Photoprotective effect of isoflavone genistein on ultraviolet B-induced pyrimidine dimer formation and PCNA expression in human reconstituted skin and its implications in dermatology and prevention of cutaneous carcinogenesis

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Genistein, the most abundant isoflavone of the soy derived phytoestrogen and inhibitor of tyrosine kinase. We previously reported the antiphotocarcinogenic effects of genistein in SKH-1 murine skin, including its capacity for scavenging reactive oxygen species, inhibiting photodynamic DNA damage and down-regulating UVB-ultra violet B)-induced signal transduction cascades in carcinogenesis. In this study we elucidate genistein’s photoprotective efficacy within the context of full thickness human reconstituted skin relative to acute challenges with ultraviolet-B irradiation. Skin samples were pre-treated with three concentrations of genistein (10, 20 and 50 μM) 1 h prior to UVB radiation at 20 and 60 mJ/cm2. Proliferating cell nuclear antigen (PCNA) and pyrimidine dimer (PD) expression profiles were localized using immunohistochemical analysis on paraffin embedded samples 6 and 12 h post UVB exposure. Genistein dose dependently preserved cutaneous proliferation and repair mechanics at 20 and 60 mJ/cm2, as evidenced by the preservation of proliferating cell populations with increasing genistein concentrations and noticeable paucity in PCNA immunoreactivity in the absence of genistein. Genistein inhibited UV-induced DNA damage, evaluated with PD immunohistochemical expression profiles, demonstrated an inverse relationship with increasing topical genistein concentrations. Irradiation at 20 and 60 mJ/cm2 substantially induced PD formation in the absence of genistein, and a dose dependent inhibition of UVB-induced PD formation was observed relative to increasing genistein concentrations. Collectively all genistein pre-treated samples demonstrated appreciable histologic architectural preservation when compared with untreated specimens. These findings represent a critical link between our animal and cell culture studies with those of human skin and represent the first characterization of the dynamic alterations of UV-induced DNA damage and proliferating cell populations relative to pre-treatment with genistein in human reconstituted skin. The implications of our findings serve as compelling validation to our conclusions that genistein may serve as a potent chemopreventive agent against photocarcinogenesis.

Introduction
Genistein (4’,5,7-trihydroxyisoflavone), the soy derived isoflavone, has garnered significant noteworthy attention from the scientific and medical communities alike in recent years (1–4). The recent heightened interest and highlighted public awareness in reference to this naturally occurring soy derivative can be attributed to the positive beneficial health effects that increased soy consumption has been proven to have on disease risk assessment. Specifically, the typical Asian diet contains 10–12 g of isoflavone rich soy protein per day, roughly 20–50 times that of the average western diet (5). Asians have significantly lower incidences in breast, colon and prostate cancers when compared to western populations, and dietary consumption may be a legitimate contributory factor to these astounding statistics (6–9). Genistein’s classification as a phytoestrogen with the capacity to bind to estrogen receptors (10) accounts for additional health benefits including the modulation of peri-menopausal symptomology without the associated dangers of hormone replacement therapy (11,12). Furthermore, scientific studies in post menopausal women indicated that continuous dietary intake of genistein not only retarded bone loss but increased mineral content and bone density with subsequent inhibition of osteoporosis (13). Soy isoflavone rich diets were also shown in human clinical trials to reduce cardiovascular disease risk via several distinct mechanisms, including preventing LDL cholesterol oxidation, increasing levels of HDL and improving arterial elasticity with an associated reduction in elevated blood pressures (14–17). More pertinent to the proposed study that we present here are the effects of genistein in cutaneous UV-induced DNA damage and subsequent cell proliferation profiles.

The incidence of skin cancer, the most common human malignancy, continues to increase, with an estimated 1.3 million new cases of non-melanoma skin cancer (NMSC) occurring in the US each year and malignant melanoma boasting the most rapid increase in incidence in the US when compared to any other type of cancer (18,19). Yet, there still remain limited options for prevention. Chronic exposure to solar radiation in humans has been implicated as the primary causation for the vast majority of cutaneous malignancies. While ultraviolet B (UVB) remains a minor component of the ultraviolet spectrum reaching the earth’s surface, it has been scientifically demonstrated to be the most efficacious in inducing photodynamic DNA damage, namely cyclobutane pyrimidine dimmers (PDs) and the subsequent progression toward cutaneous carcinogenesis (20–22). The primary cutaneous events induced by ultraviolet exposure are directly related to the absorption of these photons by cellular DNA in skin. Topical application of photoprotective compounds possessing antioxidant properties can act to regulate the initiation and promotion sequences of these events in an effort to abate the immediate effects of inflammation, erythema and cutaneous neoplastic potentiation. In previous studies we

Abbreviations: EGFR; epidermal growth factor receptor; PD: pyrimidine dimmers; PCNA; proliferating cell nuclear antigen; UVB; ultraviolet B.
have demonstrated genistein’s capacity to inhibit UV-induced oxidative DNA damage in purified DNA and cultured cells as well as block UV-induced proto-oncogene expression in mouse skin (23–30). We also showed topical application of genistein inhibited UV-induced inflammatory response cascades and erythema discomfort scores in humans along with the inhibition of tumor incidence and multiplicity in Skh-1 hairless mice. Of greater significance was the characterization of genistein’s capability to block UVB-induced epidermal growth factor and MAP Kinase phosphorylation, both of which are known to be involved in the promotion of pathways of cell proliferation and subsequent photocarcinogenesis (31).

In the proposed study, we continue to explore genistein’s antiphotocarcinogenic effect in modulating the photodynamics of UVB induced DNA damage and cell proliferation. In an attempt to conclusively translate some of our data from biochemical cell culture and animal studies to that of humans, we have employed 3-dimensional human reconstituted skin (EpiDerm™ FT) as an experimental model designed to more closely resemble the physiologic interaction of human skin with UV light. This advanced full thickness model incorporates a fibroblast containing dermis accompanied by epidermal keratinocytes designed to take full advantage of these ultra structural constituents and their role in modulating cutaneous irritancy, photodamage and cancer progression. Many studies have shown that reconstituted human skin can be used for the analysis of cutaneous irritant potentials of numerous consumer products. It has been used to screen the photoprotective efficacy of sunscreens as well as assess cytotoxicity and evaluate the expression of certain biomarkers of inflammation (32–35). Epiderm has also been used as a suitable in vitro model to evaluate the genotoxic potentials of chemical (Benza[al]pyrene) and physical agents (UV irradiation) to elucidate mutagenic and carcinogenic responses in the study of mechanism of action leading to cutaneous tumor induction. Furthermore, the inconvenience, unavailability and obvious prognostic perils associated with actual human skin biopsies warrants the usefulness of this reconstituted human skin model in photocarcinogenic research (36,37).

Evaluation of the immunohistochemical expression profiles of cyclobutane PDs and proliferating cell nuclear antigen (PCNA) in this human skin equivalent subsequently exposed to UVB enables us to gain further insight into the efficacy of genistein as a chemopreventive agent in human skin cancers. Specifically, we have determined the interactions of this soy derived isoflavone in the modulation of UVB-induced DNA damage and its associated effects upon cellular repair mechanics.

Materials and methods

**Human reconstituted skin**

EpiDerm™ FT Full Thickness (EFT-200) purchased from (MatTek Corp., Ashland, MA) is an in vitro human skin equivalent model comprising normal human keratinocytes (NHK) derived from neonatal foreskin tissue. The NHK, which are metabolically and mitotically active, are cultured to form a multilayered highly differentiated model consisting of basal, spinous, granular and cornified layers analogous to those found in vivo in human epidermal skin. This advanced full thickness model also incorporates a fibroblast containing dermis. This reconstituted skin model allows in vitro evaluation of cutaneous phenomena in which fibroblast-keratinocyte cell interactions (paracrine signaling) are important. Markers of mature epidermis-specific differentiation such as profilagrin, the K1/K10 cytokeratin pair, involucrin and type I epidermal transglutaminase have been localized in the model. Ultrastructural analysis has revealed the presence of keratohyalin granules, tonofilament bundles, desmosomes and a multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns characteristic of in vivo epidermis. A well-developed basement membrane is present at the dermal/epidermal junction. Hemidesmosomes, lamina lucida, lamina densa and anchoring fibril structures are evident by transmission electron microscopy. Immunohistochemical analysis shows the presence of basement membrane structural and signaling proteins, including collagen IV, Laminin, collagen VII and integrin α6. The dermal compartment is composed of a collagen matrix containing viable normal human dermal fibroblasts (NHDF).

Upon receipt, the reconstituted skin samples were equilibrated and maintained at 37°C, 5% CO2 in 6 well culture plates. Equilibration and maintenance of the tissues was accomplished using 2.0 ml of EFT medium (provided with EFT-200 kit). Throughout all experimentation, the tissue samples were maintained at the air liquid interface, i.e. the EFT medium in the 6 well plates was exposed to the basal side of the tissues only while the apical surface remained dry or exposed to topically applied genistein of different concentrations or DMSO-containing medium only (in the case of the control samples), as later described in greater detail.

**UVB irradiation**

Our Spectroline Phototherapy Unit consists of 2 UVB lamps from the Spectronic Corp. (Westbury, NY), predominantly emitting UVB light at the range of 280–320 nm. The average emittance was 302 nm, carefully calibrated utilizing an IL 1700 research radiometer with photodetectors. The UVB fluences used throughout the study were 20 and 60 mJ/cm², respectively. This UVB fluence range was equivalent to one minimal erythema dose (MED) in Fitzpatrick type I-IV human skin, equal to ~10–30 min of sun exposure during summer time in Central Park, New York City, based on our three-year consecutive measurements.

The six well culture plates were placed under the phototherapy unit. Each well plate contained the respective human reconstituted skin samples corresponding to specific pretreatment genistein concentrations. Tissue samples were topically exposed, via gentle pipetting, to 100 μl of either 10, 20 or 50 μM genistein prepared in 0.1% DMSO in EFT medium for 1 h prior to UVB irradiation. A 0.1% DMSO in EFT medium (the control medium) was maintained for all experiments. After topical exposure, the samples were carefully rinsed with PBS, again via gentle pipetting of the apical tissue surface to remove any non-absorbed genistein, and the EFT medium was also replaced with PBS. The six well plates containing the tissues were then irradiated with systematic rotation of the plates during irradiation to ensure an even exposure to various positions of the UVB lamps. The distance between the UV lamps and the reconstituted skin specimens was approximately 40 cm, a distance which ensured appropriate UV light transmission without temperature changes in the respective tissue samples. After UV exposure, approximately 3 × 2 mm skin sections were taken. Skin specimens were fixed in 10% neutral buffered formalin for paraffin embedding.
Samples in 10% formalin solution were paraffin-embedded, and 5 μm serial sections were mounted on poly-1-lysine (Sigma Chemicals, St Louis MO) coated glass slides for immunohistochemical evaluation.

**Evaluation of Sunscreen effect with topical genistein**

To avoid UV blocking or ‘the barrier effect’ that is often encountered with the topical application of certain compounds, we utilized an IL 1700 research radiometer with a calibrated photo-detector to measure and ensure full UV transmission to genistein treated specimens. An average UVB wave emittance length of 302 nm was used. Full transmission remained consistent for genistein concentrations between 10 and 100 μM. The blocking effect was not observed until 1 mM, a concentration far beyond the doses used in this study. Thus, the sunscreen effect of genistein would not be a confounding factor in the study. In addition, rinsing of the apical tissue surface and replacing the EFT medium with PBS before UVB irradiation further avoided the potential sun screening effect of genistein or possible photo-toxic effects of phenol red contained within the EFT medium.

**Assay for PD**

The assay for PD was well established in our previous publication (38). Briefly, slides with paraffin-embedded tissue sections were heated for 30 min at 65°C, and allowed to cool at room temperature for 20 min. Samples were deparaffinized in xylene, dehydrated in graded ethanol and then treated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidases as specified in the protocol by Vector Laboratories (Burlingame, CA) in the Vectastain Elite ABC kit. Further treatment of the sections in a 1% non-fat milk blocking solution for 30 min blocked non-specific binding. Following three rinses in PBS, sections were incubated with PCNA FL-261 (dilution 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at room temperature and then blocked with 10% normal goat serum in PBS for 40 min. The sections were then rinsed in PBS and treated with biotinylated anti-rabbit (dilution 1 : 200) for 60 min and the avidin–biotin complex for 30 min. The color was developed with 3-amino-9-ethylcarbazole and the samples were counter stained with hematoxylin (all from Vector Laboratories, Burlingame, CA). Digital micrographs were taken and the number of positively stained cells/100 keratinocytes was analyzed using NIH Image Analysis.

**Assay for PCNA**

The expression of PCNA was determined using the avidin-biotin complex immunoperoxidase technique as described in our previous publication (38). Briefly, slides with paraffin-embedded tissue sections were heated for 30 min at 65°C, and allowed to cool at room temperature for 20 min. Samples were deparaffinized in xylene, dehydrated in graded ethanol and then treated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidases as specified in the protocol by Vector Laboratories (Burlingame, CA) in the Vectastain Elite ABC kit. Further treatment of the sections in a 1% non-fat milk blocking solution for 30 min blocked non-specific binding. Following three rinses in PBS, sections were incubated with PCNA FL-261 (dilution 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at room temperature and then blocked with 10% normal goat serum in PBS for 40 min. The sections were then rinsed in PBS and treated with biotinylated anti-rabbit (dilution 1 : 200) for 60 min and the avidin–biotin complex for 30 min. The color was developed with 3-amino-9-ethylcarbazole and the samples were counter stained with hematoxylin (all from Vector Laboratories, Burlingame, CA). Digital micrographs were taken and the number of positively stained cells/100 keratinocytes was analyzed using NIH Image Analysis.

**Statistical analysis**

Data were expressed as the mean ±SD. Statistical Analyses were performed using a two-tailed Student’s t-test. A P-value of 0.05 was considered significant. All experiments were performed at least three times and were scored by more than one investigator. A microscope with a calibrated grid was used to calculate the number of epidermal stained cells per square millimeter, with at least 5 random fields examined.

**Results**

UVB substantially induced PD formation in human reconstituted skin

Immunohistochemical analysis performed on the non UV irradiated (sham control) skin samples revealed no detectable PD formation as would be expected from these specimens that were devoid of the resultant DNA damage associated with ultraviolet exposure (Figure 1A).

However, when human reconstituted skin samples were exposed to a single dose of UVB at 20 and 60 mJ/cm², an appreciable upregulation in PD formation was observed after

![Figure 1](image-url)
12 h. The skin specimens irradiated with 20 mJ/cm² of UVB conveyed prominent PD formation with associated epidermal hyperplasia (Figure 1B). Moderate rearrangement in histological architecture was appreciated with small areas of separation in the epidermis. At 60 mJ/cm² of UVB irradiation (Figure 1C), PD immunoreactivity displayed a higher rate of formation when compared to the 20 mJ/cm² exposure samples (Figure 1B) with increased suprabasal positivity. This increase in positively stained PD cells was indicative of the increased severity of DNA damage imposed upon these samples by the enhanced intensity of the UV fluence. Marked disruption in the histologic architecture of the epidermal component was also appreciated at this exposure with more extensive acanthosis and spongiosis.

Genistein inhibits pyrimidine dimer formation in human reconstituted skin exposed to 20 and 60 mJ/cm² of UVB

One hour prior to ultraviolet exposure at 20 and 60 mJ/cm² human reconstituted skin samples were pretreated with 10, 20 and 50 μM of genistein respectively, diluted in 0.1% DMSO and growth medium. Genistein was carefully rinsed off the skin samples with PBS prior to ultraviolet exposure. Reconstituted human skin samples were harvested at 12 h post UV exposure. In both the 20 and 60 mJ/cm² UVB exposure skin samples, pretreatment with genistein prior to ultraviolet exposure demonstrated a dose dependent reduction in PD formation (Figures 2 and 3). The observable ultraviolet-induced DNA damage that ensued subsequent to ultraviolet exposure in the absence of genistein (Figures 2A and 3A) had been significantly reduced (Figures 2B–D, Figures 3B–D) in the genistein treated skin samples. This dose dependent downregulation in PD formation is well observed in the genistein concentrations from 10 to 50 μM with an appreciable preservation in histologic architecture with increasing genistein preapplication concentrations (Figure 2C and D). At 20 μM of genistein, PD formation demonstrated sparse intermittent immunoreactivity (Figure 2C) and would continue to decrease to virtually undetectable levels at 50 μM of genistein (Figure 2D). In addition, the observable atypia in keratinocytes including vacuolization and hydropic changes characteristic of the UVB-vehicle samples (Figures 2A and 3A) was less severe in the skin samples pretreated with 20 and 50 μM of genistein, respectively.

At 60 mJ/cm² of UVB (Figure 3) the resultant DNA damage was predictably more severe in comparison to the lower fluence at 20 mJ/cm² (Figure 2A). Evidence of this increased severity in DNA damage could be observed in the immunoreactivity expression profiles of PD formation at this higher fluence of UV radiation. Although the DNA damage pattern intensified with the 60 mJ/cm² skin samples, genistein still conveyed significant photoprotective efficacy. At 10 μM of genistein (Figure 3B), PD immunoreactivity had been significantly reduced when compared to the UVB-vehicle skin samples (Figure 3A). While positively stained PD cells had decreased, there was still pronounced cellular atypia with destruction in histological architecture that would progressively decrease with increasing genistein concentrations. The resultant vacuolization and hydropic changes associated with this 60 mJ/cm² fluence persisted in the pretreatment groups at 10, 20 and 50 μM concentrations. However, with increasing genistein concentrations at 20 μM (Figure 3C), PD staining demonstrated a dramatic reduction in immunoreactivity with relative preservation in histological architecture, when compared to the lower concentration of 10 μM (Figure 3B). At 50 μM concentrations of genistein PD immunoreactivity was once again virtually undetectable with marked paucity in PD formation and relative preservation in histology, with some evidence of epidermal hyperplasia and ultra structural histologic modifications, including disruption in the epidermal architecture (Figure 3D).

Figure 2. Effects of Genistein on the immunolocalization of UV induced PD formation in human reconstituted skin (EpiDerm™ FT) exposed to 20 mJ/cm² of UVB. Magnification (40X). (Inset) represents sham control. (A) Vehicle (0.1% DMSO) + UVB at 20 mJ/cm², (B) 10 μM Genistein + UVB, (C) 20 μM Genistein + UVB, (D) 50 μM Genistein + UVB. Scale Bar = 50 microns. Arrows demarcate areas of positively stained cells consistent with nuclear immunoreactivity.
UVB diminishes PCNA expression in human reconstituted skin

Sham control groups were systematically maintained in human reconstituted skin samples without exposure to UVB. Immunohistochemical analysis performed on the sham (non UV) irradiation skin samples revealed PCNA expression confined to the basal layer of the epidermis arranged with uniform continuity. This observation was consistent with our recent findings in mouse skin (38). The UV irradiated skin samples stained for PCNA were harvested at 6 h post ultraviolet exposure for the purposes of illustrating the damaging effects of UV irradiation on proliferating cell populations early on. These skin samples demonstrated a substantial reduction in PCNA immunoreactivity (Figure 4B) when compared to the sham irradiation (Figure 4A). This trend continued in the 60 mJ/cm² tissue samples which also demonstrated distinct diminished immunoreactivity in PCNA. The basal keratinocytes, which are represented by the oval shaped cells in the basal layer, were clearly devoid of the nuclear reactivity (Figure 4A) that is classically associated with PCNA expression (Figure 4A). The 20 mJ/cm² tissue samples (Figure 4B) demonstrated considerable histologic disruption in the epidermal component with accompanying acanthosis. Severe hydropic changes including ultrastructural modifications were easily appreciated in the 60 mJ/cm² tissue samples.

Genistein preserves PCNA Expression in human reconstituted skin irradiated with UVB at 20 and 60 mJ/cm² UVB

One hour prior to ultraviolet exposure at 20 and 60 mJ/cm² human reconstituted skin samples were pretreated with 10, 20 and 50 μM of genistein, respectively. Subsequently, genistein was carefully rinsed off with PBS prior to ultraviolet exposure. Human reconstituted skin samples were subjected to a one-time single exposure of UVB at 20 and 60 mJ/cm², respectively. Immunohistochemical analysis of PCNA expression at

Figure 3. Effects of Genistein on the immunolocalization of UV induced PD formation in human reconstituted skin (EpiDerm™ FT) exposed to 60 mJ/cm² of UVB. Magnification (40X). (Inset) represents sham control (A) Vehicle (0.1% DMSO) + UVB at 60 mJ/cm², (B) 10 μM Genistein + UVB, (C) 20 μM Genistein + UVB, (D) 50 μM Genistein + UVB. Scale Bar = 50 microns. Arrows demarcate areas of positively stained cells consistent with nuclear immunoreactivity.

Figure 4. PCNA expression in 3-dimensional human reconstituted skin (EpiDerm™ FT). Magnification (40X). (A) Sham irradiation, (B) 20 mJ/cm² of UVB plus vehicle (0.1% DMSO), (C) 60 mJ/cm² of UVB plus vehicle (0.1% DMSO). Scale Bar = 50 microns. Arrows demarcate areas of positively stained cells consistent with nuclear immunoreactivity.
20 mJ/cm² (Figure 5 A), in the absence of genistein, revealed a significant reduction in PCNA positive stained cells when compared with sham (non UV) irradiation tissue samples (Figure 4 A). This reduction in nuclear immunoreactivity was representative of the early deleterious effect that ultraviolet exposure renders on cutaneous proliferation and repair mechanics. Samples at 10 µM of genistein (Figure 5B) revealed appreciable preservation in PCNA nuclear immunoreactivity as evidenced by the relative preservation in PCNA positive stained cells in these samples. Genistein demonstrated a dose dependent capacity to maintain PCNA immunoreactivity with increasing concentrations of genistein. At 20 and 50 µM concentrations (Figure 5C and D), genistein showed an increased propensity for preserving PCNA expression and thus protecting against UV induced cutaneous immunosuppression of proliferation and repair mechanics. PCNA nuclear immunoreactivity for these successive concentrations was preserved (Figure 5C and D).

At 60 mJ/cm² of UVB, in the absence of genistein, considerable damage had been rendered to the human reconstituted skin samples (Figure 6A). With obvious hyperplasia in the epidermal and dermal components, and the UV associated damaged tissue. Genistein at 10, 20, and 50 µM (Figure 6B, C, D) show a remarkable preservation in PCNA expression as evident by the relative preservation of PCNA positive stained cells in these samples.

**Figure 5.** Effects of Genistein on PCNA expression in human reconstituted skin samples (EpiDerm™ FT) exposed to 20 mJ/cm² of UVB. Magnification (40X). (Inset) represents sham control (A) Vehicle (0.1% DMSO) + UVB at 20 mJ/cm², (B) 10 µM Genistein + UVB, (C) 20 µM Genistein + UVB, (D) 50 µM Genistein + UVB. Scale Bar = 50 microns. Arrows demarcate areas of positively stained cells consistent with nuclear immunoreactivity.

**Figure 6.** Effects of Genistein on PCNA expression in human reconstituted skin samples (EpiDerm™ FT) exposed to 60 mJ/cm² of UVB. Magnification (40X). (Inset) represents sham control (A) Vehicle (0.1% DMSO) + UVB at 60 mJ/cm², (B) 10 µM Genistein + UVB, (C) 20 µM Genistein + UVB, (D) 50 µM Genistein + UVB. Scale Bar = 50 microns. Arrows demarcate areas of positively stained cells consistent with nuclear immunoreactivity.
hydropic changes, PCNA nuclear immunoreactivity had diminished to undetectable levels. But the theme of dose dependent preservation of proliferating cell populations would once again hold true for these 60 mJ/cm² skin samples treated with genistein. At 10 μM of genistein, an observable increase in PCNA stained cells was immediately noted (Figure 6B). Genistein treatment concentrations of 20 μM (Figure 6C) resulted in a continued increase in the nuclear immunoreactivity of PCNA, which now expressed along the basal layer with uniform continuity, very similar to that of non-irradiated skin. Treatment samples at 50 μM concentrations (Figure 6D) further illustrated the fact that even at a higher UV fluence, genistein can be protective against the damaging effects of UVB on cutaneous proliferation and repair mechanics. All samples, while dose dependently preserving PCNA immunoreactivity, demonstrated some degree of histologic modification and atypia as a result of ultraviolet exposure.

Graphical quantification of PCNA and PD in human reconstituted skin

In comparatively analysing genistein’s photoprotective efficacy in human reconstituted skin we included graphical quantification of its effects on PCNA and PD relevant to the respective UV doses (Figure 7). The graph illustrated genistein’s capacity to regulate resultant UV-induced damage in the expression profiles of these biomarkers. Genistein dose dependently preserved the formation of PD at both UV fluences. Genistein also mitigated the associated UVB-induced decrease in PCNA immunoreactivity. Collectively the graph conveyed genistein’s capacity to regulate UVB-induced photodamage and accordingly protect against photocarcinogenesis.

Discussion

We previously characterized the intricacies of acute and chronic ultraviolet exposure in relation to PCNA and PD immunohistochemical expression profiles and their implications on DNA damage and the immunosuppression of integumentary repair mechanics in murine skin (38). Briefly, we demonstrated the immediacy of the deleterious effects that UVB imposed on cutaneous mechanics by capturing the initial downregulation in PCNA expression at an early onset, subsequent to ultraviolet exposure. We correlated this sequence in events with simultaneously increased PD expression for corresponding skin samples at the same time point. We observed that as PCNA regained immunoreactivity corresponding PD immunoreactivity diminished dramatically, owing to the integral role of PCNA’s action with p21 in the processes of nucleotide excision repair (39). Specifically, p21 has been well established as an inhibitor of the short gap filling action of PCNA during DNA nucleotide excision repair. Accordingly, the resultant p21 degradation that occurs subsequent to ultraviolet irradiation functions to promote PCNA dependent repair (40). This diminished expression of PCNA was indicative of UV-induced damage on host immunoresponse and repair mechanics. The resultant PD formation provided quantitative evidence for the suspected DNA damage. The subsequent decrease in PD immunoreactivity with time and the removal of the previously formed dimers indicated that nucleotide excision repair had commenced. The corresponding upregulation in PCNA immunoreactivity provided quantitative validity to this correlate as it is integrally involved in this process.

The process of nucleotide excision repair is paramount in maintaining normal integumentary functioning and in avoiding the potential mutations associated with ultraviolet damage sites in the skin. It serves to repair the resultant bulky DNA photo products primarily represented as cyclobutane PD and 6–4 photo products (41,42).

The significance of nucleotide excision repair in preventing UV-induced skin cancer and inhibiting the promotion of tumorigenesis is also very well established clinically and evidenced by the marked increased incidence in cutaneous malignancies in patients with Xeroderma Pigmentosum (XP). Genetic and molecular analyses have revealed that the repair of ultraviolet (UV)-induced DNA damage is impaired in XP patients owing to mutations in genes that form part of the DNA repair pathway that deals primarily with nucleotide excision repair (43). Patients with XP have severely defective nucleotide excision repair pathways (44). These patients have an increased predilection for the development of melanoma and non melanoma skin cancer, with incidences that are 1000 times greater than that of the normal population before the age of 20 (45). The relationship between UV-induced immunoreactivity expression profiles of PD and PCNA, and the derived implications on DNA damage and immunosuppression of host immunity and repair mechanics (nucleotide excision repair) is classified throughout the scientific literature (38,46–48). It is in this milieu that we have illustrated genistein’s photoprotective efficacy in modifying UVB-induced cutaneous DNA damage in human reconstituted skin.

Evidence of genistein’s role in the modification of UVB-induced DNA damage was observed at both fluencies of UVB at 20 and 60 mJ/cm². The obvious induction of PD formation in UVB irradiated skin samples demonstrated the extent of DNA damage that was imposed on the tissue specimens. Pretreatment with genistein demonstrated a dose dependent decrease in PD formation at both UVB fluences. The 50 μM pretreatment samples showed the greatest protective efficacy against PDs, reducing formation to undetectable levels at 20 mJ/cm² of UVB with sparse intermittent hints of immunoreactivity at 60 mJ/cm². The appreciable reduction in the formation of PDs, one of the most abundant cytotoxic and mutagenic UV-induced DNA damage photoproducts in the skin represented...
quantitative evidence of genistein’s photoprotective efficacy in human reconstituted skin.

In relation to photo repair mechanics, the evaluation of proliferating cell populations in this model demonstrated confluent PCNA immunoreactivity in the sham irradiation sample as expected. Upon ultraviolet radiation, in the absence of genistein, PCNA nuclear immunoreactivity demonstrated a significant reduction in expression to undetectable levels for the 20 and 60 mJ/cm² tissue samples indicative of the damaging effects of ultraviolet light on cutaneous repair mechanics. There are various molecular and cellular changes that occur in the skin as a result of UV irradiation. These modifications include changes in the proteins involved in cell cycle activity, cellular repair, proliferation and apoptosis. UV exposure leads to cutaneous neoplasia by inflicting severe and sometimes irreparable damage to these very processes. PCNA is a 36 kDa acidic nuclear protein and a cofactor of DNA polymerase that exists in the nucleus of all eukaryotic cells. PCNA is integral to nucleic acid synthesis and metabolism and plays an essential role in recombination, replication, excision repair and chromatin assembly. PCNA transcription can be stimulated in non-dividing cells by UV irradiation. PCNA related DNA repair in response to such environmental stressors and possible mutagens is critical to cellular maintenance of genomic integrity. UVB also has the capacity to impose initial damaging effects on host repair pathways in which PCNA plays a critical role (38,49,50). UV exposure suppresses the function of the integumentary immune system, impeding on the skin’s capacity for repair, thereby creating a more favorable environment for the development and growth of tumors (46). Genistein proved to preserve the immunoreactivity of PCNA at both UV fluencies in a dose dependent manner, although comparatively, skin samples irradiated with 60 mJ/cm² of UVB realized a more extensive reduction in PCNA immunoreactivity; this was particularly obvious at the 10 and 20 μM concentrations. The 50 μM genistein samples demonstrated the strongest preservation in PCNA and aided in the inhibition of UV-induced damage on cutaneous cellular repair mechanics. The beneficial effects of genistein on the immunoreactivity expression profile of these biomarkers represents a significant role for this isoflavone in the modification of UV-induced human skin cancers. Cellular DNA is one of the key targets for ultraviolet DNA damage (50). This DNA damage, present in the form of CPD, if left unrepaird, will interfere with DNA transcription and replication. Oftentimes, this can lead to a misread genetic code, subsequent mutation and hence the initiation of photocarcinogenesis. Accurate transmission and sharing of genetic information between cells is critical to the survival of the integumentary organ system. The precise transmission of genetic information primarily necessitates a system that operates with extreme accuracy in the replication of DNA with meticulous distribution of the respective chromosomes. Secondarily, however, not any less significant, this system must be able to sustain itself and survive spontaneous or induced DNA damage while simultaneously minimizing the number of inherited mutations. To accomplish this goal the skin has a sophisticated repair mechanism in place along with specialized proteins (one of which is PCNA) (51). The implications of genistein’s capacity to modify induced cellular DNA damage and aid the subsequent repair and proliferation process in this reconstituted model may represent significant steps toward preventing cutaneous cancers in sun exposed human skin. Berton et al. established the theory that, by reducing the amount of DNA damage, it may be possible to reduce skin cancer formation by UV radiation (51). Although that study focused on the use of different topical antioxidants to retard DNA damage, the principle of reducing UV carcinogenesis held true. There are some concerns raised as to the genotoxic and clastogenic effect of genistein due to its inhibition of topoisomerases II (52–54). However, most studies of genistein’s effect on topoisomerases II and chromosomal damage were performed in lymphoproliferative cell lines in vitro and occurred at a high concentration threshold (55,56). Two recent studies have comprehensively investigated genetic toxicities of genistein and consistently demonstrated no in vivo mutagenicity and clastogenicity in three species of mice and rats (53,54). These studies have been corroborated by our skin carcinogenesis studies in which neither carcinogenicity nor genotoxicity was observed in mouse skin chronically treated with topical genistein (24,31).

In conclusion, genistein presents compelling photoprotective efficacy and potently minimizes the detrimental effects of UVB irradiation in human reconstituted skin. Its capacity for regulating these events is likely to be attributable to the scavenging of reactive oxygen species, the inhibition of oxidative and photodynamic damage to DNA and the inhibition of tyrosine kinase as previously reported in other experimental models (24–31,52,57). Future investigation as to the exact mechanism in this model is required.

Conflict of Interest Statement: None declared.

References


20. D’Errico, M., Teson, M., Calcagnile, A., Nardo, T., De Luca, N., Lazzari, C.,
Bohm, M., Wolff, I., Scholzen, T.E., Robinson, S.J., Healy, E., Luger, T.A.,

17. Figtree, G.A., Griffiths, H., Lu, Y.Q., Webb, C.M., MacLeod, K. and


p21WAF1 in the induction of oxidative stress and apoptotic biochemical changes

31. Wei, H., Saladi, R., Lu, Y., Wang, Y., Palep, S.R., Moore, J., Phelps, R.,
Shyong, E. and Lebwohl, M.G. (2003) Isoflavone genistein: photoprotection and
clinical implications in dermatology. J. Nutr., 133, 3811S–3819S.


and skin equivalent models for identifying photocotoxic compounds in vitro.
Photodermatol Photoinmunol Photomed, 13, 27–36.

New epidermal model for. dermal irritancy testing. Toxicology in Vitro, 8, 889–891.


Wang, Z.Y. (1999) Green tea protects against psoralen plus ultraviolet


expression of proliferating cell nuclear antigen in murine skin.

expression of cell cycle inhibitors in human skin and hairless mouse.

downregulation is required for efficient PCNA ubiquitination after UV irradiation. Oncogene, [Epub ahead of print].

cyclobutane dimers in mammalian cells: evidence from a xeroderma pigmentosum revertant.

42. van Steeg, H. and Kraemer, K.H. (1999) Xeroderma pigmentosum and the


Proc., 1, 143–146.


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