Protective Effect of Dietary *Buchu* (Chinese chives) Against Oxidative Damage from Aging and Ultraviolet Irradiation in ICR Mice Skin

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Abstract

Protective effect of skin by antioxidative dietary *buchu* (Chinese chives, *Allium tuberosum Rottler*), was evaluated in ICR mice fed diets containing 2% or 5% *buchu* for 12 months. Lipid peroxidation and protein oxidation in skin, with or without ultraviolet B (UVB) irradiation, activities of antioxidative enzymes, total glutathione concentrations, and non-soluble collagen contents were measured. Dietary *buchu* decreased significantly in TBARS and protein carbonyl levels in skin compared to the control group, and were lower in those fed 5% than 2% *buchu* diet group. ICR mice exhibited an age-dependent decrease in antioxidative enzyme activities and total glutathione concentrations on the control diet, but in the groups fed *buchu* diet the enzyme activities and glutathione concentrations remained at youthful levels for most of the study. SOD, glutathione peroxidase, and catalase activities as well as total glutathione concentrations increased with time in the skins of the mice fed *buchu* diets. Lipid peroxidation and protein oxidation provoked by UVB irradiation on ICR mice skin homogenates were also significantly inhibited by dietary *buchu*. The *buchu* diets also decreased the formation of non-soluble collagen in mice skin, compared to the control group. These results suggest that antioxidative components and sulfur-compounds in *buchu* may confer protective effect against oxidative stress resulting from aging and exposure to ultraviolet irradiation.

Key words: *buchu*, antiaging, lipid peroxidation, protein oxidation, antioxidative enzyme, UVB irradiation, collagen

INTRODUCTION

Buchu (Chinese chives, *Allium tuberosum Rottler*), a member of the lily family, is a perennial edible plant commonly cultivated in South Korea. The Chinese Medicine Dictionary (1) lists numerous medicinal uses for *buchu* including: tonic, peptic, antidote, and in the treatments of burns, pain, diarrhea, detoxification and asthma. *Buchu* is also an anticoagulant and effective against cellular toxicity in breast, lung, and rectal cancers (2). *Buchu* exhibited a strong antioxidative effect *in vitro* study using linoleic acid emulsion as well as *in vivo* study using rats (3). It has been suggested that the antioxidant and antiaging properties of *buchu* are derived from its β-carotene (4), chlorophyll (5), vitamin C (6), phenols (7), and sulfuric compounds (8).

Skin is considered as an appropriate tissue for studying aging since it is easily accessible, includes various types of cells, and changes due to aging, genetics or environmental factors are easily observed. Skin aging can be di-

vided into two categories by mechanism: one is endogenous aging which is caused by changes in biological factors, and the other is aging caused by ultraviolet light exposure. Skin aging has physiological consequences that go beyond cosmetic appearance, and can result in physical impairments (9,10).

Ultraviolet rays are comprised of UVC (190~290 nm), UVB (290~320 nm) and UVA (EVA I, 340~400 nm, and EVA II, 320~340 nm), depending on the wavelength. Among these spectra, UVB is included within sunlight and is responsible for skin burn, mutagenesis, cell apotosis, skin cancer, etc. Ultraviolet rays from the sun penetrate the epidermal and dermal layers of the skin resulting in genetic degeneration leading to melanoma (11,12), skin cancer (13,14), skin aging and hyper-sensitivity to solar exposure. Currently, the incidence of skin cancer is increasing more rapidly than most other cancers, with the exception of breast cancer in women. UV radiation initiates skin damage through the formation of free radicals which

overwhelm the antioxidant system in skin, leading to the oxidation of proteins, lipids and DNA (15,16). Previous studies have found that antioxidants in the diet may confer protection damage from UV radiation in skin. Huachen et al. (17) demonstrated that a genistein-supplemented diet confers protection against oxidative damage in skin tissue of hairless mice exposed to UVB irradiation. Another study reported that skin cancer incidence was decreased in pheophorbide a-injected mice (18).

In the present study, we investigated whether a long-term *buchu* diet can prevent skin oxidation in the mice. The study also sought to clarify the effect of dietary *buchu* on the formation of collagen, which is the main protein of the skin. Finally, effect of dietary *buchu* in reducing total oxidative stress from ultraviolet radiation was investigated

MATERIALS AND METHODS

Materials

Buchu was obtained from Daedong-Myun, Kimhae City, Kyungnam in South Korea. The *buchu* was freeze-dried, powdered, and stored at -70°C until used.

Animal experiment

Male ICR mice (4 weeks of age, 130 animals), weighing 19.5~21.0 g, were purchased from the Experimental Animal Center in South Korea, and assigned to one of the three dietary groups: control, 2% *buchu* diet, or 5% *buchu* diet. The mice were fed their respective diets for 12 months, after which some animals from each group were sacrificed at 2 months intervals. The diet composition and animals housing conditions were the same as previously reported (3).

Animal sacrifice and sample collection

The animals were anesthesized by CO_2 from dry ice and their fur removed using an electrical shaver. Skin tissue was then removed, washed with 0.9% saline solution, and stored at -70°C.

Preparation of skin homogenate

The skin tissue including epidermis and dermis was cut by iris scissors, diluted with 1.15% KCl solution at 1:10, and homogenized with a Polytron homogenizer. The homogenate was centrifuged at 800 × g for 10 minutes and the supernatant collected for skin tissue homogenate.

Assays of skin lipid peroxides, protein oxidation, antioxidant enzyme activities and total glutathione levels

Lipid peroxide and protein carbonyl levels in skin tissue were assayed using as previously reported (3). Superoxide dismutase (SOD) activity was measured by the

McCord method (19), and glutathione peroxidase (GSH-Px) and catalase activities were also assayed as previously reported (3). Protein concentration in skin homogenate was measured by the Lowry method (20), using bovine serum albumin as the standard. Total glutathione concentration was also measured as previously reported (3).

Insoluble collagen measurement in skin

Insoluble collagen level was determined by the method of Reddy et al. (21). Briefly, skin tissue was dehydrolyzed with acetone, and then defatted in chloroform : methanol (2:1, v/v) solution. After homogenizing in 20% NaCl/0.05 M Tris-HCl (pH 7.5), the homogenate was centrifuged at 10,000 × g for 5 minutes. The pellet was extracted at 4°C for 24 hours, homogenized, and centrifuged again at 10,000 × g for 45 minutes, after which the pellet was extracted again with 0.5 M acetic acid at 4°C for 24 hours. Again, it was centrifuged at 40,000 × g for 60 minutes, yielding a pellet which was insoluble collagen. The insoluble collagen was hydrolyzed with 6 N HCl at 110°C for 24 hours and then concentrated under pressure. The concentrate was neutralized with 6 N KOH and then divided into 2 mL aliquots to which 1.0ml 0.7% chloramin T/15% methylcellosolve/0.5 M citrate buffer (pH 6) was added, and held at room temperature for 20 minutes. One mL of 19% perchlorate was then added and held for 5 minutes at room temperature. Twenty percent ρ -dimethylamino-benzaldehyde / methylcellosolve (1 mL) was added and samples incubated at 60°C for 20 minutes. Absorbance was measured at 560 nm. Trans-4-hydroxy- L-proline was used as the standard to calculate a standard curve for determination of hydroxyproline concentrations.

Ultraviolet irradiation of skin homogenate

Skin homogenate (1 mL) was poured into petridishes and irradiated with UVB (280~320 nm) using an ultraviolet irradiator (National Biologocal Corporation, Ohio, USA) at a rate of 1.0 mW/sec for 20 miniutes.

Statistical analysis

Values are shown as means ± SEM. Significance of differences among treatments were analyzed by one-way ANOVA. Once significance was established, Fisher's least significant difference test was used to evaluate differences between groups at $p < 0.05$.

RESULTS AND DISCUSSION

Skin lipid peroxidation and protein oxidation

Thiobarbituric acid reactive substances (TBARS) were assayed for determination of lipid peroxidation, and carbonyl concentrations were detected for protein oxidation in the ICR mice skins (Fig. 1). TBARS levels in the skins
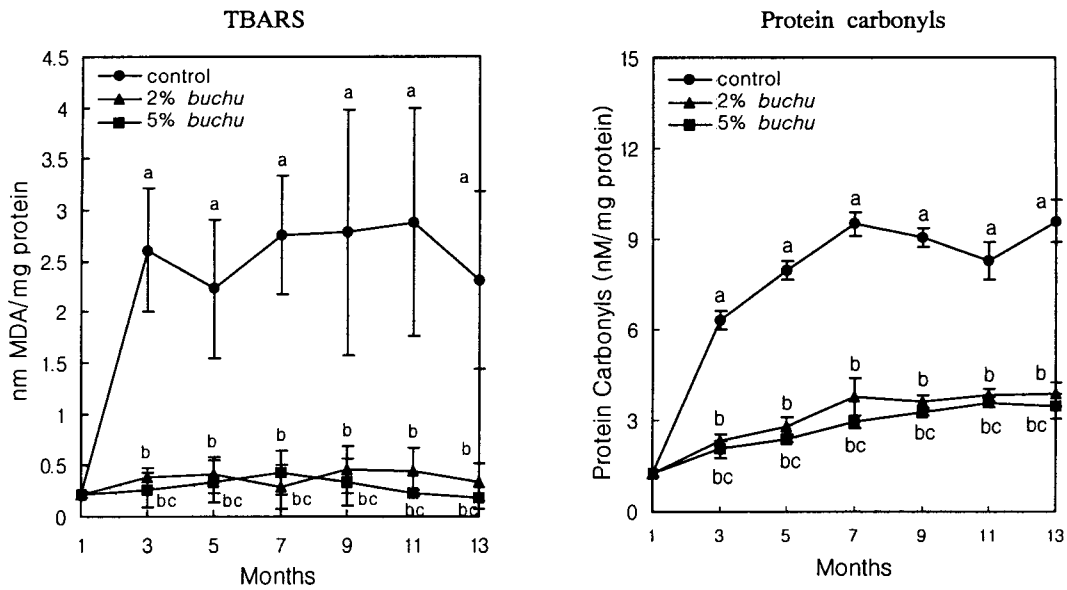
Fig. 1. Age-related TBARS and protein carbonyl concentrations in skin homogenate of mice fed *buchu*.

of 1 month old ICR control mice averaged 0.21 ± 0.03 nM MDA/mg protein, which was half of the level in the liver (0.44 ± 0.18 nM MDA/mg protein). After 3 months, TBARS levels of control mice had increased to 2.61 ± 0.06 nM MDA/mg protein, which remained constant throughout the remainder of the study. In the mice fed *buch* diets, lipid peroxidation increased much more slowly, with TBARS concentrations in 2% and 5% diet groups of 0.37 ± 0.06 and 0.26 ± 0.05 nM MDA/mg protein, respectively; an 80 ~90% decreased rate in the *buchu* groups as compared to the control group. Therefore, adding *buchu* to the diet strongly inhibited the accumulations of lipid peroxidation in the skin of mice. It is believed that sulfur-containing amino acid byproducts such as allyl sulfide, dimethyl disulfide, dimethyl trisulfide, and taurine; and flavonoids, such as linalool, kaempherol in *buchu* deactivate free radicals produced during the age-related processes of primary oxidation. This inhibition may also prevent secondary oxidation of lipids of skin cell membranes. It is also known that lipid peroxides combine with protein, RNA and DNA, thereby compromising the function of mitochondria and inducing biochemical changes in cells which, in turn, lead to increased incidences of disease and accelerated aging. In contrast to the previous study (3), in which long-term *buchu*-diet had no effect on TBARS in liver, in this study lipid peroxidation in aging skin was significantly reduced by the *buchu* diet. Therefore, skin is good model for the study of antioxidative protection against lipid peroxidation.

Protein carbonyl concentrations in control 1 month old ICR mice skins were 1.23 ± 0.01 nM/mg protein, which, like TBARS, were lower than in the liver (1.64 ± 0.02 nM/mg protein). In the control group, skin protein carbonyl concentrations increased rapidly with age. However, protein carbonyl concentration increased much less rapidly in the *buchu* diet group; suggesting that adding *buchu* to the diet might inhibit skin protein oxidation. There was no statistical significance between 2% and 5% *buchu* groups. In ICR mice skins aged 13 months, the 2% and 5% *buchu* groups had protein carbonyl concentrations of 3.86 ± 0.04 and 3.46 ± 0.03 nM/mg protein, respectively, which represented a 60% and 64% decrease compared to the control group. This decrease in protein carbonyl concentrations might be the result of antioxidative activities of phenolic compounds and/or β-carotene. It has been reported that carbonyl measurements are an useful indicator for oxidative degradation of cellular proteins. The cascade of events begins with oxidation of lipids, the products of which react with proteins forming carbonyl compounds. Typically, this protein degradation is associated with hydroperoxide or malondialdehyde production, which are products of lipid oxidation (22). Agarwal and Sohal (23) reported that x-ray oxidation of proteins and DNA increases with aging, and Oliver et al. (15) observed that protein carbonyl concentrations increase in human red blood cells aged from 9 to 75 years.

Antioxidant enzymes activities in skin

The effects of the *buchu* diets on the activities of the antioxidive enzymes: SOD, GSH-Px, and catalase in mice skin, are shown in Fig. 2. Mice skin SOD activity after 1 month in the control group was 26.82 ± 1.58 unit/mg protein, which was one-third of that in the liver (64.76 ± 5.67 unit/mg protein). SOD activities in ICR mice skin aged 5 months from animals on 2% and 5% *buchu*-added diets were 47.72 ± 2.34 unit/mg protein and 66.03 ± 3.27 unit/
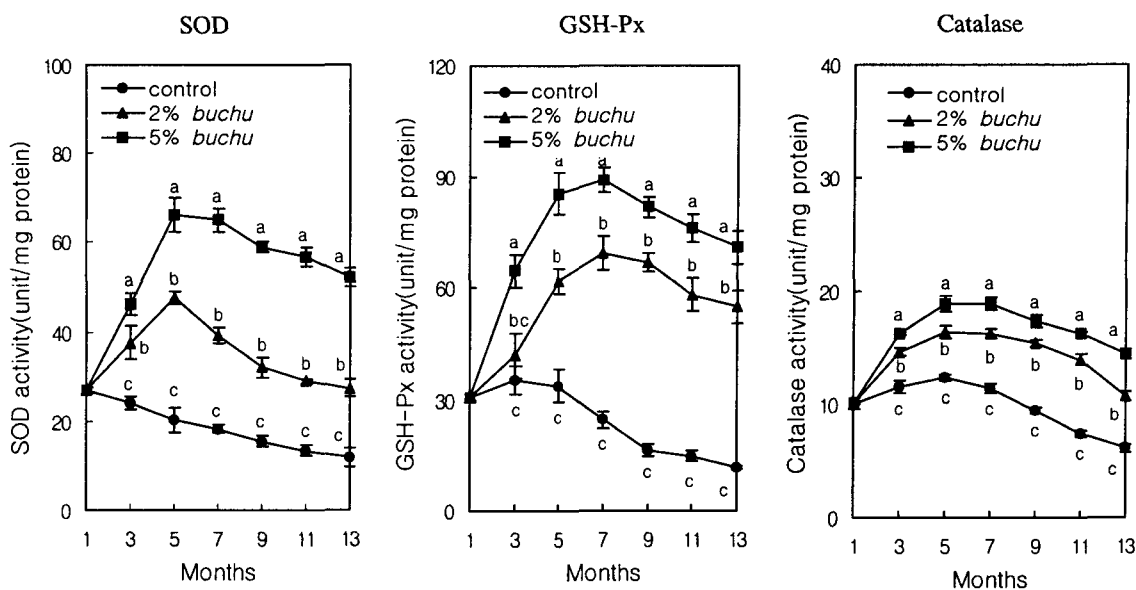
Fig. 2. Age-related SOD, GSH-Px and catalase activities of skin of mice fed *buchu*.

mg protein, respectively, which was the highest for the entire study; activity decreased in older mice. SOD activity in the control group decreased linearly with time. SOD activities in mice aged 13 months in control, 2% and 5% *buchu*-added diet groups were 11.84 ± 1.26, 27.43 ± 1.42, and 52.13 ± 3.99 unit/mg protein, respectively. SOD activity in skin in the 5% *buchu* group was 440% higher, than in the control group. However, liver SOD activities were 85% and 72% lower in the 2% and 5% *buchu* groups than in skin: 27.43 ± 1.42 vs 176.50 ± 10.91 unit/ mg protein and 52.13 ± 3.99 vs 189.10 ± 8.88 unit/mg protein, respectively.

GSH-Px activities in ICR mice skins aged 1 month in all three dietary treatment groups were 30.71 ± 1.25 unit/mg protein, which were almost the same level as in the liver (32.30 ± 2.72 unit/mg protein). GSH-Px activities in ICR mice skins reached a peak at age 7 months in 2% and 5% *buchu* diet with levels of 64.57 ± 3.28 and 85.35 ± 5.64 unit/mg protein, respectively and decreased thereafter. GSH-Px activities in ICR mice skins aged 13 months in the 2% and 5% *buchu* diet groups, and control group were 54.79 ± 4.25, 70.73 ± 4.58, and 11.81 ± 0.30 unit/mg protein, respectively, which were 460% and 600% higher for the treatment groups compared to control, and statistically significant. GSH-Px activities in the skin of the 2% and 5% *buchu* dietary groups were 62% (142.97 ± 9.20 unit/mg protein) and 58% (169.50 ± 3.98 unit/mg protein) lower than the GSH-Px activity in liver. GSH-Px is present in the cytosol where it reacts with hydroperoxide to convert GSH to GSSG. GSH-Px activity is high in liver, lower in heart, lung, and brain and is lower still in muscle (24, 25). The higher GSH-Px activity in the *buchu* dietary

groups may be the result of sulfur-containing compounds in *buchu*, which are required for the biosynthesis of glutathione.

Catalse activities in ICR mice skins aged 1 month were 10.09 ± 0.30 unit/mg protein in all groups, and were similar to levels in liver (13.52 ± 2.70 unit/mg protein). Catalase activities in ICR mice skins reached their peaks at 5 months at 12.37 ± 0.28, 16.37 ± 0.57, and 18.86 ± 0.65 unit/mg protein, respectively, for control and 2% and 5% *buchu* dietary groups; and decreased thereafter, especially in the control group. Catalase activities in ICR mice skins aged 13 months in 2% and 5% *buchu* dietary groups were 10.75 ± 0.40 and 14.42 ± 0.40 unit/mg protein, respectively; which were significantly higher (75% and 134%) than the control group (6.15 ± 0.34 unit/mg protein). Skin catalase activities of 2% and 5% *buchu* dietary groups were 48% (20.54 ± 3.38 unit/mg protein) and 40% (24.07 ± 3.62 unit/mg protein) lower than GSH-Px activity in liver. As shown above, the addition of *buchu* to the mice diets increased antioxidant enzyme activities. Antioxidant enzyme activities in skin showed the same pattern as in the liver; however, skin activities were lower than in the liver.

Glutathione concentration in skin

As shown in Fig. 3, total glutathione concentrations in mice skins aged 1 month in all three dietary treatment groups were 14.89 ± 0.39 μM/mg protein, which were half the level in liver (27.38 ± 2.97 μM/mg protein). Total glutathione concentrations in ICR mice skin in the 2% *buchu* diet group reached a peak at 7 months (23.79 ± 0.81 μM/ mg protein), and in the 5% *buchu* group it peaked months (42.42 ± 0.50 μM/mg protein). After reaching the peaks,
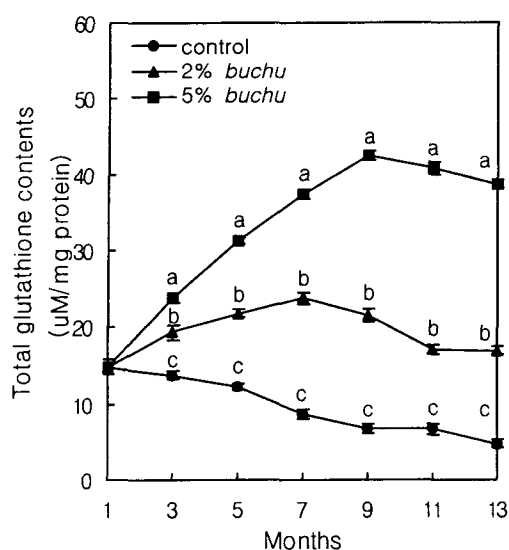
Fig. 3. Age-related total glutathione concentrations in skin of mice fed *buchu*.

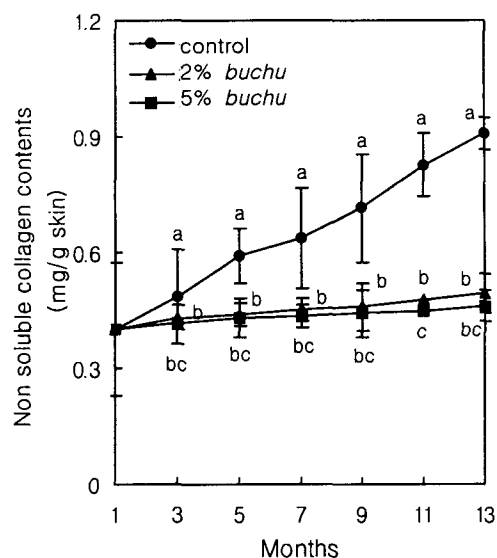
Fig. 4. Age-related non-soluble collagen concentrations in skin of mice fed *buchu*.

total glutathione concentrations decreased with age in all groups, but most rapidly in the control group. Total glutathione concentrations in ICR mice skins aged 13 months in 2% and 5% *buchu* dietary groups were 17.02 ± 0.50 and 38.75 ± 0.85 μM/mg protein, respectively; which were significantly higher (358% and 816%) than the control group (4.75 ± 0.47 μM/mg protein). Skin total glutathione concentrations in the 2% and 5% *buchu* groups were 80% and 68% lower than in liver (84.65 ± 1.20 and 121.18 ± 3.57 μM/mg protein, respectively). It is believed that high total glutathione concentrations in skin tissue of mice fed *buchu* might be due to the high concentrations of sulfur compounds in *buchu*. Antioxidant nutrients, such as vitamin C, can spare glutathione, reducing losses by 50% when 500 mg of vitamin C is supplemented for 2 weeks in healthy non-smoking men and women (26). Glutathione is a tripeptide component composed of glutamate, cysteine and glycine and functions as an antioxidant in combination with other protective ones. Cell degeneration, cell death, and compromised cellular protection against free radical toxicity occur when 20~30% of total glutathione is lost (27). These results suggest that dietary *buchu* contributes substantially to glutathione status, either by supplying precursors for glutathione biosynthesis or by providing other antioxidants which have a sparing effect on glutathione.

Insoluble collagen concentration in skin

Insoluble collagen concentrations in the skins of mice fed the *buchu* diets are shown in Fig. 4. Insoluble collagen concentrations in the skins of mice aged 1 month were 4.02 ± 0.17 mg/g in all groups. Collagen levels were not affected by aging in mice fed the *buchu* diets, however, they were up to 125% higher in the control group. Insoluble collagen concentrations in the ICR mice skins aged

13 months were 4.94 ± 0.05 and 4.61 ± 0.04 mg/g skin in the 2% and 5% groups, respectively, which were significantly lower (46% and 49%) than the control group which was 9.07 ± 0.04 mg/g. Skin consists of about 70~80% collagen. Collagen has a slow turnover rate, but is easily deformed by aging when cross-link bonds are formed (28). Crosslinking during the growth period results in physical and chemical stability in skin. However, with aging, excessive collagen crosslink causes a loss of elasticity and function (29). Kim (30) reported that pyridinoline, which prevents the movement of nutrients and wastes, was decreased by glutathione. As shown in the present study, total glutathione levels are increased dose-dependently with increased dietary *buchu*. It is likely that pyridinoline in *buchu* prevents collagen bridge-formation, which decreases collagen insolubility.

Effect of *buchu*-diet on UVB irradiated skin

The protection of dietary *buchu* against ultraviolet irradiation damage in skin was investigated by assaying markers of lipid and protein oxidation in UVB irradiated mice skin homogenate. As shown in Fig. 5, dietary *buchu* significantly inhibited lipid and protein oxidation. TBARS concentrations from mice at 1 month of age were the same for all dietary treatment groups at 0.39 ± 0.01 nM MDA/mg protein. At 13 months of age, TBARS concentrations in mice skins of the 2% and 5% *buchu* dietary groups remained low at 0.49 ± 0.02 and 0.40 ± 0.02 nM MDA/mg protein, respectively; however the control group experienced a highly significant increase of about 90% (4.73 ± 0.07 nM MDA/mg protein). These results support the hypothesis that antioxidants in *buchu* inhibit lipid peroxidation caused by UVB through scavenging of free radicals.
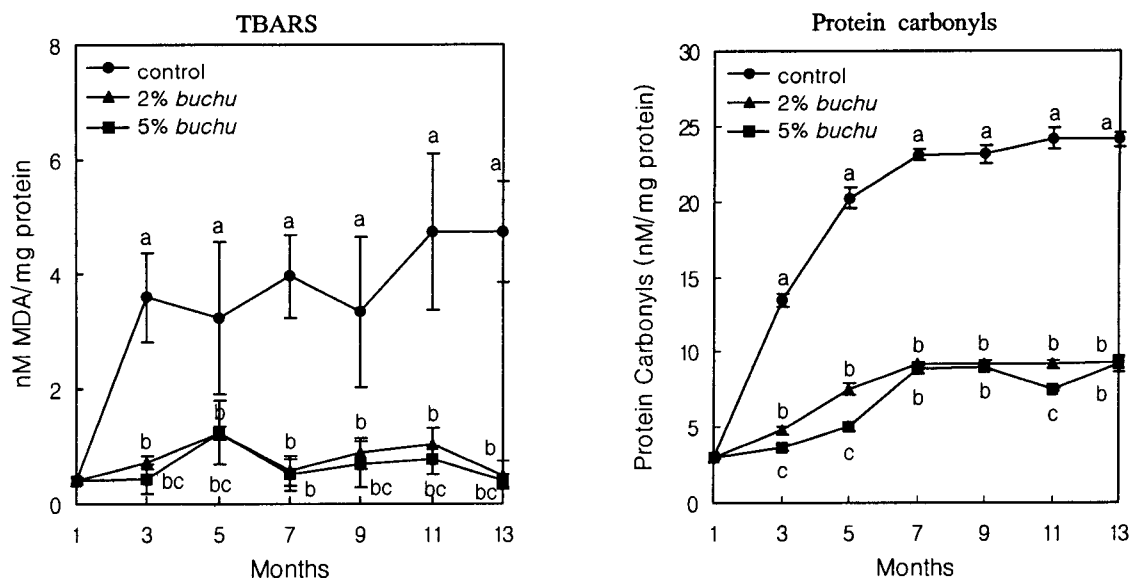
Protein oxidation, as protein carbonyl concentrations, in

Fig. 5. Age-related TBARS and protein carbonyl concentrations in UVB irradiated skin homogenates of mice fed *buchu*.

skin homogenate irradiated by UVB were 2.95 ± 0.01 nM/ mg protein in all three dietary treatment groups in mice aged 1 month. Protein oxidation values at 7 months in the 2% and 5% *buchu* diet groups were 9.18 ± 0.01 and 8.86 ± 0.03 nM/mg protein, respectively, which were significantly lower (60% and 62%) than the control group (23.09 ± 0.04 nM MDA/mg protein). It is well documented that solar UVB radiation increases free radial production and damage leading to skin cancer (31). When skin is irradiated with solar UVB, free radicals are produced, fiber cells are cut, and DNA structural changes resulting from dimer formation, occur. Mathews-Roth reported that β-carotene- or canthazanthin-supplemented diets (3.3%) can decrease the number of skin cancer cells in UVB irradiated hairless mice (32). The present study demonstrated that dietary *buchu* also prevents UVB degeneration of the skin.

CONCLUSION

This study investigated the antioxidative and anti-aging protective effect of dietary *buchu* on skin. *Buchu* was added to the diets of ICR mice over the course of twelve months. Mice were sacrificed at different time points for measurements of markers of oxidative damage in skin. TBARS, protein carbonyl concentrations, antioxidant enzymes activities, total glutathione, and insoluble collagen level were measured. TBARS and protein carbonyl concentrations were significantly lower in mice fed *buchu*, as compared to those on the control diet. SOD activity in skin was decreased in controls, however, it was increased in mice on the *buchu* diet for up to 5 months, after which it declined. The mice with the highest dietary content of *buchu* (5%) also had the highest SOD activity.

GSH-Px activity in skin was decreased in the control group after 3 months. However, it was increased in mice fed the *buchu* diet, up to 7 months, after which it declined. Catalase activity in skin was increased in all three dietary group up to 5 months, after when it decreased. Catalase activity in skin was higher in mice fed the *buchu* diet than in controls. Total glutathione concentrations decreased with age in controls, but increased in mice fed 2% and 5% *buchu* up to 7 months and 9 months, respectively. After that, total glutathione concentrations decreased, but remained higher than in control. Dietary *buchu* also prevented lipid and protein oxidation in UVB-irradiated skin homogenate. Insoluble collagen concentrations were increased in control, but not in mice fed *buchu*. This study suggests that sulfur compounds in *buchu* may prevent skin oxidation resulting from aging or UVB irradiation in ICR mice.

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