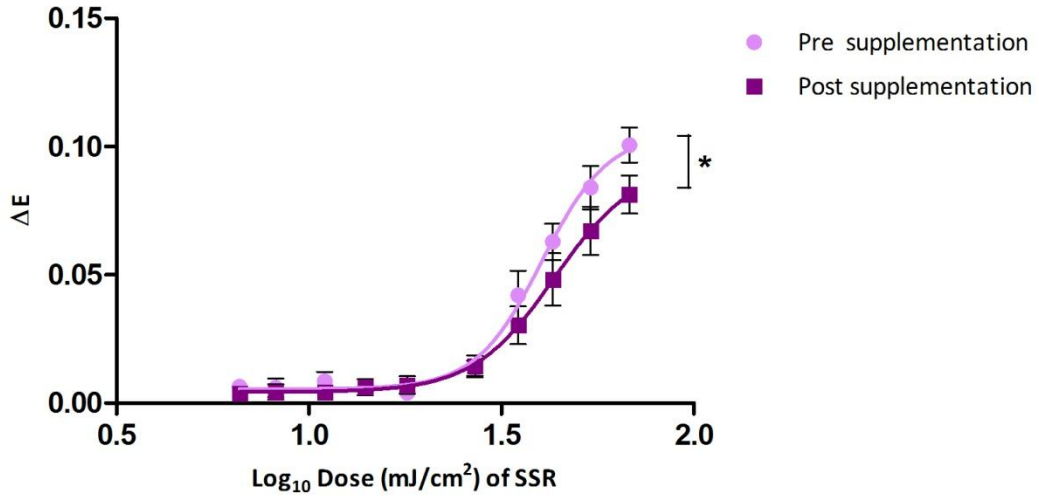


Figure 3.6 UVR-erythema dose response curve pre and post 12 weeks supplementation. Data are presented as mean ± SEM, n=10, * P = 0.033 for difference in the AUC pre- and post-supplementation.

3.5.5 Eicosanoid production

Two of the most abundant eicosanoids seen at the peak of the sunburn response are the potent pro-inflammatory mediators PGE_2 and 12-HETE; hence these were the most appropriate to analyse in this study.

3.5.5.1 PGE_2

3.5.5.1.1 Impact of UVR on PGE_2:

A significant increase in PGE_2 was seen following skin exposed to 3x MED of SSR compared to baseline: mean 49.1 ± SEM 11.02 pg/µL (unexposed skin) and mean 115.3 ± SEM 20.67 pg/µL (exposed skin), $P = 0.003$. Post-supplementation the UVR-induced rise was still seen: mean 47.48 ± SEM 9.65 pg/µL (un-exposed skin) and mean 111.1 ± SEM 13.19 pg/µL (exposed skin), $P = 0.001$.

3.5.5.1.2 Impact of GTC supplementation on PGE_2

There was no significant difference in PGE_2 concentration between the unexposed skin pre- and post-supplementation ($P = 0.89$), or between the skin exposed to UVR pre and post supplementation with GTC ($P = 0.85$) [Figure 3.7].

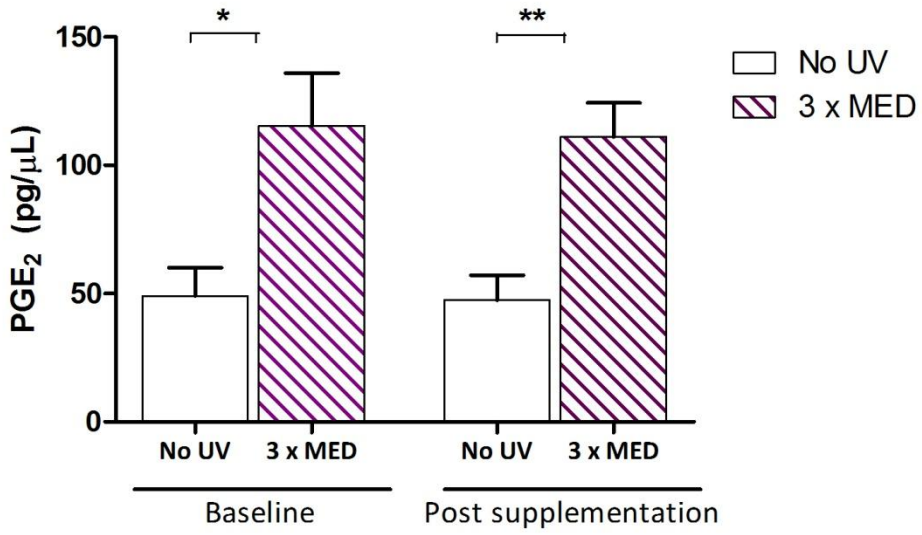


Figure 3.7 PGE_2 concentration (pg/µL) in skin blister fluid in un-exposed and UVR exposed skin pre and post-supplementation with GTC, * $P = 0.003$, ** $P = 0.001$ Data presented as mean ± SEM (n=10)

3.5.5.2 12-HETE

3.5.5.2.1 Impact of UVR on 12-HETE:

There was a significant increase in 12-HETE production following exposure to SSR at baseline: mean ± SEM of 13.28 ± 1.98 pg/µL increasing to 64.44 ± 11.28 pg/µL post-UVR (*P*=0.0005). A significant rise following UVR was also seen post-supplementation: 15.24 ± 3.8 rising to 41.25 ± 8.6 (*P* =0.012).

3.5.5.2.2 Impact of GTC supplementation on 12-HETE:

A significantly lower concentration of 12-HETE was seen in UVR-exposed skin following GTC supplementation when compared to baseline UVR-exposed levels (*P*=0.015). No significant difference in 12-HETE concentration seen in unexposed skin following GTC supplementation compared to baseline levels in unexposed skin (*P*=0.51) [Figure 3.8].

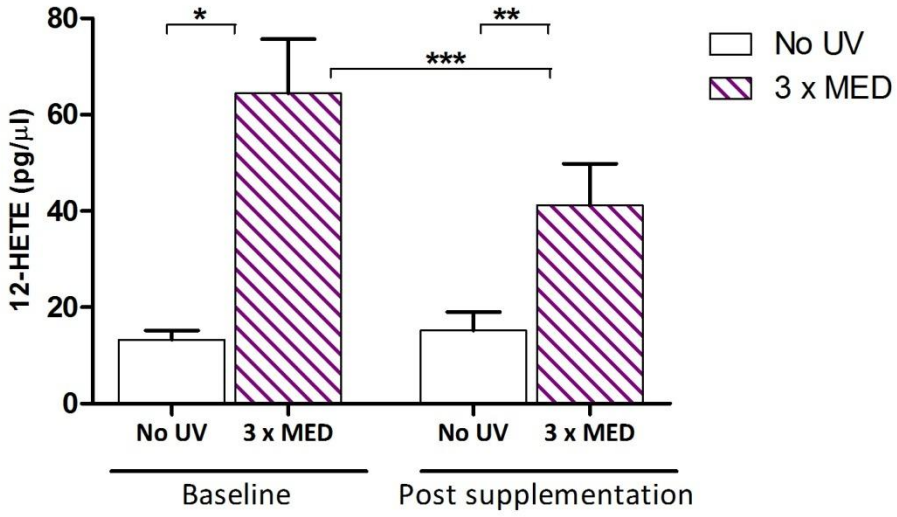


Figure 3.8 Concentration of 12-HETE (pg/µL) in skin blister fluid in un-exposed and UVR exposed skin pre and post GTC supplementation, **P* = 0.0005, ***P* = 0.01, ****P* = 0.015 Data presented as mean ± SEM (n=14).

3.6 Correlation of data

Datasets showing statistically significant changes were examined for correlation. It was of particular relevance to examine whether the reduction of UVR-induced erythema (i) at

the highest UVR dose (68mJ/cm^2) and (ii) evidenced by reduction in AUC, were correlated with the reduction of 12-HETE concentration in UVR-exposed skin [Table 3.3]. However, no significant correlations were found. This suggested that reduction of 12-HETE may not be responsible for the reduction in erythema, but could also be in part due to the small number of subjects participating in the study, and the variability in UVR-induced 12-HETE levels.

Table 3.3 Correlation of erythema and 12-HETE data

Correlation of data			Number of subjects	Spearman r	P value
Reduction in EI at 68 mJ/cm^2	versus	Reduction in 12-HETE in UVR-exposed skin	n=10	0.15	0.68
% change in AUC	versus	% change in 12-HETE in UVR-exposed skin	n=10	0.5	0.15

In skin samples (blister fluid and dermal tissue) there were qualitative differences in metabolites identified between individuals, which together with the small n number, generally made any possible (relevant) associations amenable to descriptive rather than to statistical analysis.

Benzoic acid and 4-OH-benzoic acid were consistently observed in all participants in both blister fluid and skin biopsies pre- and post- supplementation. There was no significant difference in percentage change from baseline levels observed in skin biopsies; however, the percentage change in benzoic acid levels from baseline in blister fluid samples was found to be statistically significant at P=0.031. These metabolites are general breakdown products of polyphenols, hence they were observed in all participants (n=10) at baseline and therefore no correlative analyses have been performed on this data. However, the significant increase seen in benzoic acid does indicate that subjects experienced an increase in polyphenol metabolites in the target area (skin) as a result of GTC intervention.

In blister fluid, the intact catechin EGC; its derivative M4-valerolactone and the EC derivative M6-valerolactone were observed in the same subject following supplementation with GTC (not present at baseline). The same subject also expressed 4'-*O*-Methyl EGC in their skin biopsy although no other intact catechins were observed. A

further subject expressed 4'-*O*-Methyl EGC, EGC and EC in their skin tissue samples although no further intact catechins were present in their corresponding blister fluid. Another participant expressed EGC in blister fluid at week 12 and its methylated form 4'-*O*-Methyl EGC in their skin sample, again post supplementation. Interestingly, a reduction in both erythema and 12-HETE production following 12-week supplementation with GTC were identified for all three of the above participants, suggesting a range of catechin metabolites may be bioactive [Table 3.4].

Of the three subjects where no intact catechins were observed in either blister fluid or dermal tissue post supplementation, two volunteers still demonstrated a decrease in erythema. However, the remaining participant showed an apparent small increase in erythema at the highest UVR dose (68mJ/cm^2) following supplementation although a reduction in 12-HETE in UVR-exposed skin was observed (64.62pg/µL -16.06pg/µL). The complexity and huge inter-individual differences in oral bioavailability and metabolism of polyphenols may provide an explanation as to why intact catechins were not observed in all participants.

Table 3.4 Change in erythema at 68mJ/cm^2 and 12-HETE (UVR-exposed skin) in subjects expressing intact catechins and their derivatives

Catechins in blister fluid	Catechins in skin biopsy	Erythema pre supp 68mJ/cm^2	Erythema post supp 68mJ/cm^2	Reduction in erythema from baseline	12-HETE (pg/µL) in UVR-skin at baseline	12-HETE (pg/µL) in UVR-skin post supp	Reduction in 12-HETE (pg/µL) from baseline
EGC	EGC-4-ME	79.8	48.5	31.3	136.62	64.31	72.31
EGC M4 M6	EGC-4-Me	139.5	102.5	37	147.83	81.36	66.47
-	EGC-4-ME EGC EC	97.3	96.2	1.1	79.94	26.23	53.71
-	EGC-4-ME	128.8	121.7	7.1	94.79	92.40	2.39
-	EGCG	79.3	48.7	30.6	51.90	22.06	29.84
M4 M6	-	92.0	79.2	12.8	21.21	45.22	-
-	EC	106.0	63.0	43.0	67.25	29.51	37.74

3.7 Interpretation and discussion of results from the oral GTC intervention study

This study evaluated the cutaneous uptake of catechins and their metabolites, measured the impact of low dose green tea (~ 2.5 cups/day) on pro-inflammatory UVR challenges to the skin and assessed the potential of GTC to reduce PGE_2 and 12-HETE production.

3. 7.1 Skin and blister fluid polyphenol content

The analysis of catechin uptake in the skin highlighted the complexity of these molecules and their metabolites. There was much inter-individual variability in bioavailability which is line with the current literature (Lee et al., 2002). Of the 10 participants who provided skin blister fluid and dermal biopsies for the skin uptake analyses, 100% had benzoic acid, its 4-hydroxyl form and its glycine conjugate hipppuric acid present with a significant increase in benzoic acid observed following supplementation with GTC (P=0.03). Intact catechins and their associated hydroxyl-phenyl-valerolactone metabolites were present in some, but not all, subjects [Table 3.1]. Overall, despite variability between subjects there was an increased presence of polyphenol metabolites reaching human skin as a result of GTC intervention, and this is a novel finding.

Previous limited work carried out on green tea and its effects on UVR-induced skin inflammation has mainly focused its attention on EGCG. Systemic and topical administration of GTC and EGCG were found to protect against the sunburn response in mice (Katiyar et al., 1995) while a further study showed topically applied green tea extract to human skin resulted in dose-dependent inhibition of the UVR-induced erythemal response, with EGCG and ECG found to be the most effective catechins (Elmets et al., 2001). However, in our study EGCG was only detected in the skin biopsy of one subject following 12 weeks supplementation. One explanation for this could be the complex biotransformation and metabolism catechins undergo within the body following ingestion (section 1.6.4). Our skin sample analyses identified EGC along with its methylated form 4'-*O*-Methyl EGC to be the most abundant catechins, with their presence observed in both the blister fluid and dermal tissue of two subjects [Table 3.4]. Therefore, these results may suggest EGC could play a role in UVR-erythema reduction, in addition to ECG and EGCG, despite the earlier work of Elmets, et al (2001) where EGC and EC apparently had little effect in inhibiting erythema.

3.7.2 Urinary polyphenol content

Polyphenol content in urine was used as a compliance measure. All four major catechins present in green tea were seen consistently in all but one participant from baseline, at day 1, week 6 and week 12. Therefore, this subject was considered non-compliant, thus their results are not included in the analyses. Benzoic, hippuric and syringic acids are general polyphenol breakdown products and were found to be significantly increased following 12 weeks GTC supplementation compared to baseline levels. Hippuric acid has previously been reported as the major urinary metabolite following both green and black tea intervention (Mulder et al., 2005) and our results were consistent with this finding. However, it must be noted that hippuric acid is the terminal metabolite of benzoic acid, a colonic breakdown product common to various polyphenols and therefore not solely related to green tea consumption. In contrast, hydroxyphenyl-valerolactones are exclusive catechin metabolites which are produced by colonic ring fission. M4 and M6' are predominantly derived from EGC whilst M6 is metabolised from EC. Hydroxyphenyl-valerolactone levels were significantly increased at day 1 and weeks 6 and 12 compared to baseline and thus may be a determinant of EC and EGC intake.

3.7.3 Impact of GTC on UVR-induced erythema

Analyses were performed on the impact of GTC on the UVR-erythema dose response in human subjects. There was no change seen in the MED following 12 weeks supplementation with GTC compared to baseline [Figure 3.2]. As the MED is a visual assessment it is a subjective measurement which may be affected by a number of factors including room temperature and lighting. In addition, an interval scale is used to identify the MED which may result in loss of sensitivity.

Using the reflectance instrument to objectively measure UVR-induced erythema allows more subtle changes to be measured and assessed. $D_{0.03}$ is used as an approximation of the MED (Diffey and Farr, 1991), but unlike the MED it uses a continuous scale. Although the UVR-erythema dose response data indicated a small increase following GTC intervention at the $D_{0.03}$ level this was not found to be statistically significant.

Using the UVR dose range enables the potential impact of GTC on higher UVR-dose erythema than the erythema threshold dose to be assessed. Accordingly, in this study,

the erythema induced by the highest UVR-dose given (68mJ/cm^2) and the AUC of the entire UVR-erythema dose response was analysed. It was revealed that the GTC intervention had the greatest effect at higher UVR doses, with a significant reduction in erythema at the highest UVR-dose following supplementation (*P*=0.006) [Figure 3.5]. These results were supported by a significant reduction in the AUC of the response to SSR post-GTC (*P* = 0.033) [Figure 3.6]. The above evidence of supplementation with oral GTC having the ability to offer protection against UVR-erythema in human subjects is supported by recent literature. An oral intervention study found GTC offered protection against the threshold erythema induced by the single UVR dose tested; however the volunteers consumed a much higher dose (1402mg catechins/d for 12 weeks) (Heinrich et al., 2011) when compared to 540mg GTC/d for 12 weeks used in this study. Potentially the combination with a small dose of vitamin C in the current study enhanced the gut stability and uptake of GTC, which was our rational for using the combination. Previous work has shown that vitamin C alone has no effect on UVR-erythema in humans (McArdle et al., 2002), and therefore it is unlikely, although cannot be completely excluded, that the antioxidant vitamin C may have separately contributed to the study findings.

3.7.4 Impact of GTC on skin blister fluid eicosanoids

With the above findings providing evidence of GTC being taken up into the skin and thus having the ability to reduce UVR-induced inflammation, we looked at its potential to offer this systemic protection through the reduction of pro-inflammatory eicosanoid production. PGE_2 is a potent mediator of UVR-erythema with upregulation peaking in the first 24hr of the sunburn response (Rhodes et al., 2009). Therefore, we looked at whether GTC, which is reported to be a COX-inhibitor in some systems (Hussain et al., 2005, Hou et al., 2007) impacted on this COX-2 metabolite. However, the results showed no significant reduction in PGE_2 concentration in blister fluid from unexposed or UVR-exposed skin following the 12 week intervention.

We then turned our attention to 12-HETE, the most abundant pro-inflammatory eicosanoid induced in human skin by UVR exposure (Kendall and Nicolaou, 2013). 12-HETE has previously been reported to induce a dose-related erythema when applied directly to human skin *in vivo* (Wollard et al., 1989), and to be a potent leucocyte chemo-attractant associated particularly with the later stages of the sunburn response (Rhodes

et al., 2009). Therefore, we looked at whether GTC, which is reported to be a LOX-inhibitor in some models (Hong et al., 2001a, Schewe et al., 2001) impacted on this 12-LOX metabolite. Our results indicated that 12-week supplementation with relatively low dose GTC has the ability to significantly reduce 12-HETE concentration (P=0.015) in UVR-irradiated skin [Figure 3.8]. No evidence was found from correlation analysis that this was the mechanism of reduction of UVR-induced erythema on GTC, but this could reflect factors including the complexity of the mediation of UVR-induced erythema, which also involves UVR-induced prostanoids and nitric oxide (Rhodes et al., 2001a) and the relatively small subject number.

Chapter 4 Conclusion

4.1 Overview of findings

Acute exposure of human skin to UVR results an inflammatory response (sunburn). The eicosanoid PGE_2 is a potent mediator of UVR-erythema (Rhodes et al., 2009), while 12-HETE is the most abundant pro-inflammatory eicosanoid induced in human skin by UVR exposure (Kendall and Nicolaou, 2013), and may thus also play a role in the inflammation. Previous research has shown GTC possesses both potent antioxidant and anti-inflammatory properties which may have the potential to provide systemic photoprotection (Afaq et al., 2003b, Katiyar et al., 2000b, Heinrich et al., 2011). We hypothesised that oral GTC are bioavailable in human skin and through COX and LOX inhibition may reduce the production of these pro-inflammatory eicosanoids and the UVR-induced erythemal response. In an oral GTC intervention study, a reproducible and quantitative system of UVR-induced erythema was employed in humans *in vivo* alongside UVR-stimulated eicosanoid expression in skin, with assessments at baseline and following 12 weeks of GTC supplementation. Further novel assessments were made of skin GTC bioavailability, in this pilot study.

Overall findings from this study showed 12-week supplementation with a modest dose of encapsulated GTC (~2.5 cups/d), when combined with low dose vitamin C (50mg/d) as a stabiliser, showed GTC were bioavailable in human skin (blister fluid and dermal tissue) albeit with considerable inter-individual variation. The UVR-erythema dose response data indicated little change in the erythema threshold dose ($D_{0.03}$), but protection against UVR-induced skin erythema elicited by higher UVR doses (68mJ/cm^2; P=0.006), and reduction in the AUC of the response to SSR post-GTC (P=0.033). Moreover, the possibility was examined that the bioactives might reduce UVR-inflammation including the erythemal response through the reduction of pro-inflammatory eicosanoid expression. The results showed no significant reduction in PGE_2 concentration in blister fluid from unexposed or UVR-exposed skin following the 12 week intervention, but supplementation with GTC and vitamin C was accompanied by a significantly reduced 12-HETE concentration in UVR-exposed skin (P=0.015), which is consistent with LOX inhibition by GTC.

4.2 Relevance of findings

Overexposure to UVR results in adverse effects on human skin, including sunburn and skin cancer. The limitations of topically applied sunscreens, particularly inadequate application, have given rise to research to develop systemic sun protection. It is thought that systemic photoprotection may provide a constant low level of protection to the entire body surface (section 1.5.2). There has been little research on the effect of oral supplementation with GTC on UVR-induced skin inflammation in human subjects.

The results reported in this thesis further support the findings of Heinrich *et al* (2011) which show supplementation with GTC offers protection against UVR-induced skin erythema (Heinrich et al., 2011). It must be taken into consideration our study used a combination of bioactives (GTC and vitamin C). Vitamin C has been shown to provide a stabilisation effect, increasing catechin bioavailability through enhancing accessibility and intestinal uptake (Peters et al., 2010) (section 1.6.5). Previously, vitamin C alone was found to have no effect on UVR-induced erythema in humans (McArdle et al., 2002), and therefore it is unlikely, although cannot be completely excluded, that the antioxidant vitamin C may have separately contributed to the study findings. Conversely, the use of this combination of bioactives may in turn provide greater photoprotection than GTC alone.

Previously, in terms of pro-inflammatory eicosanoids, much focus has been on the involvement of PGE_2 in UVR-mediated erythema. However, COX-2 inhibitors only partially suppress UVR-induced erythema (Kozuka et al., 1983). Our findings suggest the possibility that 12-HETE may be involved in the later erythema, and hence suppression of the LOX-derived mediator 12-HETE may operate an anti-inflammatory effect reflected in the reduced erythema, as well as a potential effect through reduced leukocyte attraction (not examined in this thesis). However, it must be noted, mediation of UVR-induced erythema is complex and involves many mediators including UVR-induced prostanoids and NO (Rhodes et al., 2001a). Previous research found the antioxidant capacity of green tea provided direct scavenging activity against NO *in vitro* (Nakagawa and Yokozawa, 2002) and moreover, EGCG inhibited UVB-induced activation of NFκB, sequentially blocking inducible iNOS preventing production of NO (Mukhtar and Ahmad, 2000, de Mejia et al., 2009). Therefore, further research is warranted to look at the effect of this combination

of bioactives on a wider range of pro-inflammatory mediators involved in the sunburn response.

This pilot study focused on the acute cutaneous effects of UVR on skin (sunburn). However, a more chronic effect from repeated exposure is skin carcinogenesis. Both sunburn erythema and skin cancer may be initiated through UVR-induced DNA damage, and epidemiological studies are supportive of sunburn erythema as a risk factor for skin cancer (Armstrong and Kricker, 2001, Berwick and Vineis, 2000). In mouse models, topically applied GTC were found to inhibit tumour initiation and promotion (Huang et al., 1992). Interestingly, 12-HETE is a recognised tumour promoter, including of skin cancer (Winer et al., 2002). Thus it may be of further significance that our results indicate GTC in combination with vitamin C appear to have the ability to reduce UVR-induced 12-HETE, in addition to the sunburn protection.

Although this is early data and warrants further investigation, these findings suggest that the bioactives have potential to be a preventative measure against skin cancer in humans and continuous low level sun protection through dietary GTC might act as a useful adjunct to the more intermittently applied higher protection topical sunscreens.

4.3 Strengths and weaknesses of the work

The oral GTC intervention study was performed as pilot work, with an open (uncontrolled) before-after design and a relatively low subject number (n=16). This number of participants was appropriate for an initial pilot study of this design. However, before-after studies can be misleading due to the possibility that there are other variables influencing study outcomes, such as seasonal changes in UVR resistance, or assessment variables. Hence the gold standard design of a double blind randomised placebo-controlled (RCT) would be required to confirm the impact of GTC with vitamin C on UVR-induced erythema and 12-HETE levels. This would need to be sufficiently powered to show a difference between active and control supplement groups.

Although a strength of this pilot study was all subjects received the same dose of GTC and vitamin C (550mg/d and 50mg/d respectively) it would be useful to assess the impact of varying doses of these bioactives to examine for a dose-response relationship in outcomes and to identify the optimum effective dose. This could be achieved by

randomising the subjects into three separate trial arms i.e: 1) high dose supplementation, 2) low dose supplementation and 3) placebo.

The participants who took part in this study were white Caucasian volunteers with skin phototype I/II (skin that burns easily and never tans or skin that usually burns and tans minimally with difficulty, (Fitzpatrick, 1988)). Therefore, it could be argued that the relevance of these findings is limited to a sector of the general population. Further research could be performed to assess the impact on subjects with different skin phototypes such as those who burn less easily and tan readily (section 1.4.1) to observe the effect this combination of bioactives has on a broader range of subjects. On the other hand, it can be argued that this study has examined the impact of the bioactives on those who are at highest risk of sunburn and skin cancer, and therefore the most in need of photoprotection.

A further strength of the study was the collection of 24h urine samples at baseline, day 1 and weeks 6 and 12 following supplementation as a measure of compliance. Often compliance is measured by counting the number of supplements returned following completion of the study. In this instance you are solely relying on each subject fully completing the course of supplements and reporting honestly if they have missed any of the doses. However, by measuring the amount of catechins and metabolites found in the urine samples we were able to assess the intake of unique GTC in each subject. Subsequently, one completing volunteer failed to demonstrate the four main catechins found in green tea and thus data were excluded from the final analyses.

UVR-induced erythema was assessed both visually (MED) (section 2.3.2.1) and by the use of the reflectance instrument (section 2.3.2.2). The MED is a subjective measure which uses an interval scale to measure erythema intensity compared to the reflectance instrument which is more objective. The latter is measured on a continuous scale allowing more subtle changes to be detected. Therefore, quantification of UVR-erythema and the construction of the UVR-dose response curves using dose-response modelling software (section 2.4.4.1) allow the more subtle effects to be captured which would not have been detected upon visual inspection alone. The other side of this coin is that the changes in erythema witnessed in this study were modest.

It should be noted that huge advances in technology enabled this original and pioneering work which has demonstrated catechins and their metabolites are able to be measured in small skin samples obtained in humans *in vivo*.

4.4 Future research

Future research could be performed as follows:

- A double blind RCT is warranted to further explore the use of oral GTC with vitamin C in human photoprotection. A sufficient number of subjects must be recruited for the study to be statistically powered to show a difference between trial arms.

- The impact of GTC with vitamin C versus GTC alone versus vitamin C alone could be examined, to clarify the contribution of the bioactives. Different doses of GTC could also be examined, including a higher dose to examine for a potentially larger effect on UVR-erythema.

- Our results found GTC with vitamin C offered protection against UVR-induced erythema at higher UVR doses and thus it may be advantageous to increase the UVR dose range for future studies.

- With regards to the complexity of UVR-induced erythema, the impact of bioactives on a wider range of pro-inflammatory mediators including NO could be assessed.

- Other outcome measures could be examined, i.e. the impact on the leukocyte infiltration component of the sunburn response could be evaluated histologically, and ultimately the impact on skin cancer prevention could be assessed.

- Our results highlighted the huge inter-individual variation for catechin bioavailability. Therefore, factors influencing the catechin uptake and metabolism by skin warrants further exploration.

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