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Inhibition of UV light- and Fenton reaction-induced oxidative DNA damage by the soybean isoflavone genistein

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We have investigated the effect of the soybean isoflavone genistein on 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation in calf thymus DNA exposed to either UV irradiation or the Fenton reaction system. Under the conditions used we observed that UV light and the Fenton reaction significantly increase 8-OHdG formation in DNA. Co-incubation with genistein inhibits the formation of 8-OHdG induced by either UV light irradiation or the Fenton reaction in a dose-dependent manner. The quenching effect of genistein on 8-OHdG formation induced by UV light is much more potent than that by the Fenton reaction, suggesting that the mechanisms of 8-OHdG formation may differ between the two systems. We further compared the antioxidant activities and quenching effect on 8-OHdG formation of genistein with biochanin A. Genistein potently scavenges both hydrogen peroxide in the medium and superoxide anion generated by xanthine/xanthine oxidase, whereas biochanin A has either a weak or no scavenging effect on these reactive oxygen species. However, both genistein and biochanin A display a similar quenching effect on UV light-induced 8-OHdG formation. These results suggest that the quenching effect of genistein and biochanin A on UV light-induced 8-OHdG formation is different from their ability to scavenge hydrogen peroxide and superoxide anion. The potent inhibition of UV light-induced oxidative DNA damage by genistein suggests its potential anticarcinogenic role in photocarcinogenesis.

Introduction

Epidemiological studies have shown that a high soybean intake is associated with low incidence rates of human cancers (1–6). One of the hypothesized candidates against cancer in soybeans is genistein, a most abundant isoflavone in soya (structure shown in Figure 1). In recent years genistein has received wide attention because of its potential anticancer properties (5,6). Genistein is a potent inhibitor of the activity of protein tyrosine kinases and modulates cell proliferation and transformation (7). In addition, genistein inhibits DNA topoisomerase II (8) and ribosomal S6 kinase (9), subsequently leading to protein-linked DNA strand breaks and tumor cell growth arrest. Genistein was also shown to stabilize the transient DNA–DNA topoisomerase complex and induces DNA strand breaks during replication (10,11). More recently genistein has been shown to destroy B cell precursor leukemia by its targeting to CD19-associated tyrosine kinases (12). In addition, genistein exhibits antioxidant properties by preventing hemolysis of red blood cells by dialuric acid or H_2O_2 and by protecting against microsomal lipid peroxidation induced by a Fe^{2+}–ADP complex (13–15). We have shown that genistein scavenges phorbol ester-type tumor promoter-induced H_2O_2 formation both *in vitro* and *in vivo*, inhibits 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA*)-induced proto-oncogene expression (c-*fos*) in mouse skin and suppresses TPA-promoted skin tumorigenesis (16,17).

Increased oxidative stress is known to cause oxidative DNA damage. One of the abundant oxidized DNA bases is 8-hydroxy-2′-deoxyguanosine (8-OHdG), a well-established biomarker relevant to carcinogenesis and aging (18,19). Increased levels of 8-OHdG have been found in cancerous tissues (20,21) and are responsible for DNA base mutations (22,23) and activation of certain oncogenes, such as H-*ras* and K-*ras* (24,25). The formation of 8-OHdG can be induced by various environmental factors, such as chemical modification, ionizing irradiation and UV irradiation with a sunlamp (18,19). In view of the potential carcinogenicity of 8-OHdG, the development of an approach to reduce this type of DNA damage may contribute to the prevention of cancer or other degenerative disorders in humans. In the present study we have determined the quenching effect of genistein on the formation of 8-OHdG by UV light irradiation and the Fenton reaction and demonstrate that genistein, as well as its derivative isoflavone biochanin A, potently inhibits UV light-induced 8-OHdG formation.

Materials and methods

Chemicals and reagents

Genistein (5,7,4′-trihydroxyisoflavone), calf thymus DNA, H_2O_2 (30%), $FeCl_2$, dimethyl sulfoxide (DMSO), phenol red, horseradish peroxidase, xanthine, xanthine oxidase and ferricytochrome c were purchased from Sigma Chemical Co. (St Louis, MO). Nuclease P1 and alkaline phosphatase were purchased from Boehringer Mannheim Co. (Indianapolis, IN). Biochanin A was purchased from Research Plus Inc. (Bayonne, NJ).

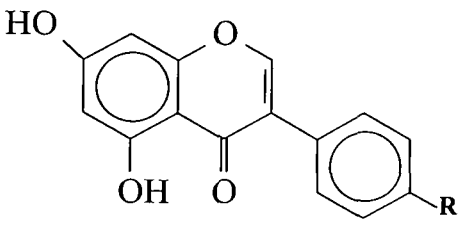
Exposure of DNA to $FeCl_2/H_2O_2$ (Fenton reaction system)

Calf thymus DNA was solubilized in 10 mM Tris–HCl, pH 7.0, to a concentration of ~400 μg/ml. Reaction mixtures (2 ml) containing 400 μg DNA, 25 μM $FeCl_2$, 0.03% H_2O_2 and different concentrations of genistein in DMSO were incubated at 37°C for 30 min by gentle shaking in the dark. Since genistein has poor solubility in aqueous solution, DMSO was used as the vehicle to dissolve genistein in the reaction mixture. The final DMSO concentration was adjusted to 1% for all samples. The reaction was terminated by adding 5 M NaCl to a final concentration of 1 M and then 2 vol. ice-cold ethanol. The DNA was precipitated by being placed at –20°C overnight, followed by centrifugation at 12 000 *g* for 10 min. The DNA pellet was washed with 70% ethanol, dried in air and solubilized in 0.5 ml 10 mM Tris–HCl, pH 7.0, for analysis of 8-OHdG.

Exposure of DNA to UV light

The solution containing 400 μg DNA and different concentrations of genistein or biochanin A in 1% DMSO in a 6-well plastic cell culture flask (3.4 cm diameter) was exposed to UV light from a germicidal lamp 20 cm from the solution surface for 10 min. The UV light contains 85% UV-C (254 nm) and 15% UV-A (365 nm). The radiation energy was ~0.5 J/cm^2, measured with a

*Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; DMSO, dimethyl sulfoxide, dG, deoxyguanosine; ROS, reactive oxygen species.



Genistein R = OH
Biochanin A R = OCH_3

Fig. 1. Structures of genistein and biochanin A.

Model IL 1700 research radiometer/photometer from International Light Inc. (Newburyport, MA). After irradiation DNA was precipitated as described above.

Determination of 8-OHdG and deoxyguanosine (dG)

The amount of 8-OHdG and dG in the DNA was measured using HPLC with electrochemical detection as described by Floyd *et al.* (26), with a slight modification. Briefly, 100 μg DNA in 200 μl 10 mM Tris–HCl, pH 7.0, were incubated with 10 μl 0.5 M sodium acetate, pH 5.1, and 6 U nuclease P1 at 37°C for 30 min, followed by incubation with 80 μl 0.4 M Tris–HCl, pH 7.5, and 7 5 U alkaline phosphatase at 37°C for 1 h. The DNA hydrolysates was filtered through a 0.2 μm syringe filter (Alltech) and 10–100 μl filtrate injected onto a reverse phase C18 HPLC column (5 μm, 250×4.6 mm column; Whatman, Clifton, NJ). The eluent buffer contained 15% methanol, 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NaOH, 10 mM acetic acid, pH 5.1, at a flow rate of 1 ml/min. A model 400 EC detector (EG&G, Princeton Applied Research, NJ) and a UV spectrophotometric detector (Waters, MA) were linked in line with a computer using Millennium 2010 Chromatography Manager for data processing The amounts of 8-OHdG and dG were calculated from the peak area based on their corresponding standards and the results expressed as the number of 8-OHdG/10^5 dG.

Analyses of H_2O_2 and measurement of $O_2^{\bullet-}$ production

The assay for H_2O_2 was conducted using horseradish peroxidase-mediated oxidation of phenol red as previously described (16). The superoxide dismutase-inhibitable reaction of cytochrome c assay was performed to measure $O_2^{\bullet-}$ production by xanthine/xanthine oxidase as described by O'Brien (27), with a slight modification. All experiments were conducted in duplicate in a final volume of 1 ml reaction mixture containing different concentrations of genistein and biochanin A in 1% DMSO, 2.5 mM xanthine and 0.3 mM ferricytochrome c in 50 mM phosphate buffer, pH 7.4. The reaction was initiated by adding 0.1 U xanthine oxidase and $O_2^{\bullet-}$ production was spectrophotometrically quantitated by measuring absorbance alteration at 550 nm.

Results

Formation of 8-OHdG in calf thymus DNA exposed to $FeCl_2$/H_2O_2 and UV light

Under the experimental conditions described in Materials and methods the formation of 8-OHdG in calf thymus DNA exposed to $FeCl_2/H_2O_2$ and UV light was quantitated by HPLC with electrochemical detection. Figure 2 illustrates a typical elution profile for dG with UV detection and 8-OHdG with electrochemical detection. Table I shows that under the experimental conditions specified $FeCl_2/H_2O_2$ (Fenton reaction system) and UV light significantly increase formation of 8-OHdG by 21- and 144-fold respectively over the background level. These two systems provide a suitable model to examine the effects of tested compounds on oxidative DNA damage *in vitro*.

Effect of genistein on 8-OHdG formation by UV light and the Fenton reaction

As shown in Figure 3, genistein inhibits both Fenton reaction- and UV light-induced 8-OHdG formation in a dose-dependent manner. For the purpose of comparison the results are normalized as the relative values (percentage) to controls. The

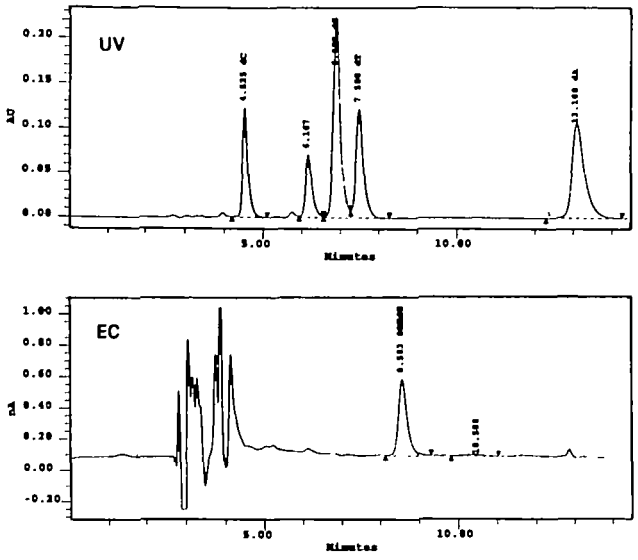


Fig. 2. Representative profile of UV detection (upper) and electrochemical detection (lower). 8-OHdG and dG were quantitated as described in Materials and methods.

quenching effect of genistein on 8-OHdG formation induced by UV light is much more pronounced than that by the Fenton reaction. The IC_{50} of the quenching effect of genistein on 8-OHdG is ~0.25 μM for UV light and 25 μM for the Fenton reaction, accounting for a 100-fold difference. At a concentration of 5 μM >95% of UV light-induced 8-OHdG was inhibited, whereas Fenton reaction-induced 8-OHdG formation was only quenched by ~20%. The different quenching effects of genistein in the two systems suggest that the mechanism by which UV light-induced 8-OHdG may be different from that of the Fenton reaction.

Effect of genistein and biochanin A on H_2O_2 scavenging, $O_2^{\bullet-}$ production, and UV light-induced 8-OHdG formation

We have determined the scavenging effect of genistein and biochanin A on 5 μM H_2O_2 added in the reaction mixture. Genistein and biochanin A are structurally related isoflavones, as shown in Figure 1. The two compounds share the same isoflavone core structures, but genistein has a hydroxy group at position 4′, which is replaced by a methoxy group in biochanin A. Figure 4A shows that genistein potently scavenges H_2O_2, whereas biochanin A exhibits only weak scavenging activity. At a concentration of 50 μM genistein scavenges 71% of H_2O_2, whereas biochanin A scavenges only 12% of H_2O_2.

We further compared the effect of genistein and biochanin A on $O_2^{\bullet-}$ generation by xanthine/xanthine oxidase. Figure 4B shows that genistein inhibits $O_2^{\bullet-}$ formation in a dose-dependent manner and $O_2^{\bullet-}$ formation is completely quenched at 10 μM genistein. In contrast, biochanin A has no inhibitory effect on $O_2^{\bullet-}$ formation. Consistent with scavenging of H_2O_2, only gensitein has an inhibitory effect on $O_2^{\bullet-}$ production by xanthine/xanthine oxidase.

Figure 4C shows that genistein and biochanin A have similar quenching effects on UV light-induced 8-OHdG. Although the quenching effect of biochanin A is slightly weaker than that of genistein at lower concentrations of 0.1–1 μM, the difference narrows to zero as the concentrations increase up to 10 μM. Comparison of the quenching efficiency of genistein on three parameters projects a decreasing order: inhibition of UV light-induced 8-OHdG > quenching of $O_2^{\bullet-}$ production by xanthine/

Table I. Formation of 8-OHdG in calf thymus DNA exposed to $FeCl_2/H_2O_2$ or UV light

Treatment	8-OHdG/10^5 dG	
	Control	Treated
UV light	3.70±0.30 (6)	533.13±39.48 (6)
$FeCl_2/H_2O_2$	4.96±0.29 (4)	103.23±10.22 (4)

Data represent mean ± SD (*n*) of three to four experiments. The amounts of 8-OHdG and dG were determined by HPLC with electrochemical detection and data quantitiated by the Millennium 2010 Chromatography Manager.

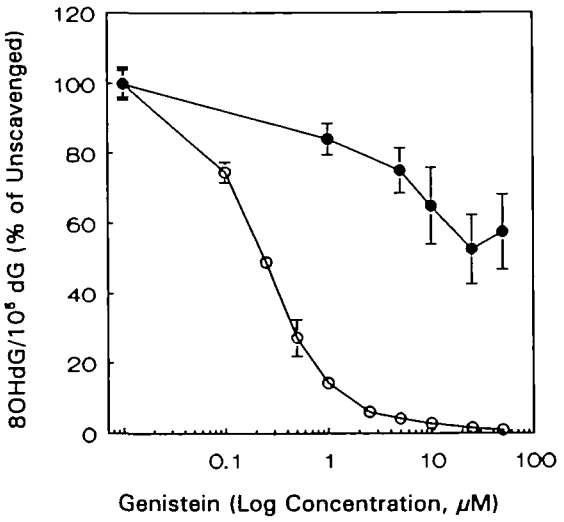


Fig. 3. Effect of genistein on 8-OHdG formation by UV light irradiation and the Fenton reaction. Values represent mean ± SD of three to six samples from three to four experiments. Results are expressed as the percentage 8-OHdG unscavenged by genistein on UV light irradiation (○) and in the Fenton reaction (●).

xanthine oxidase > scavenging of H_2O_2 in the medium. For example, at a concentration of 5 μM genistein 8-OHdG formation is quenched by 96%, $O_2^{\bullet-}$ generation inhibited by 92% and H_2O_2 in the medium scavenged by 22%.

Discussion

Reactive oxygen species (ROS) have been known to damage many biological macromolecules, of which DNA is the most significant target. Oxidative DNA damage is considered to be a good biomarker relevant to carcinogenesis and aging (18,19). One of the most abundant and frequently measured oxidized DNA bases is 8-OHdG, which has been widely implicated in liver (28), kidney (29) and skin carcinogenesis (30,31). Various studies have shown that 8-OHdG can be induced by the Fenton reaction, ionizing irradiation, UV light and methylene plus visible light, implying that 8-OHdG can be produced by either $OH^{\bullet}$ or $^1O_2$ (18,19).

Generally $OH^{\bullet}$ is the most common and deleterious ROS and highly reactive in attacking any macromolecules encountered. Thus in the Fenton reaction system and with ionizing irradiation 8-OHdG is produced through generation of hydroxy radicals (19). In systems containing a photosensitizer, like methylene blue or riboflavin, 8-OHdG is formed via production of $^1O_2$ (32,33). Recently Fisher-Nielsen *et al.* (34,35) reported that exposure of calf thymus DNA and V79 Chinese hamster cells to sunlight (consisting of the unfiltered output of a

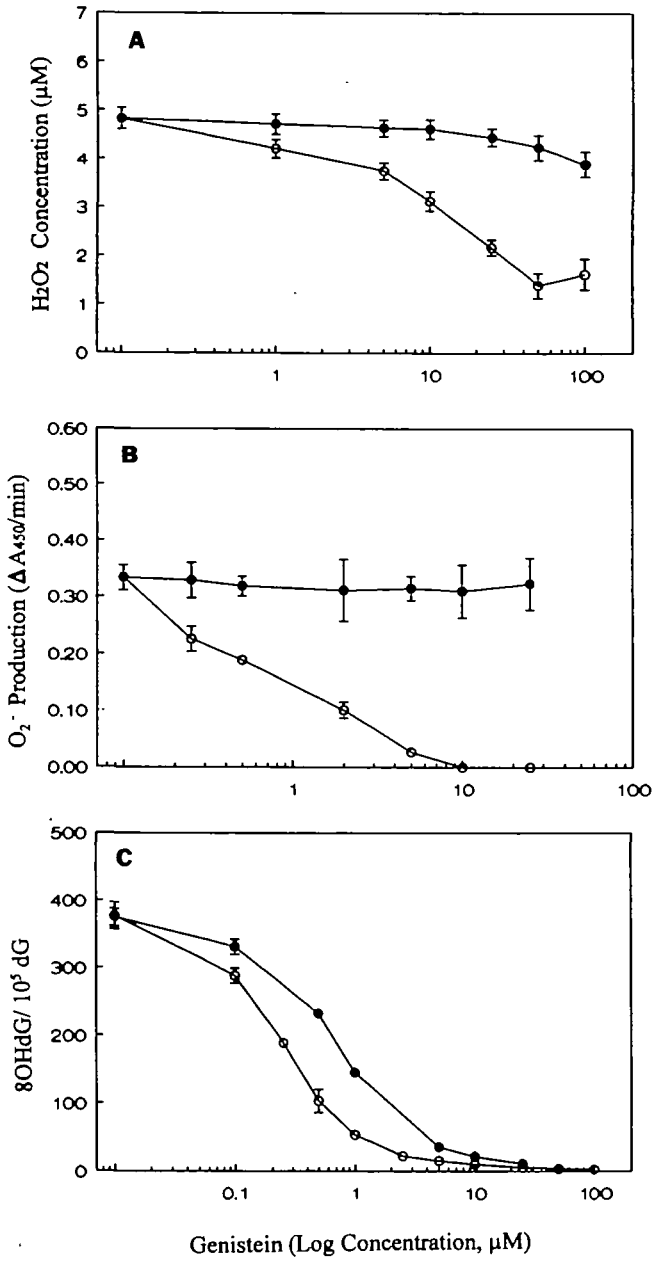


Fig. 4. Effect of genistein and biochanin A on scavenging of H_2O_2 in the medium, $O_2^{\bullet-}$ production by xanthine/xanthine oxidase and UV light-induced 8-OHdG formation. (**A**) Assay for H_2O_2 in the medium was conducted by measuring horseradish peroxidase-mediated oxidation of phenol red. The data are from three experiments with each assay performed in triplicate Results are expressed as the amount of H_2O_2 (μM) in the medium containing genistein (○) and biochanin A (●). (**B**) The assay for $O_2^{\bullet-}$ production was performed by measuring oxidation of ferricytochrome c in a reaction mixture incubated with genistein (○) and biochanin A (●). Data are from three experiments with each assay performed in duplicate and the results expressed as Δ_{450}/min oxidized ferricytochrome c. (**C**) Values represent mean ± SD from two experiments with each assay in duplicate. Results are expressed as 8-OHdG/10^5 dG in calf thymus DNA incubated with genistein (○) and biochanin A (●).

mercury lamp or sun lamp) significantly increases the formation of 8-OHdG in DNA. However, what ROS are responsible for 8-OHdG formation by UV light irradiation remains speculative. In the present study we used a germicidal lamp with 85% UV-C (254 nm) and 15% UV-A (365 nm). Under the experimental conditions specified elsewhere UV light

irradiation significantly increases 8-OHdG in calf thymus DNA.

Compared with the Fenton reaction system, UV light is more potent in inducing 8-OHdG formation (Table I). Although genistein inhibits the formation of 8-OHdG in both systems (Figure 3), the inhibition of UV light-induced 8-OHdG formation by genistein is much more pronounced than that of Fenton reaction-induced 8-OHdG. These results suggest that there is a different mechanism of 8-OHdG formation in the two systems. In a preliminary study 1% DMSO inhibited most 8-OHdG formation by the Fenton reaction and ionizing irradiation, but had little effect on that formed by UV light irradiation (unpublished data). The Fenton reaction is well known to generate $OH^{\bullet}$, which induces 8-OHdG formation in DNA. UV light irradiation appears to be independent of $OH^{\bullet}$, because DMSO does not quench UV light-induced 8-OHdG formation. Recently we generated certain preliminary data to support this hypothesis. The results showed that UV-induced 8-OHdG is largely dependent on singlet oxygen, since deuterium oxide (D_2O) plus oxygen dramatically increased UV-induced 8-OHdG, whilst sodium azide (a potent scavenger of singlet oxygen) almost totally quenched UV-induced 8-OHdG (unpublished data). Fisher-Nielsen *et al.* (34,35) reported that UV light-induced 8-OHdG was inhibited by glutathione, ascorbate and 5-aminosalicylic acid. On a comparable molar basis genistein has a much more potent inhibitory effect on UV light-induced 8-OHdG formation than other classic antioxidants, such as ascorbic acid and glutathione, suggesting that the quenching of UV light-induced 8-OHdG formation by genistein is independent of antioxidant activity.

To confirm the relationship between antioxidant activity and quenching effect on 8-OHdG we compared genistein and its structurally related isoflavone biochanin A. Biochanin A shares the same isoflavone core structure as genistein, but has a methoxyl group at position 4', instead of the hydroxy group in genistein. The results show (Figure 4A and B) that genistein exhibits a significant scavenging effect on H_2O_2 and $O^{\bullet-}$ produced by xanthine/xanthine oxidase, whereas biochanin A has little or no effect. Therefore, the hydroxy group at position 4' is important for isoflavones to maintain antioxidant activity. On the other hand, biochanin A and genistein have similar quenching effects on UV light-induced 8-OHdG formation. Although genistein is slightly more potent in quenching 8-OHdG than biochanin A at low concentrations, both compounds have the same effect when the concentrations increase up to 10 μM. These experiments suggest that the quenching effect on UV light-induced 8-OHdG formation occurs by a mechanism independent of the antioxidant activity of genistein. Therefore, it is our hypothesis that genistein inhibits UV light-induced oxidative DNA damage via either scavenging of $^1O_2$ or binding to a specific site to prevent DNA from oxidative damage.

Yamamoto *et al.* (32) reported that visible light increased 8-OHdG formation in mouse lymphoma cells by 2.5–400 times. The increase in 8-OHdG was positively correlated with the concentration of riboflavin in the incubation medium. The conclusion was made that $^1O_2$ from irradiation of a photosensitizer like riboflavin is involved in formation of 8-OHdG. Floyd *et al.* (33) corroborated this phenomenon in a system consisting of methylene blue plus visible light. In a recent study we demonstrated that DMSO, a scavenger of $OH^{\bullet}$, has little effect on UV light-induced 8-OHdG formation, but denaturation of DNA by boiling or deoxygenation of a DNA solution dramatically decreased 8-OHdG in DNA exposed to UV light (unpublished data). This experiment implies that the formation of 8-OHdG by UV light irradiation is dependent on an alteration of DNA structural integrity in the absence of photosensitizers. A study from Hendry's group has shown that genistein intercalates into the supercoiled structure of DNA (personal communication). The binding of genistein and biochanin A may block attack by ROS generated in the medium or photosensitization by UV light irradiation.

It must be emphasized that there is a concern regarding a filter effect of genistein, rather than a quenching effect on UV-induced 8-OHdG formation. Indeed, genistein may serve as a filter to absorb the UV light energy if its concentration in the incubation buffer is high. In the case of the present study 1 μM genistein quenches ~90% of UV-induced 8-OHdG at a genistein:DNA molar ratio of 6.25×10^{-4} (1600 DNA versus 1 genistein molecules). At such a low concentration the potent quenching of UV-induced 8-OHdG formation by genistein is less likely attributed to a filter effect.

The soybean isoflavone genistein exhibits a variety of antioxidant properties (7–12) and has been implicated in the prevention and therapy of cancer (6). In the current study we present a novel discovery that genistein quenches UV light-induced oxidative DNA damage more potently than conventional ROS scavengers like ascorbic acid and glutathione. The potent inhibition of UV light-induced oxidative DNA damage suggests a potential role of genistein in the prevention of photocarcinogenesis and photoaging of skin.

In summary, we have demonstrated that the soybean isoflavone genistein significantly inhibits formation of 8-OHdG by UV light irradiation and the Fenton reaction. However, the quenching effect of genistein on 8-OHdG formation by UV light irradiation is much more potent than that by the Fenton reaction. Compared with a structurally related isoflavone biochanin A, the quenching effect on UV light-induced 8-OHdG formation appears to be independent of antioxidant activity. It is our hypothesis that the binding of genistein to DNA molecules may exert site-specific protection against 8-OHdG formation by UV light irradation. The quenching effect on UV light-induced oxidative DNA damage by genistein may be implicated in the prevention of photocarcinogenesis in skin.

Acknowledgements

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