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# Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation

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**The use of dietary botanicals is receiving considerable interest in the protection of skin from the adverse biological effects of solar ultraviolet (UV) radiation. Dietary feeding of proanthocyanidins extracted from grape seeds (GSP) (0.2 and 0.5%, w/w) in AIN76 control diet to SKH-1 hairless mice resulted in prevention of photocarcinogenesis in terms of tumor incidence (20–95%), tumor multiplicity (46–95%) and tumor size (29–94%) against UVB-induced complete (both initiation + promotion), initiation and promotion stages of photocarcinogenesis. Feeding of GSP (0.5%, w/w) also resulted in prevention of malignant transformation of UVB-induced papillomas to carcinomas in terms of carcinoma incidence (45%), carcinoma multiplicity (61%) and carcinoma size (75%) compared with non-GSP treated mice following UVB-induced complete carcinogenesis protocol at the end of 30 weeks. Biochemical analysis revealed that treatment of GSP *in vivo* and *in vitro* systems significantly inhibited UVB- or $Fe^{3+}$-induced lipid peroxidation by 57–66% ($P < 0.01$) and 41–77% ($P < 0.05$–$0.001$), respectively, thus suggesting the antioxidant mechanism of photoprotection by GSP. Long-term feeding of GSP did not show apparent signs of toxicity in mice when determined in terms of body weight, diet consumption and physical characteristics of internal body organs like spleen, liver and kidney. Feeding of GSP also did not show apparent signs of toxicity when determined in terms of total body mass (mass of lean + fat), total bone mineral density and total bone mineral content by employing dual-energy X-ray absorptiometry (DXA). DXA analysis also revealed that feeding of GSP significantly decreased tissue fat level (24–27%, $P < 0.05$) without changing the total body mass of the animals compared with non-GSP-fed animals. This can be attributed to increased lipolysis or decreased synthesis of fat due to administration of GSP. Together, it can be suggested that inhibition of photocarcinogenesis by GSP treatment may be associated with the reduction in UVB-induced oxidative damage and tissue fat content.**

**Abbreviations:** DMBA, 7,12-dimethylbenz[*a*]anthracene; DXA, dual-energy X-ray absorptiometry; GSP, grape seed polyphenols; LPO, lipid peroxidation; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TBMD, total bone mineral density; TBMC, total bone mineral content; UV, ultraviolet.

## Introduction

Chronic exposure of solar ultraviolet (UV) radiation to human skin, particularly UVB wavelengths (290–320 nm), is primarily responsible for the incidence of melanoma and non-melanoma skin cancer, which has a tremendous impact on public health and healthcare expenditures. The incidence of skin cancer has been increasing dramatically, and this increase is expected to continue as the population ages and greater amounts of UV radiation reach the surface of the Earth because of depletion of the ozone layer (1–3). Additionally, the increased tendency of individuals to obtain a rapid tan and the use of tanning booths are also implicated in the high risk of melanoma and non-melanoma skin cancers (1–3). It has also been suggested that melanoma is considerably more life threatening than the other skin cancers (1–3). It has been expected that ~1.3 million new cases of basal cell carcinoma and squamous cell carcinoma were diagnosed in 2001 (4) with 1200–1500 deaths in the USA (4–6). Therefore, it is desirable to develop newer and effective chemopreventive agents, which can reverse, inhibit or slow down the incidence of UV-induced melanoma and non-melanoma skin cancer in the human population.

Chemoprevention refers to the prevention of diseases through dietary manipulation or pharmacological intervention. It is important to mention that worldwide interest is considerably increasing on the use of naturally occurring dietary supplements, which can be used as chemopreventive agents and/or as complementary and alternative medicine. Ideally, such chemopreventive agents should be non-toxic in nature. In our continuing efforts to identify newer and effective dietary botanicals, which can inhibit solar UV radiation-induced skin cancers, studies were performed to assess the anti-photocarcinogenic efficacy of proanthocyanidins isolated from grape seeds (hereafter referred to as GSP) using animal skin tumor model. Grapes are widely consumed all over the world and are rich in polyphenolic compounds also called procyanidins or proanthocyanidins (7). Preliminary studies have shown that proanthocyanidins from grape seeds have anti-inflammatory (8), and anti-oxidant properties (9–11). Arii *et al.* (12) have shown that oral administration of 1% grape seed extract in the diet to *Min* mice inhibits APC mutation-associated intestinal adenoma formation. Topical treatment of GSP has been shown to inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated CD-1 mice skin (13). Inhibition of tumor promotion by GSP treatment was also associated with the inhibition of TPA-induced ornithine decarboxylase and myeloperoxidase activities (13), which indicates the anti-tumor promoting properties of GSP. Zhao *et al.* (14) have shown that topical treatment of GSP to SENCAR mice skin significantly inhibited tumor incidence, tumor multiplicity and tumor size in TPA-promoted and DMBA-initiated mouse skin model.

Since the incidence of solar UV radiation-induced skin cancer (photocarcinogenesis) is higher among American people, particularly in Caucasians, compared with chemical carcinogen-initiated skin cancers, we tested the chemopreventive efficacy of GSP on photocarcinogenesis *in vivo* animal model. Moreover, in recent years, the human population is constantly and increasingly using complementary and alternative medicine prepared or developed from naturally occurring dietary botanicals. Therefore, the present study was designed and conducted to determine whether dietary feeding of GSP would prevent UVB radiation induced (i) photocarcinogenesis in terms of tumor incidence, tumor multiplicity and tumor size and (ii) malignant conversion of benign papillomas to carcinomas in SKH-1 hairless mouse model. To accomplish this study, GSP (0.2 and 0.5%, w/w) was given in an AIN76 control diet to mice. In these experiments, the effect of GSP was determined on different stages of photocarcinogenesis, such as, (i) UVB-induced tumor initiation stage, (ii) UVB-induced tumor promotion stage and (iii) UVB-induced complete carcinogenesis stage (included both UVB-induced tumor initiation and promotion protocols). The rationale for the selection of these three different protocols was to dissect out the protective effects of GSP at the different stages of photocarcinogenesis. Additionally, we were also interested in determining whether (iv) the feeding of GSP to animals prevents UV radiation-induced oxidative damage (e.g. lipid peroxidation) and (v) long-term intake of GSP in the diet has any apparent toxic effect in animals. For this purpose, the effect of dietary feeding of GSP was determined on the body weight and *in vivo* body composition like bone free soft-lean tissue mass (LTM), fat mass (FM), total bone mineral content (TBMC) and total bone mineral density (TBMD) in experimental mice and compared with that of non-GSP-fed animals. These parameters were determined by using dual-energy X-ray absorptiometry (DXA) (GE-Lunar PIXImus, software version 1.44, Madison, WI).

## Materials and methods

*Animals and diet*

Six 7-week-old female SKH-1 hairless mice were used in this study and obtained from Charles River Laboratories (Wilmington, MA). These mice were housed five per cage and were acclimatized for at least 1 week before use in the animal facility, and were maintained at standard conditions of 12 h light/12 h dark cycle, 24 ± 2°C temperature and 50 ± 10% relative humidity. Animals were fed an AIN76 control diet (Harlan Teklad, Madison, WI) with or without GSP (0.2 and/or 0.5%, w/w) and water *ad libitum*.

*Chemicals and GSP*

DMBA and TPA were purchased from Sigma Chemical Co. (St Louis, MO). All chemicals employed in this study were of analytical grade and purchased from Sigma Chemical Co. Purified GSP was provided as a gift by Kikkoman Corporation, Japan, for this study. Chemical composition of GSP was analyzed by Kikkoman Corporation and given in Table I.

*Preparation of GSP containing experimental diet*

GSP containing 0.2 and 0.5% experimental diet was prepared by mixing 0.2 or 0.5 g of GSP separately in 99.8 or 99.5 g of AIN76 powdered diet. Ingredients were mixed well for at least 4 h in a rotating pan so that both GSP and AIN76 powdered diet mixed uniformly. After mixing both ingredients, water was added (~1/10th of the diet) and further mixed very well with hands. Usually 100 ml of water in 1 kg of diet is sufficient. After mixing with water, the diet was kept in shallow trays, spread uniformly by hands and made a compact texture. Thereafter, cut into small pieces by a knife and kept at room temperature inside the fume hood to dry and solidify. When it was dried, it was then given to the animals and the rest of the diet was stored at 4°C. Usually, the diet was prepared once a week and changed twice a week when given to the animals.

**Table I.** Chemical composition of grape seed extract or proanthocyanidins from grape seeds (GSP) used in this study

| Components | Percent of total GSP |
|---|---|
| Total proanthocyanidins | 89.3 |
| Dimers[a] | 6.6 |
| Trimers[b] | 5.0 |
| Tetramers | 2.9 |
| Oligomers | 74.8 |
| Total monomeric flavanols | 6.6 |
| (+)-Catechin | 2.5 |
| (−)-Epicatechin | 2.2 |
| (−)-Epigallocatechin | 1.4 |
| (−)-Epigallocatechin-3-gallate | 0.5 |
| Moisture | 2.2 |
| Protein | 1.1 |
| Ash | 0.8 |

[a]Dimers containing procyanidin B1, procyanidin B2, procyanidin B3, procyanidin B4 and procyanidin B5.
[b]Trimers containing procyanidin B5-3′-gallate and procyanidin C1.

*UVB irradiation of mice and UVB light source*

UVB irradiation of mice was performed as described previously (15). Briefly, the dorsal skin area of the mice was exposed to UVB radiation from a band of four FS-20 fluorescent lamps from which short wavelengths of UVB (280–290 nm) and UVC normally not present in natural solar light were filtered out using Kodacel cellulose film (Eastman Kodak Co., Rochester, NY). After filtration with a Kodacel film, the majority of the resulting wavelengths of UV radiation were in UVB (290–320 nm) and UVA range with peak emission at 314 nm as monitored. The UVB emission was monitored with an IL1700 phototherapy radiometer equipped with an IL SED 240 detector fitted with a W side angle quartz diffuser and a SC5 280 filter (all from International Light, Newburyport, MA).

*Photocarcinogenesis experiment and protocol*

One week after their arrival in the animal facility, the mice were divided into different treatment groups with 20 mice in each group. Three long-term tumorigenesis protocols were employed to determine the photoprotective effect of GSP: (i) UVB-induced complete carcinogenesis protocol (included both UVB-induced tumor initiation and promotion stages), (ii) UVB-induced tumor initiation and (iii) UVB-induced tumor promotion as detailed previously (15,16). Briefly, these protocols are summarized below.

*Anti-complete photocarcinogenesis protocol*

For the studies to determine whether dietary feeding of GSP will inhibit UVB-induced complete carcinogenesis, mice in group 1 were given AIN76 control diet while groups 2 and 3 were given GSP at the dose of 0.2 and 0.5% (w/w), respectively, in the AIN76 diet throughout the experimental protocol to determine the dose-dependent effect of GSP on complete photocarcinogenesis protocol. Mice in groups 2 and 3 were given GSP for 14 days, thereafter on day 15 the mice belonging to groups 1, 2 and 3 were irradiated every day with UVB (180 mJ/cm$^2$) and continued for a total of 10 days. Thus, the total UVB-irradiation dose during tumor initiation was 1800 mJ/cm$^2$ fractionated equally in 10 days. One week after the last UVB exposure of initiation, the mice were again UVB irradiated with the same dose (180 mJ/cm$^2$) three times a week for total 30 weeks from the last UVB exposure, which served as the tumor promoter. Tumor yield and size stabilized at 24 weeks, but to determine the effect of GSP on malignant conversion of papillomas to carcinoma, this experiment was continued until week 30. The fourth group of mice received 1% GSP in diet but did not receive any other treatment to determine whether dietary feeding of GSP alone has any effect on tumor formation.

*Anti-initiation protocol*

For studies to determine the effect of GSP on UVB-induced initiation stage of photocarcinogenesis, one group of mice was given the AIN76 control diet and a second group was given GSP (0.5%, w/w) in the AIN76 diet. As 0.5% GSP in diet was more chemopreventive than 0.2% GSP to prevent skin tumorigenesis, in further experiments 0.5% dietary feeding of GSP was used. Fourteen days after GSP feeding regimen, on day 15, mice in both groups were UVB irradiated (180 mJ/cm$^2$) everyday for a total of 10 days. Thus, the total UVB dose of 1800 mJ/cm$^2$ was delivered on the dorsal skin of each mouse in 10 days. One week after the last UVB exposure, animals in both groups were treated

topically with 10 nmol of TPA as a tumor promoter in 100 µl acetone per mouse per application (15). The TPA treatment was given thrice a week up to the end of 24 weeks of the experiment following the last UVB exposure.

*Anti-promotion protocol*

For studies to determine the effect of dietary feeding of GSP on UVB-induced tumor promoting stage of photocarcinogenesis, one group of mice was given the AIN76 control diet, and the second group of mice was given GSP (0.5%, w/w) in the AIN76 diet only during tumor promotion stage of photocarcinogenesis. Before tumor promotion, the mice in the second group (GSP + AIN76) received only the AIN76 diet as in group 1. The mice in this protocol were topically treated with a single application of 200 nmol (51.2 µg) of DMBA (Aldrich Chemical Co., Milwaukee, WI) in 100 µl of acetone per mouse (15). One week after tumor initiation with DMBA, the mice in both groups were UVB irradiated (180 mJ/cm$^2$) three times a week to achieve tumor promotion phase until 24 weeks from the start of the UVB irradiation.

During the experimental protocols, mice were regularly monitored for food and water consumption and any apparent signs of toxicity, such as weight loss or mortality. The body weight of mice in each group was recorded fortnightly throughout the experimental protocol and their dietary intake was also recorded weekly. Skin papillomas or suspected carcinomas were recorded weekly. The tumors >1 mm in diameter that persisted for 2 weeks or more were recorded. Tumor data were recorded until 24 weeks when their yield and size were stabilized. At this time point, the dimensions of all of the tumors on each mouse were recorded. The tumor volumes were calculated by the hemiellipsoid model formula: tumor volume = 1/2 $(4\pi/3) \times (l/2) \times (w/2) \times h$, where $l$ = length, $w$ = width and $h$ = height, as followed earlier (15). The carcinoma incidence and multiplicity were recorded until 30 weeks of the experimental protocol. At this time, carcinoma incidence and multiplicity were stable. The diagnosis of carcinoma was confirmed histologically either at the time when carcinoma bearing mice died or at the termination of the experiment at 30 weeks. Moreover, because of ulcerations and larger size of carcinomas, the Institutional Animal Care and Use Committee (IACUC) at University of Alabama at Birmingham, Birmingham, did not allow these experiments for a longer time, therefore, experiments were stopped at this stage.

*Assay of lipid peroxidation*

To determine whether anti-carcinogenic activity of GSP is mediated through the inhibition of UVB-induced oxidative damage of the skin, lipid peroxidation (LPO) was estimated in the epidermal microsomal fraction of the skin as described previously (17,18). Briefly, LPO was measured in terms of the formation of thiobarbituric acid, and the color intensity was measured at 530 nm on a spectrophotometer. In *in vitro* LPO estimation with GSP and other antioxidants, these agents were dissolved in equal amount of acetone and added to the incubation mixture prior to the start of the incubation. Data were calculated and presented as percent inhibition of lipid peroxidation by the treatment of GSP or other antioxidants.

*Evaluation of non-toxicity of dietary feeding of GSP*

Physico-chemical observations were recorded to determine the non-toxic nature of dietary feeding of GSP to the animals. To evaluate the non-toxic nature of GSP in the diet, we determined weekly consumption of diet and fortnightly body weight per mouse and compared with the control, non-GSP-fed group of mice. At the termination of the photocarcinogenesis experiment at 24 or 30 weeks, mice were killed, internal body organs like spleen, liver and kidney were dissected out and observations were recorded to test the apparent toxicity of GSP, if any, in terms of organ weights and/or lengths.

*Determination of in vivo body composition of mice by DXA*

To assess the non-toxicity of long-term dietary feeding of GSP in mice, we also determined the effect of dietary feeding of GSP on the bone free soft LTM, FM, TBMC and TBMD in experimental mice and compared with non-GSP-fed animals. At the end of photocarcinogenesis experiment at week 30, mice were killed and kept at −80°C until they were analyzed for these parameters. These parameters were determined by using DXA as described previously (19). The head of the mouse was not included in any of these measures, and the DXA analysis was done on thawed animals.

*Statistical analysis*

In tumorigenesis experiments, the statistical significance of difference between UVB alone and GSP + UVB treatment groups in terms of tumor incidence and tumor multiplicity was evaluated by the $\chi^2$ analysis and Wilcoxon rank sum test, respectively. An advantage of Wilcoxon rank sum test is that its validity does not depend on any assumption about the shape of the distribution of tumor multiplicities. Kinetics of tumor multiplicity was analyzed by using Fisher–Irwin exact test. Student's $t$-test was used to determine the statistical significance of difference in lipid peroxidation, tumor volume and fat content data analysis between UVB alone irradiated and GSP + UVB-irradiated groups of animals.

## Results

*Composition of GSP utilized in this study*

Grape seeds are a particularly rich source of proanthocyanidins, and only the procyanidin-type of proanthocyanidins has been detected in the seeds (20). Thus, the proanthocyanidins from grape seeds contain procyanidin oligomers and polymers. Proanthocyanidins are a class of phenolic compounds, which acquire the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (−)-epicatechin (21). The chemical composition of GSP, as analyzed by Kikkoman Corporation, Japan, indicated that it contained 89% total proanthocyanidins, which were present in the form of dimers (6.6%), trimers (5.0%), tetramers (2.9%) and oligomers (74.8%). Monomeric flavanols were 6.6%, as detailed in Table I (7).

*Dietary feeding of GSP prevents photocarcinogenesis*

The chemopreventive effect of dietary feeding of GSP was evaluated in three different protocols of photocarcinogenesis, as described below.

*Prevention against complete photocarcinogenesis*

Dietary feeding of GSP (0.2 and 0.5%, w/w) during complete photocarcinogenesis protocol resulted in a dose-dependent reduction in photocarcinogenesis when expressed in terms of percent of mice with tumors and tumor multiplicity compared with that of non-GSP-fed but UVB-irradiated control animals, as shown in Figure 1 (top row). Feeding of GSP at the dose of 0.2 and 0.5% resulted in 20 (not significant) and 35% ($P < 0.05$) inhibition of tumor incidence (percent of mice with tumors), respectively, at the termination of the experiment at 24 weeks as compared with non-GSP-fed animals (Figure 1, top row, left panel). Non-GSP-fed animals achieved 100% tumor incidence at the 16th week of tumor promotion while GSP-fed animals could not achieve 100% tumor incidence up to the end of the 24th week when tumor incidence was stabilized. Further, feeding of GSP increased the latency period of tumors by 2 weeks during a 24 week long tumor protocol. In complete photocarcinogenesis protocol, a total of 360 tumors (18 ± 2.5 tumors/tumor bearing animal) were recorded in the non-GSP-fed group of 20 animals while 192 (12 ± 2 tumors/tumor bearing animal) and 126 tumors (9.7 ± 2 tumors/tumor bearing animal) were recorded with dietary feeding of 0.2 and 0.5% of GSP, respectively, in the animals. Thus, feeding of 0.2 and 0.5% GSP significantly inhibited tumor multiplicity by 46 ($P < 0.05$) and 65% ($P < 0.005$), respectively, as compared with non-GSP-fed animals, as shown in Figure 1 (top row, right panel) and Table II. Further, the kinetics of tumor multiplicity in GSP-fed (0.5%) animals was significantly prevented ($P < 0.05$, Fisher-Irwin exact test) throughout the experimental protocol compared with the non-GSP-fed control group. As shown in Table II, dietary feeding of 0.2 and 0.5% GSP significantly inhibited tumor size when expressed in terms of total tumor volume per group (66–78%, $P < 0.001$) or tumor volume per tumor bearing mouse by 57–66% ($P < 0.001$). When data were compared in terms of average tumor volume per tumor between GSP-fed and non-GSP-fed groups, a significant reduction was observed by 0.2 and 0.5% GSP (35%, $P < 0.05$) as shown in Table II. Further, a group of animals

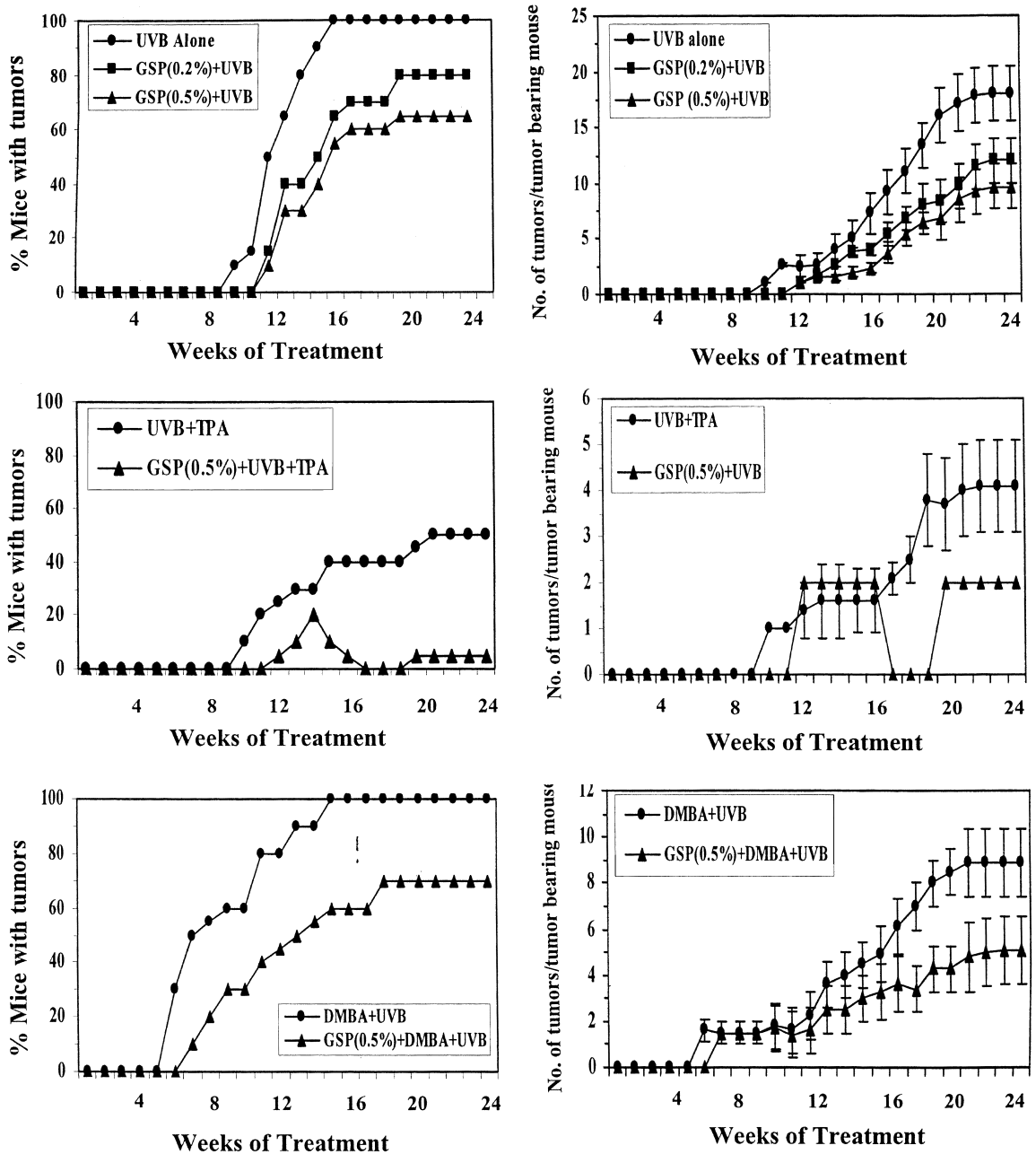


**Fig. 1.** Chemopreventive effects of dietary feeding of GSP (0.2 and/or 0.5%, w/w) in AIN76 diet on UVB-induced complete (top row), tumor initiation (middle row) and tumor promotion (bottom row) in SKH-1 hairless mouse skin. The details of all three experimental protocols are described in Materials and methods section. The percent of mice with tumors (left panel), and number of tumors per tumor bearing mouse (right panel) were plotted as a function of the number of weeks of treatment. Each treatment group contained 20 mice, and the number of tumors per tumor bearing mouse (right panel) has been shown as means ± SD. Tumor yield was stabilized at 24 weeks of tumor promotion treatment.

was subjected to dietary feeding of GSP (1.0%, w/w) alone for the whole experimental protocol to determine whether feeding of GSP had any effect on tumor incidence. At the termination of the experiment at 24 weeks, we were unable to record any tumor appearance on these animals. Thus, these observations indicated that long-term feeding of GSP alone did not induce a carcinogenic effect in laboratory animals.

*Prevention against UVB-induced tumor initiation*

Following antitumor initiation protocol, 50% mice were recorded with tumors on their skin in the non-GSP-fed group at the end of 24 weeks of treatment whereas only 5% of mice developed tumors in the GSP-fed group (Figure 1, middle row, left panel). This indicated that out of the 20 mice in the GSP-fed group, only one mouse developed tumor on its skin. Thus, a highly significant reduction in tumor incidence (95%, $P < 0.0005$) was observed in GSP-fed animals. Latency period of tumors was also found to increase by 2 weeks in GSP-fed animals. At one stage, between 13 and 15 weeks of tumor promotion, 10–20% mice developed tumors in the GSP-fed group, but most of the tumors regressed later probably due to the anticarcinogenic effect of GSP. When data were analyzed

**Table II.** Protective effect of dietary feeding of GSP on the physical characteristics of skin tumors at the termination of the photocarcinogenesis protocols[a]

| Physical characteristics | Photocarcinogenesis protocols[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Complete | | | Initiation | | Promotion | |
| | UVB alone | GSP (0.2%) + UVB | GSP (0.5%) + UVB | UVB + TPA | GSP (0.5%) + UVB + TPA | DMBA + UVB | GSP (0.5%) + DMBA + UVB |
| Number of tumors/group | 360 | 192 (46)[c] | 126 (65)[d] | 41 | 2 (95)[e] | 178 | 71 (60)[d] |
| Tumor volume/group ($mm^3$) | 7302 | 2501 (66)[f] | 1598 (78)[f] | 168 | 9 (94)[e] | 1276 | 337 (74)[f] |
| Tumor volume/tumor bearing mouse ($mm^3$) | 365 ± 44[g] | 156 ± 24 (57)[f] | 123 ± 16 (66)[f] | 17 ± 5 | 9 ± 2 (47)[c] | 64 ± 12 | 24 ± 5 (63)[f] |
| Tumor volume/tumor ($mm^3$) | 20 ± 8 | 13 ± 5 (35)[c] | 13 ± 5 (35)[c] | 4 ± 2 | 4 ± 2 | 7 ± 3 | 5 ± 2 (29)[c] |

[a]Total number of tumors and tumor volume in different treatment groups were recorded at 24 weeks when tumor yield and size were stabilized. The values in parentheses indicate the percent inhibition.
[b]Three different photocarcinogenesis protocols, such as UVB-induced complete, UVB-induced initiation and UVB-induced promotion, were used as described in Materials and methods section. The indicated doses of GSP were given in AIN76 control diet.
[c]Significant versus UVB alone, $P < 0.05$.
[d]Highly significant versus UVB alone, $P < 0.005$.
[e]Highly significant versus UVB alone, $P < 0.0005$.
[f]Highly significant versus UVB alone, $P < 0.001$.
[g]Mean ± SD obtained from 20 animals in each group at the time of data recording.

in terms of multiplicity of tumors or total number of tumors per group at the termination of the experiment, only two tumors (on only one animal) were recorded in the GSP-fed group of animals in comparison with 41 tumors (4.1 ± 1 tumors/tumor bearing animal) in the non-GSP-fed group, as shown in Figure 1 (middle row, right panel). Thus, dietary feeding of GSP inhibited tumor multiplicity by 95% ($P < 0.0005$) at the termination of the experiment. In addition to tumor incidence and multiplicity, dietary feeding of GSP resulted in significant reduction in tumor volume when analyzed in terms of total tumor volume/group (94%, $P < 0.0005$) or tumor volume/tumor bearing mouse (47%, $P < 0.05$), as shown in Table II.

*Prevention against UVB-induced tumor promotion*

Tumor promotion stage is a reversible stage of multistage carcinogenesis, therefore, is most suitable for anticarcinogenic agents to prevent, reverse or slow down the process of carcinogenesis. Following DMBA-initiation and UVB-induced tumor promotion protocol, latency period of tumors was reduced by 3 weeks compared with anti-complete and anti-initiating protocol as shown in Figure 1. The animals in the control group (non-GSP-fed) achieved 100% tumor incidence at 15th week while GSP-fed animals achieved only 60% tumor incidence at this time, and 70% tumor incidence was recorded at the end of this protocol at 24 weeks, as shown in Figure 1 (bottom row, left panel). Thus, dietary feeding of GSP prevented tumor incidence by 30%. When tumor data were analyzed in terms of tumor multiplicity, a total of 178 tumors (8.9 ± 2 tumors/tumor bearing animal) were recorded in the non-GSP-fed group of animals compared with 71 tumors (5.1 ± 2 tumors/tumor bearing animal) in the GSP-fed group of animals. Thus, dietary feeding of GSP resulted in a 60% reduction ($P < 0.005$) in the total number of tumors per group. Additionally, tumor data were also analyzed in terms of tumor size in both treatment groups. As shown in Table II, dietary feeding of GSP resulted in 74% reduction ($P < 0.001$) in terms of total tumor volume per group or 63% reduction ($P < 0.001$) in terms of tumor volume per tumor bearing mouse compared with the non-GSP-fed group of animals. Further feeding of GSP to UVB-irradiated mice also resulted in a 29% reduction ($P < 0.05$) in terms of average tumor volume per tumor compared with non-GSP-fed and UVB-irradiated group of control mice.

*Prevention of malignant conversion of papillomas to carcinomas*

After observing that dietary feeding of GSP significantly prevented UVB radiation-induced skin tumorigenesis in different photocarcinogenesis protocols employed, we were interested to look at the chemopreventive efficacy of GSP on malignant conversion of papillomas into carcinomas. Again, because dietary feeding of 0.5% GSP was more highly chemopreventive than that of 0.2% GSP, we continued these experiments with only 0.5% GSP treatment, and only against complete photocarcinogenesis protocol. This experiment was continued up to 30 weeks of tumor promotion. As shown in Figure 2, histological observations indicated that papillomas had started transforming into carcinomas at 21 weeks of tumor promotion. As evidenced by Figure 2, 70% mice developed carcinoma in non-GSP-fed control mice compared with only 25% in GSP-fed group of mice. Thus, 45% ($P < 0.05$) prevention in terms of carcinoma incidence was observed by dietary feeding of GSP, as shown in Figure 2 (top panel). The weekly progression of carcinoma multiplicity in terms of total number of carcinomas per group and number of carcinomas per carcinoma bearing mouse is shown in the middle and bottom panels, respectively, of Figure 2. When the data were analyzed in terms of carcinoma multiplicity, we found that a total 18 papillomas were converted into carcinomas in non-GSP-fed but UVB-irradiated group of mice in comparison to only seven in GSP + UVB-irradiated group of mice. Histologically, these carcinomas were distinguished as keratoacanthomas and squamous cell carcinomas (data not shown). The data indicated that dietary feeding of GSP resulted in prevention of UVB-induced transformation of benign papillomas to carcinomas by 61% ($P < 0.005$), as shown in Table III. But when the data were analyzed in terms of number of carcinomas per carcinoma bearing mouse, the protective effect of GSP was not evident (Figure 2, bottom panel). Further, dietary feeding of GSP inhibited the growing size of carcinoma. As evidenced by the analysis of physical characteristics of the carcinomas shown in Table III, feeding of GSP to UVB-irradiated mice

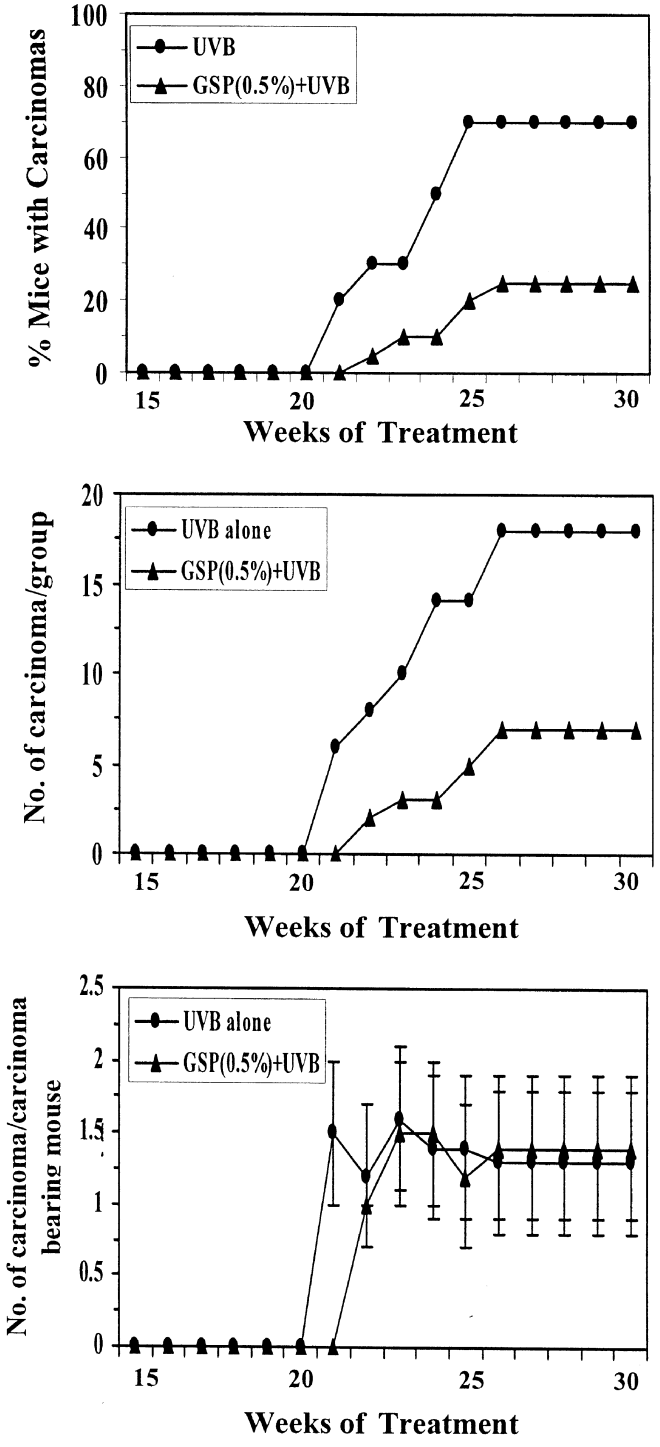


**Fig. 2.** Chemopreventive effects of dietary feeding of GSP (0.5%, w/w) on UVB-induced malignant conversion of papillomas to carcinomas during complete carcinogenesis protocol in SKH-1 hairless mouse skin. The details of experiments are described in Materials and methods section. The percent of mice with carcinoma (top panel), number of carcinomas per group (middle panel), and number of carcinomas per carcinoma bearing mouse (bottom panel) were plotted as a function of number of weeks of treatment. Each treatment group contained 20 mice, and the number of carcinoma/carcinoma bearing mouse has been shown as means ± SD.

resulted in a significant reduction in total carcinoma volume per group and average carcinoma volume per carcinoma by 75 ($P < 0.001$) and 36% ($P < 0.05$), respectively, compared with the UVB alone-irradiated group of mice.

**Table III.** Protective effects of dietary feeding of GSP on physical characteristics of carcinomas in complete photocarcinogenesis protocol in mice[a]

| Physical characteristics | Treatment groups | | % Inhibition |
|---|---|---|---|
| | UVB alone | GSP (0.5%) + UVB | |
| % Mice with carcinoma | 70 | 25 | 45[b] |
| Number of carcinoma/group | 18 | 7 | 61[c] |
| Carcinoma vol/group ($mm^3$) | 8010 | 1996 | 75[d] |
| Carcinoma vol/carcinoma ($mm^3$) | 445 ± 29[e] | 36[b] | |

[a]Total number of carcinoma and carcinoma volume were recorded at the termination of the experiment at 30 weeks when carcinoma yield and size were stabilized.
[b]Significant versus UVB alone, $P < 0.05$.
[c]Significant versus UVB alone, $P < 0.005$.
[d]Highly significant versus UVB alone, $P < 0.001$.
[e]Mean ± SD obtained from 20 animals in each group at the time of data recording.

*Dietary feeding of GSP inhibited UVB-induced LPO*

After evaluating that dietary feeding of GSP inhibited UVB-induced photocarcinogenesis in animals, we were interested to determine whether the anticarcinogenic effect of GSP is mediated through prevention of UVB radiation-induced oxidative damage of the macromolecules like lipids in the skin. Therefore, we determined the effect of GSP (0.5% in the AIN76 diet) on UVB-induced LPO *in vivo* and *in vitro* systems. Exposure of UVB (180 $mJ/cm^2$) to mouse skin resulted in a >3-fold increase in LPO at 24 and 48 h after UVB irradiation when compared with non-UVB exposed mouse skin, as shown in Figure 3 (top panel). Dietary feeding of GSP resulted in 66 and 57% ($P < 0.01$) reduction in UVB-induced epidermal LPO when measured, respectively, at 24 and 48 h after UVB irradiation. Moreover, dietary feeding of GSP alone to mice did not result in epidermal LPO and was found almost equal to non-GSP-fed control mice (Figure 3, top panel).

As LPO is one of the hallmarks of oxidative damage, we further determined the antioxidant potential of GSP using inhibition of LPO as a marker. The experiment was conducted *in vitro* using epidermal microsomes. Treatment of GSP (5–80 mg/ml) *in vitro* to epidermal microsomes resulted in significant inhibition (41–77%, $P < 0.05$–$0.001$) of $Fe^{3+}$-induced LPO in a dose-dependent manner, as shown in Figure 3 (middle panel). Further, we determined and compared the antioxidant potential of GSP with other well-known antioxidant compounds like ascorbic acid, vitamin E, silymarin, BHT and (−)-epigallaocatechin-3-gallate from green tea. Treatment of equal doses (10 μg/ml) of EGCG, ascorbic acid (vitamin C), silymarin, BHT, vitamin E and GSP to epidermal microsomes *in vitro* resulted in the inhibition of $Fe^{3+}$-induced LPO by 44, 58, 44, 67, 44 and 59%, respectively. Treatment of BHT showed maximum inhibition of LPO (67%) while GSP was the next best antioxidant to inhibit LPO in comparison with other known antioxidants, and thus better than vitamin E, silymarin and EGCG in terms of inhibition of LPO when data were analyzed in terms of equal doses (10 μg/ml), but not in terms of equimolar doses, as shown in Figure 3 (bottom panel). The data obtained from LPO experiments suggested that prevention of photocarcinogenesis in mice by GSP treatment could be associated with the prevention of UVB-induced oxidative damage to lipids.

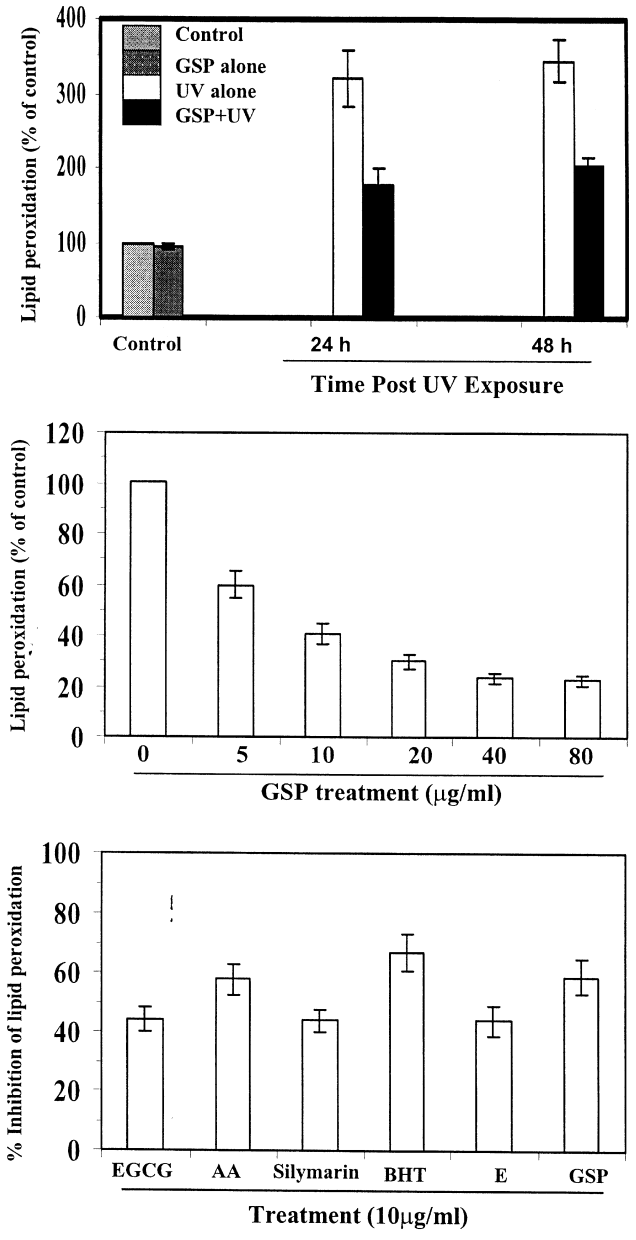


**Fig. 3.** Treatment of GSP inhibits LPO *in vivo* and *in vitro* systems. Dietary feeding of GSP (0.5%, w/w) to SKH-1 hairless mice significantly inhibits UVB-induced LPO by 66 ($P < 0.01$) and 57% ($P < 0.01$), respectively, at 24 and 48 h after UVB exposure (top panel). Treatment of GSP *in vitro* to epidermal microsomes inhibited (41–77%, $P < 0.05$–$0.001$) $Fe^{3+}$-stimulated LPO in a dose-dependent manner (middle panel). Comparative inhibition of $Fe^{3+}$-stimulated LPO by different known antioxidants in epidermal microsomes *in vitro*. Treatment of BHT showed maximum inhibition of LPO (67%) while GSP was the next superior antioxidant to inhibit LPO (59%) among the known antioxidants tested in this experiment. Antioxidant effects were determined by using equal doses of 10 μg/ml each, but not in terms of equimolar concentration (bottom panel). AA = ascorbic acid; E = vitamin E; EGCG = (−)-epigallocatechin-3-gallate; BHT = butylated hydroxytoluene. Each *in vivo* and *in vitro* experiment was repeated at least three times and data represented as mean ± SD. In case of *in vivo* experiment, skin biopsies at least from three mice were pooled together to prepare microsomal fraction of the epidermis, and the experiment was repeated three times.

*Dietary feeding of GSP is non-toxic to the animals*

Further, we were interested to determine whether long-term feeding of GSP has any apparent toxic effect in animals. For this assessment, we determined and compared physico-chemical characteristics of animals between GSP-fed and non-GSP-fed groups of mice. To determine any adverse effect caused by dietary feeding of GSP treatment on body weight loss or extra weight gain as a marker of apparent toxicity, animals in each group, with and without GSP treatment, were weighed fortnightly. We observed that there was no apparent loss in body weights or extra weight gain in those animals, which were given dietary feeding of GSP compared with non-GSP-fed animals over the whole period of the experiment (data not shown). These observations demonstrated that mice treated with GSP (both 0.2 and 0.5%, w/w) did not produce toxicity in them as it would have been manifested by loss in their weight or gain in body weight. Additionally, there was no significant difference in water or total diet consumption per mouse per day among the animals of different treatment groups (data not shown), further suggesting that dietary feeding of GSP has no adverse effect on diet consumption. The data also reflected that experimental animals did not dislike the feeding of GSP in the diet and therefore it did not affect their appetite. Further, we evaluated the adverse physical effect of dietary feeding of GSP, if any, on internal body organs of the animals. At the termination of the experiment at week 30, animals were killed and internal body organs like spleen, liver and kidney were dissected out and their weights or lengths were measured. There was no significant difference in weights or lengths of these internal body organs among GSP-fed and non-GSP-fed groups of animals (data not shown). However, UVB irradiation alone to animals increased spleen length and weight when compared with non-UVB-irradiated animals. It appeared that UVB irradiation did induce a kind of toxicity in the spleens of experimental animals, which resulted in larger size and weight of spleen. Dietary feeding of GSP alone to animals did not affect the size of spleen but at the same time feeding of GSP to UVB-irradiated animals resulted in reduction of UVB-induced increase in weight of the spleens (24–31%, $P < 0.05$) but did not significantly reduce the length of the spleen when compared with non-GSP-fed but UVB-irradiated animals. These observations suggested that long-term feeding of GSP to the animals has no apparent toxic effect on the internal body organs. Similar observations of dietary feeding of GSP (0.5%, w/w) on body weight, and weight and length of internal body organs were also found in mice under anti-initiating and anti-promoting protocols of photocarcinogenesis (data not shown).

*Dietary feeding of GSP has no apparent toxicity to bone mineral density and bone mineral content but significantly reduced fat content in the body*

To further confirm that long-term feeding of GSP has no apparent toxic effect in animals, animals were subjected to assess whether dietary feeding of GSP (0.5%, w/w) has any apparent adverse effect on lean (bone-free tissue mass), FM, TBMD and TBMC compared with non-GSP-fed mice. These parameters were assessed at the end of photocarcinogenesis protocols at week 24 or 30 by using DXA. The data obtained from DXA demonstrated that there was no significant change in TBMD, TBMC, lean and total body mass (mass of lean + fat) in GSP-fed mice compared with non-GSP-fed mice (data not shown), but at the same time feeding of GSP to UVB-irradiated animals resulted in a significant reduction of tissue fat content (27%, $P < 0.05$) which tempted to suggest its possible role, at least in part, in prevention of photocarcinogenesis in animals by GSP treatment. Together, cumulative observations on non-toxicity of GSP in diet to animals

indicated that long-term dietary feeding of GSP has no apparent toxic effect in the animals. Similar observations of dietary feeding of GSP (0.5%) on TBMD, TBMC, lean and fat were also noted in mice under anti-initiating and anti-promoting protocols of photocarcinogenesis (data not shown).

## Discussion

Our experimental data demonstrated that dietary feeding of purified proanthocyanidins from grape seeds to mice prevented photocarcinogenesis in terms of tumor incidence, tumor multiplicity and tumor size (Figure 1 and Table II). This study also reveals that dietary feeding of GSP affords protection against UVB-induced skin tumorigenesis in all the three stages of multi-stage carcinogenesis. It is important to mention that UVB radiation has both initiating and promoting activity as every UV treatment produces both DNA damage and epigenetic events. Therefore, it is not possible to design 'clean' UVB-induced initiation or promotion protocols, however, to study the causes and mechanisms of UV carcinogenesis and to determine the preventive effects of any chemopreventive agents, multi-stage photocarcinogenesis protocols are generally employed (15,16,30,32). It may be possible that putative anti-initiating activity of GSP may be due to removal of the promoting activities from the 'initiating' UV exposures in the anti-initiation experiment. Further, it is worth mentioning that dietary feeding of GSP inhibited UV-induced global DNA hypomethylation in the mouse skin (29). Therefore, inhibition of UV-induced epigenetic events in DNA may be one of the mechanisms of prevention of photocarcinogenesis in animals by GSP. The information on a new chemopreventive agent (proanthocyanidins) is encouraging because it is well documented that chronic exposure of solar UV radiation to human skin is the major etiological agent for the development of melanoma and non-melanoma skin cancers. Several studies have shown that naturally occurring botanical supplements found in herbs, fruits and vegetables, and beverages with substantial antioxidant properties are a promising class of cancer chemopreventive agents (22–25). As skin exposure of UV radiation induces the generation of ROS and exert oxidative damage, the dietary botanical supplements with substantial antioxidant activity should be more effective against the photodamaging effect of UV radiation.

The present study provides additional information on the effectiveness of GSP as an inhibitor of malignant conversion of benign skin papillomas to carcinomas in SKH-1 hairless mice (Figure 2 and Table III). It is well known that transformation of benign papillomas to carcinomas further requires genetic and epigenetic changes in the tumor cells and this can be achieved by using free radical-generating agents (26,27) or genotoxic substances (28). In this photocarcinogenesis protocol, it appeared that chronic exposure of UVB radiation performed these alterations/functions. Although, the exact mechanism of prevention afforded by GSP is not clearly understood by the present study but it appeared that the inhibition of UVB-induced oxidative damage of lipids (Figure 3), and global DNA hypomethylation in the skin by dietary feeding of GSP, at least in part, are the possible determinant factors (29). It has been shown that the increase in the rate of malignant transformation by free radical-generating compounds may be related to free radical-mediated enhancement of genetic instability, ultimately increasing the progression rate of non-malignant lesions (30). Therefore, the results of this study suggest that feeding of GSP might prevent UVB-induced free radical-mediated enhancement of genetic instability by decreasing the total number of carcinomas per group as well as the percentage of mice with carcinomas when compared with that of non-GSP-fed and UVB-irradiated group of mice. Earlier studies suggest that the transformation of benign papillomas to carcinomas requires further genetic changes in papillomas, and it can be achieved by tumor initiating agents (28,31). In the present photocarcinogenesis protocols (complete and tumor promoting), repeated exposure of UVB radiation to papillomas would provide further genetic changes in tumor cells (32), which enhanced the transformation of benign papillomas to carcinomas. Therefore, it can be suggested that dietary feeding of GSP affords protection against genetic and epigenetic alterations caused by UVB radiation. Similar observations were also found when topical treatment of green tea polyphenols inhibited benzoyl peroxide (free radical-generating agent)- and 4-nitroquinoline-*N*-oxide (initiating agent)-induced malignant conversion of chemically induced papillomas into carcinomas (33).

Proanthocyanidins rich extract from grape seeds contains several types of polyphenolic components such as dimers, trimers, tetramers, oligomers and monomeric flavanols like catechins and epicatechin derivatives (Table I) (7). Zhao *et al*. (14) suggested that dimerization and trimerization of monomers (catechins and epicatechins) could lead to anticarcinogenic effects in DMBA-initiated and TPA-promoted skin tumorigenesis possibly due to increase in their antioxidant activity. It has also been shown that dimeric procyanidins are absorbed into the blood stream (34,35), and some of the products of hydrolytes of the higher oligomers and polymers were presumed to be absorbed through the intestinal membrane and then the absorbed procyanidins and/or hydrolytes of procyanidins might display antioxidative activity *in vivo* (36,37). Thus, antioxidative activity of GSP might be responsible for the protection against photocarcinogenesis. Similar to GSP, resveratrol is another antioxidant found mainly in the skin of grapes in small amounts and has been shown to prevent UVB-induced markers of tumor promotion, such as edema, COX-2 expression and LPO, when treated topically on SKH-1 hairless mouse skin (38). In comparison with resveratrol, the availability of GSP in grape seeds is quite high and the molecular structures are complex because GSP are mainly dimers, trimers, tetramers and oligomers in nature (39).

In efforts to determine the photoprotective mechanism of GSP, we found that treatment of GSP *in vivo* as well as *in vitro* systems significantly inhibited UVB-induced or $Fe^{3+}$-induced LPO (Figure 3). Lipid peroxidation in biological membranes is a free radical-mediated event and is regulated by the availability of substrates in the form of polyunsaturated fatty acids, pro-oxidants which promote peroxidation and antioxidant defenses such as α-tocopherol, glutathione, β-carotene and superoxide dismutase (40–42). LPO is highly detrimental to cell membrane structure and function. Elevated level of LPO has been linked to injurious effects such as loss of fluidity, inactivation of membrane enzymes and receptors, increased permeability of ions and, eventually rupture of cell membranes leading to release of cell organelles (40,43). Peroxidation products can also result in damage to crucial biomolecules, including DNA (44,45). Thus, inhibition of UV-induced elevated level of LPO by dietary feeding of GSP would result in reduction of the risk factors associated with UV-induced

ROS-mediated tumor promoting effects of UV radiation in cutaneous inflammatory responses and malignancies.

Further, there has not been a systematic report, which defines the toxicity or non-toxicity of proanthocyanidins *in vivo* in animals, especially when GSP is given in diet for a longer time. In the present study, mice were given GSP in diet for 30 weeks and it was observed that during this time period animals neither lose weight nor gain weight (data not shown), which may have distorted their physical or metabolic activity. Additionally, long-term feeding of GSP did not adversely affect the internal body organs of animals, which is evidenced by determining the physical characteristics, such as weights and lengths of the internal body organs like liver, spleen and kidney (data not shown). Most importantly, long-term dietary feeding of GSP did not affect the overall body mass (lean + fat), bone mineral density and bone mineral content when analyzed by DXA (data not shown). These observations provided ample evidence for the first time that dietary feeding of GSP has no apparent toxic effects in animals.

The most significant observation was that dietary feeding of GSP inhibited UVB-induced tumor incidence and multiplicity concomitant with significant reduction in tissue fat content in mice compared with that of non-GSP-fed but UVB-irradiated mice. Although several dietary modifications are known to inhibit carcinogenesis, and some may decrease body fat levels in rodents (e.g. caloric restriction) (46–49), this is the first study with proanthocyanidins from grape seeds to show a close relationship between the inhibition of photocarcinogenesis and reduction in tissue fat levels. Similar observations were also noted with green tea and black tea on the inhibition of UVB-induced skin carcinogenesis concomitant with reduction in tissue fat (50). The results of the present study suggest that the inhibitory effect of dietary feeding of GSP on photocarcinogenesis may be related—at least in part—to their effects on the reduction in tissue fat levels including the level of fat content in skin layers. This may be attributed to administration of GSP increased lipolysis or decreased the absorption or synthesis of fat without changing body weight. It may be of interest to determine the effects of dietary feeding of GSP on the profile of fatty acids in epidermal phospholipids and in the neutral fat of the parametrial fat pads.

In summary, as we are aware, this is the first study which demonstrates that dietary feeding of proanthocyanidins from grape seeds has the potential to prevent photocarcinogenesis and malignant conversion of papillomas to carcinomas in mice, and this prevention is closely associated with the inhibition of UVB-induced LPO or photo-oxidative damage of lipids, and reduction in tissue fat levels. Additional studies are needed to determine whether chemopreventive effects of GSP on photocarcinogenesis are mediated through: (i) antioxidant mechanism of GSP and/or (ii) their effects on skin fat content, and also whether treatment of GSP decreases the arachidonic acid content or modifies the levels of other fatty acids in dermal or epidermal phospholipids. Decreased levels of arachidonic acid in the epidermis could result in decreased levels of prostaglandins that have been implicated in carcinogenesis.

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