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Prevention of the photodamage in the hairless mouse dorsal skin by kojic acid as an iron chelator

Hiroaki Mitani [a], Ichiro Koshiishi [b], Tomomi Sumita [b], Toshio Imanari [b,*]

[a] *Sansho Seiyaku Co., Fukuoka, Japan*

[b] *Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi, Inage, Chiba-shi, Chiba, Japan*

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Abstract

Kojic acid, a fungal metabolic product, has been used as a skin-depigmenting agent in skin care products marketed in Japan. Iron in the skin is known to be involved in wrinkling as a result of chronic photodamage. Kojic acid was expected to have anti-wrinkling activity, since it possesses iron-chelating activity. We now evaluated the anti-wrinkling activity of kojic acid by using hairless mice exposed to chronic solar-simulating ultraviolet (UV) irradiation as model animal. At the end of a 20-week irradiation period, topical application of kojic acid before UV irradiation was observed to dramatically prevent: (1) the wrinkling, (2) hyperplasia of the epidermis, (3) fibrosis of the lower dermis, and (4) the increase of extracellular matrix components in the upper dermis. These findings indicate that kojic acid is a typical agent preventing wrinkling of the skin due to chronic photodamage.

Keywords: Photodamage; Wrinkling; Kojic acid; Iron-chelator; Radical scavenger

1. Introduction

Exposure of the skin to sunlight induces dermal changes, which may greatly alter the aspect of the skin. These changes are also accompanied by an increase in extracellular matrix components in the skin dermis (Margelin et al., 1993; Kligman et al., 1985; Trautinger et al., 1994). We have recently reported that hairless mice, which were exposed to chronic solar-simulating ultraviolet (UV) irradiation, are suitable model animals for evaluation of the photoprotective activity of substances by determination of the contents of the extracellular matrix components (Mitani et al., 1999; Koshiishi et al., 1999b).

UV irradiation-induced formation of reactive oxygen species is thought to be involved in photodamage of the skin (Black, 1987). Species such as single oxygen, superoxide, and hydrogen peroxide have been implicated as important contributors to this damage. Topical and oral antioxidants are modestly protective against skin photodamage (Bissett et al., 1990). Furthermore, in the presence of catalytic amounts of iron, these oxygen species can be converted to highly damaging oxygen radicals such as the hydroxyl radical (Dunford, 1987; Puppo and Halliwell, 1988). Species such as the hydroxyl radical are damaging to a variety of biological materials such as proteins, lipids, and nucleic acids (Braughler et al., 1986; Davies, 1987; Inoue and Kawanishi, 1987). Since the skin has a significant level of iron, which may be available to participate in oxygen radical formation and thus in the photodamage, certain metal chelators should be photoprotective. In hairless mice, topical application of certain iron chelators (e.g., 1,10-phenanthroline, 2-furildioxime) before UV irradiation has been observed to delay dramatically the onset of visible and histologic skin changes induced by long-term sub-erythemal doses of UVB radiation (Bissett and McBride, 1996; Bissett et al., 1991, 1994).

Based on these facts, we have proposed that bifunctional substances, both as antioxidants and as iron chelators, should be effective agents for photoprotection. Kojic acid, 5-hydroxy-2-(hydroxymethyl)-4-pyrone (Fig. 1), is contained in traditional Japanese foods such as soybean paste, soybean sauce, and Japanese wine, by virtue of being continuously produced in these foods by a fungus, *Aspergillus oryzae* (Niwa and Akamatsu, 1991). Furthermore, since kojic acid has a skin-depigmenting activity

* Corresponding author. Tel.: +81-43-290-2894; fax: +81-43-290-2895.

E-mail address: imanar@p.chiba-u.ac.jp (T. Imanari).

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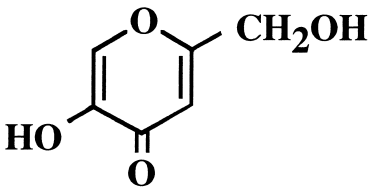

Fig. 1. Structure of kojic acid.

through the inhibition of melanocyte tyrosinase (Mishima et al., 1988), many Japanese cosmetic manufacturers have marketed cosmetic products containing kojic acid. Kojic acid is widely known to be an iron chelator (McBryde and Atkinson, 1961) as well as a radical scavenger (Niwa and Akamatsu, 1991). In the present study, we examined the efficacy of kojic acid against photodamage by using hairless mice exposed to chronic solar-simulating UV irradiation.

2. Materials and methods

2.1. Materials

Standard unsaturated disaccharides, chondroitinase ABC and chondroitinase ACII were obtained from Seikagaku Kogyo (Tokyo, Japan). Mightysil RP-18 GP 100-4.6 (3 μm) from Kanto Chemical (Tokyo, Japan), and Collagenase "Amano" (1000 units/mg, from *Clostridium histolyticum*) was purchased from Amano Pharmaceutical (Nagoya, Japan). All other chemicals were of reagent grade.

2.2. Radiation source and schedules

The solar-simulating UV source was a bank of five FL20 BL B (Toshiba, Japan) black lights (the maximum emission intensity at 352 nm; UV distribution: 300–310 nm, 0.9%; 310–320 nm, 2.0%; 320–420 nm, 97.1%). The distance from the lamps to the mice was approximately 20 cm, and air was circulated by fans. Irradiation was carried out in an air-conditioned room, so the temperature in the cage was maintained at 25–27°C. A dose of 10.8 J/cm^2, obtained with 2 h of irradiation, was given five times weekly (on weekdays) for 20 weeks, yielding a total dose of 1080 J/cm^2. Average UVA irradiance at skin level was 1.50 mW/cm^2 (measured at 365 nm with a UV radiometer [Eisai and Torex, Japan]).

2.3. Animals and treatment groups

Albino hairless female mice (Hos:HR-1), 10-weeks-old, were divided into five groups (group I–V) of eight animals each. To group I (control) and group II, a mixture of polyethyleneglycol-1000 with an equal volume of ethanol (vehicle) was applied to the dorsal trunk in 100 μl aliquots at 90 min prior to irradiation. To groups III, IV and V, 5% kojic acid in vehicle, 5% 1,10-phenanthroline in vehicle, and 5% 2-furildioxime in vehicle were applied in a similar manner, respectively. Groups II, III, IV and V were exposed to UV irradiation. They were housed individually in stainless steel cages in an air-conditioned room and were maintained on a standard laboratory diet (CR-1, Clea Japan, Tokyo, Japan). At the end of the irradiation period, all the animals were killed. Strips (2×1 cm, length × width) were collected from the dorsal skins, and then used for the histological and analytical studies.

2.4. Histology

The strips of dorsal skin, 2×1 cm, were fixed with 10% formalin neutral buffered solution, embedded in polyester wax (Kusakabe et al., 1984), and sectioned at 6 μm. The sections were treated with Masson's Trichrome staining.

2.5. Determination of unsaturated disaccharides produced from hyaluronan and dermatan sulfate by chondroitinase digestion

Unsaturated disaccharides were measured by a sensitive and specific high-performance liquid chromatograpyh (HPLC) with postcolumn derivatization (Koshiishi et al., 1999a). The chromatographic conditions were as follows: column, Mightysil RP-18 GP 100-4.6 (3 μm); eluent, 10% (v/v) acetonitrile containing 1 mM tetra-*n*-butylammonium hydrogen sulfate at a flow rate of 1.0 ml/min; column temperature, 60°C. The eluate was mixed with 0.3 M NaOH solution (0.25 ml/min) and 1% (w/v) 2-cyanoacetamide solution (0.25 ml/min), and the mixture solution was heated at 110°C for 2 min in the reaction tube. The reaction products were detected with a fluorescence detector (Ex., 340 nm; Em., 440 nm).

2.6. Determination of hyaluronan and chondroitin / dermatan sulfate in the tissue sections on glass slides

The digestion of hyaluronan and chondroitin/dermatan sulfate in the tissue sections by chondroitinase ABC and ACII was as follows (Koshiishi et al., 1999a): the polyester wax sections were dewaxed with ethanol and dried well. The tissue sections were photographed with a dark field microscope with a camera for calculating their areas by NIH Image. The sections were rehydrated in a graded series of ethanol and water. A portion of 50 mM Tris–acetic acid buffer solution (pH 8.0) containing chondroitinase ABC (0.5 unit/ml), chondroitinase ACII (0.5 unit/ml), collagenase (500 units/ml, Mandle unit), and ΔDi-UA2S (internal standard) was applied to the sections. The glass slides were put in a moisture chamber at 37°C for 2 h. The enzyme solutions were collected, and a portion was subjected to HPLC.

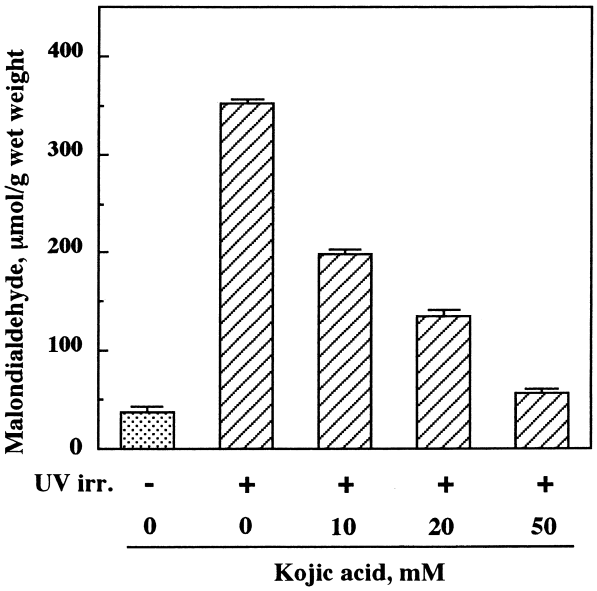

Fig. 2. Effect of kojic acid on lipid peroxidation induced in skin homogenate by UV irradiation. The experimental procedure is described in Materials and methods. Each value represents the mean ± S.D. ($n = 5$).

One hundred skin sections were prepared from each strip of the dorsal skin (5 sections/glass slide). Five sections were randomly chosen and used for quantification of hyaluronan and chondroitin/dermatan sulfates. The non-paired *t*-test was used to analyze the different groups; $P < 0.01$ was designated as significant.

2.7. Evaluation of the iron-chelating activity of kojic acid

Tris–HCl buffer solution (0.1 M, pH 7.4) containing 100 μM ascorbate, 100 μM $FeCl_3$ and 100 μM kojic acid or iron chelators was prepared and immediately incubated at 37°C for 30 min. Ascorbate and resulting dehydroascorbate in the reaction solution were determined by HPLC as follows. Post-column HPLC was used with the following chromatographic conditions (Koshiishi and Imanari, 1997; Koshiishi et al., 1998): column, Asahipak GS-320 7E; eluent, 20 mM acetic acid containing 0.5 mM EDTA (1.0 ml/min). The post-column reaction conditions were as follows: reagent 1, 0.02 M benzamidine solution (0.25 ml/min); reagent 2, 0.75 M borate buffer containing 0.2 M potassium sulfite, pH 10.5 (0.25 ml/min); reaction temperature, 100°C; reaction time, 1 min; detection, fluo-

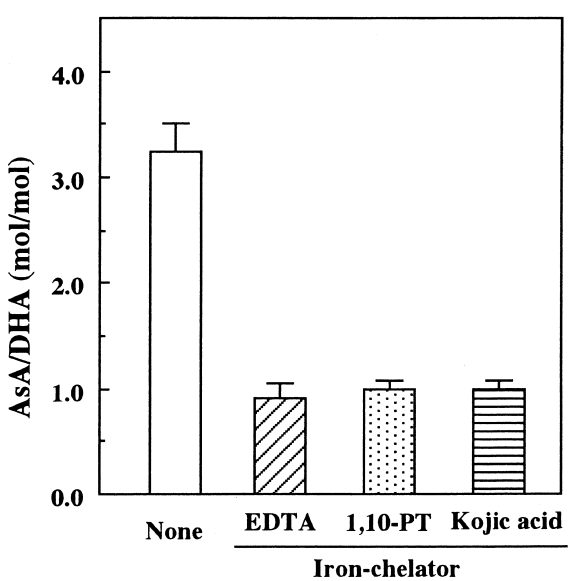

Fig. 3. Evaluation of iron-chelating activity of kojic acid. The experimental procedure is described in Materials and methods. Each value represents the mean ± S.D. ($n = 5$).

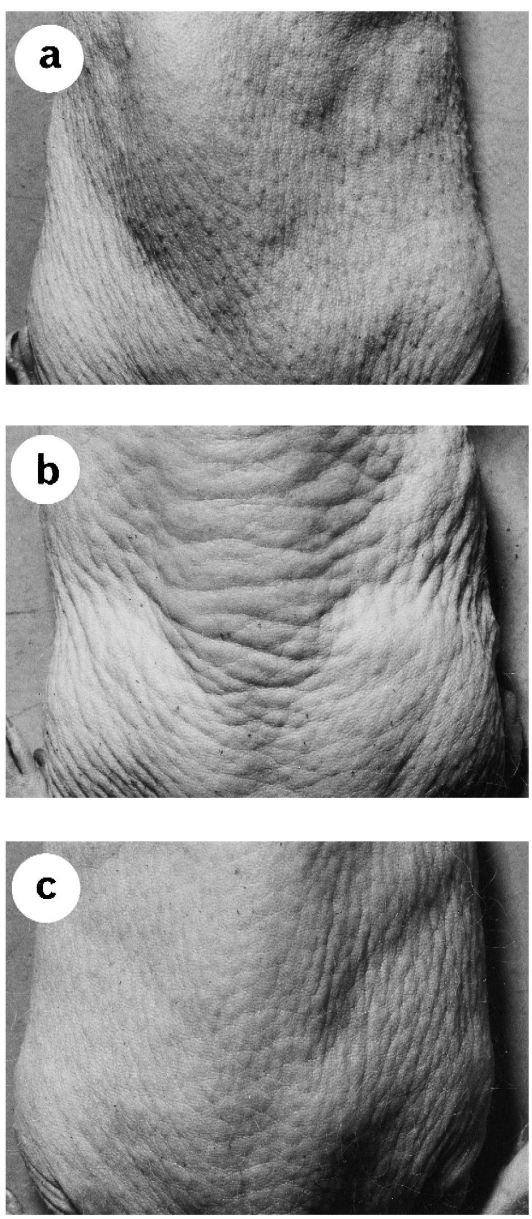

Fig. 4. Features of dorsal skin of hairless mice at the end of the irradiation period. (a) group I (vehicle, UV(−)); (b) group II (vehicle, UV(+)); (c) group III (5% kojic acid in vehicle, UV(+)).

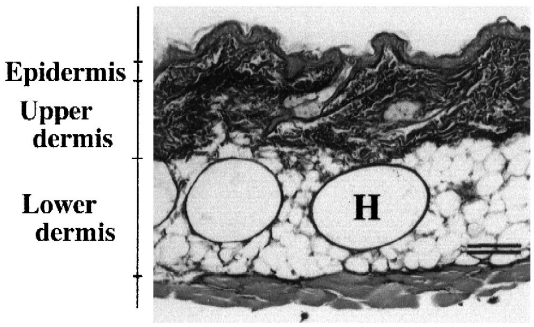

Fig. 5. Histological section of hairless mouse dorsal skin. A characteristic of hairless mouse skin is the presence of horn-containing cysts (H) in the lower dermis, which derive from embryonic hair follicles. Masson's Trichrome staining. Scale bar, 100 μm.

rescence spectrophotometer (Ex. 325 nm, Em. 400 nm). The biological sample solutions were applied directly to HPLC without pretreatment.

2.8. Assay for lipid peroxides in the hairless mouse dorsal skin homogenate exposed to UV irradiation

The hairless mouse dorsal skin homogenate was mixed with an equal volume of kojic acid solution. The mixture was put in a quartz cuvette and exposed to UV irradiation (FL20S-BLB, 5.4 J/cm^2 at 365 nm). After irradiation, 0.2 ml of the sample solution was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate solution, 1.5 ml of 20% acetate buffer (pH 3.5), and 1.5 ml of 0.8% thiobarbituric acid solution, successively. The mixture was made up to 4.0 ml with distilled water, and then heated at 95°C for 20 min. After cooling, 1.0 ml of distilled water and 5.0 ml of the mixture solution of *n*-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was removed and its absorbance at 532 nm was measured (Ohkawa et al., 1979). 1,1,3,3-Tetraethoxypropane was used as a standard, and the level of lipid peroxide was expressed as nmol of malondialdehyde.

3. Results

Kojic acid has radical scavenging activity and iron chelating activity. When the homogenate of the hairless mouse dorsal skin was exposed to UV irradiation in the presence of kojic acid, lipid peroxidation was significantly suppressed with a dependence on the kojic acid content (Fig. 2). Furthermore, to estimate its availability for the iron chelation under physiological conditions, the effect of kojic acid on the oxidation of ascorbate by the ferric ion was investigated. The effects of EDTA, 1,10-phenanthroline and kojic acid on the oxidation of ascorbate are shown in Fig. 3. EDTA and 1,10-phenanthroline are well known to be iron chelators (Bissett et al., 1991). Kojic acid facilitated ascorbate oxidation by the ferric ion, indicating that kojic acid as well as EDTA and 1,10-phenanthroline act as iron chelators under physiological conditions.

The features of the dorsal skin of hairless mice in groups I [vehicle, UV(−)], II [vehicle, UV(+)] and III [kojic acid, UV(+)] are shown in Fig. 4. At the end of the 20-week irradiation period, none of the mice developed any tumors, but all the mice in group II exhibited wrinkling. The application of kojic acid prevented wrinkling in mice irradiated with UV (group III). 1,10-Phenanthroline and 2-furildioxime also prevented wrinkling (data not shown).

Skin dermis is not of uniform construction, but consists of the upper dermis and the lower dermis (Fig. 5). The quantitative alterations of hyaluronan and chondroitin/dermatan sulfate contents in the lower dermis and the upper dermis of dorsal skin of the UV irradiated hairless mice, to which iron chelators were applied, are shown in Table 1. The values represent the content in a unit of skin area (ng/mm^2). As compared to the control (group I), chondroitin/dermatan sulfate contents both in the lower dermis and in the upper dermis of the UV irradiated hairless mouse skin (group II) were markedly higher. Kojic acid as well as 1,10-phenanthroline and 2-furildioxime strongly suppressed the increases of chondroitin/dermatan sulfate content both in the lower and the upper dermis.

Table 1
Effects of iron chelators on the glycosaminoglycan contents in hairless mouse dorsal skin exposed to chronic UV irradiation

Group	Application	UV irradiation	Upper dermis		Lower dermis	
			HA (ng/mm^2)[a]	C/DSs (ng/mm^2)[a]	HA (ng/mm^2)[a]	C/DSs (ng/mm^2)[a]
I ($N = 6$)	Vehicle	−	115 ± 30	81 ± 8	164 ± 19 (59%)	62 ± 4 (43%)
II ($N = 6$)	Vehicle	+	132 ± 31	170 ± 22[b]	230 ± 32[b] (64%)	176 ± 36[b] (51%)
III ($N = 6$)	Kojic acid	+	126 ± 15	113 ± 6[c]	158 ± 20[c] (56%)	92 ± 11[c] (45%)
IV ($N = 6$)	1,10-Phenanthroline	+	121 ± 21	104 ± 12[c]	173 ± 21[c] (59%)	87 ± 8[c] (45%)
V ($N = 6$)	2-Furildioxime	+	96 ± 19	111 ± 9[c]	137 ± 23[c] (59%)	83 ± 20[c] (43%)

Skin sections were separated into upper and lower dermis with a small surgical knife. Analytical values represent the means ± S.D. ($N = 6$). The values in parentheses represent the percentage of each glycosaminoglycan in lower dermis versus whole dermis.

[a] Amounts of glycosaminoglycans per unit area.

[b] $P < 0.01$ versus group I.

[c] $P < 0.01$ versus group II.

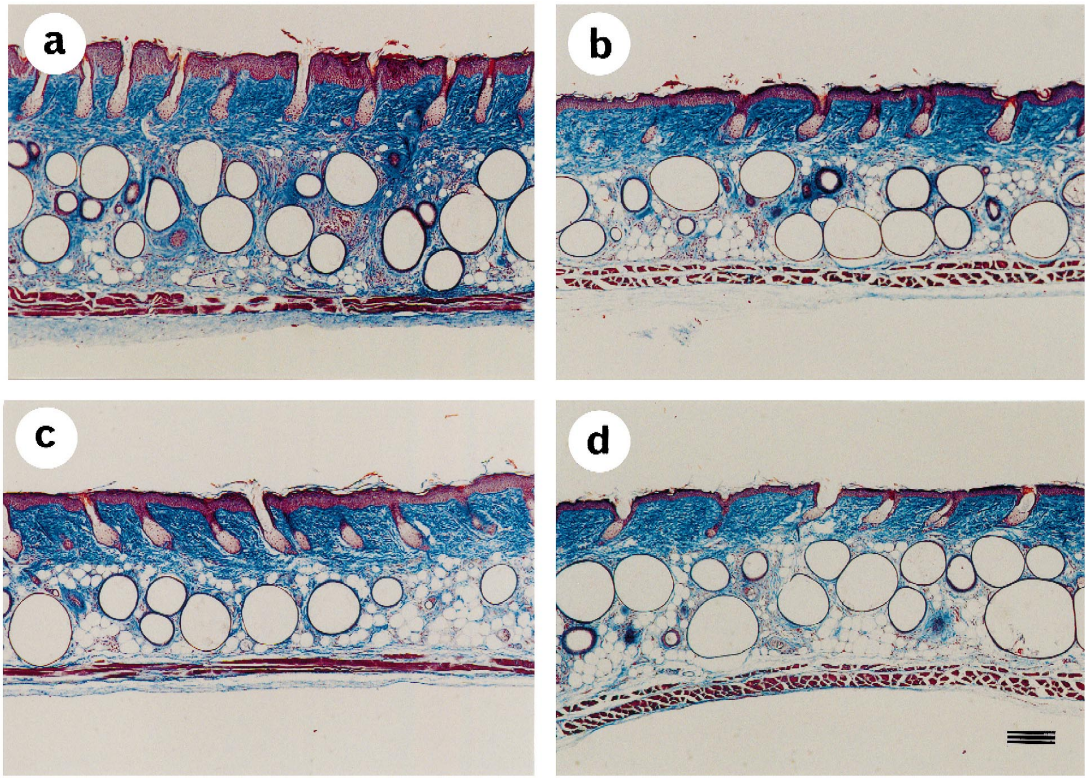

Fig. 6. Hairless mouse dorsal skin exposed to chronic UV irradiation. (a) group II (vehicle, UV(+)); (b) group III (5% kojic acid in vehicle, UV(+)); (c) group IV (1,10-phenanthroline in vehicle, UV(+)); (d) group V (2-furildioxime in vehicle, UV(+)). Masson's Trichrome staining. Scale bar, 100 μm.

At the end of the irradiation period, the skin sections were treated with Masson's Trichrome staining (Fig. 6). Hyperplasia of epidermis and the disappearance of adipocytes, and a concomitant accumulation of collagen fiber in the lower dermis (fibrosis) were observed as compared with the control animals. The application of kojic acid as well as of 1,10-phenanthroline and 2-furildioxime suppressed these characteristic alterations in the dorsal skin exposed to the chronic UV irradiation.

4. Discussion

Chronic solar-simulating UV irradiation to the hairless mouse dorsal skin induced (1) wrinkling, (2) hyperplasia of the epidermis, (3) conversion of the adipose tissue into fibrous tissue in the lower dermis, and (4) an increase of chondroitin/dermatan sulfate content in the upper dermis. Sclerosis of the skin resulted from the fibrosis of the lower dermis and the accumulation of matrix components in the upper dermis appeared to be associated with the wrinkling. These results indicate that the hairless mice exposed to chronic solar-simulating UV irradiation are suitable model animals for photodamage. The quantitative alterations of the extracellular matrix components in the skin dermis could be used as markers for the photodamage. We recently described quantitative alterations of collagen (as the content of hydroxyproline), hyaluronan, and chondroitin/dermatan sulfates in each part of the dermis, which are associated with the photodamage induced by chronic UV irradiation (Mitani et al., 1999; Koshiishi et al., 1999b). Skin dermis is not of uniform construction, but consists of the upper dermis and the lower dermis. The predominant cells in both of these parts are fibroblasts and adipocytes, respectively. Biological evaluation of photodamage should be done individually in each part. The skin dermal extracellular matrix consists of collagen (type I and III), hyaluronan and chondroitin/dermatan sulfate as major components. Among them, chondroitin/dermatan sulfate appeared to be a possible marker for the tissue fibrosis, which is a characteristic of the photodamage (Mitani et al., 1999; Koshiishi et al., 1999b).

Kojic acid has both radical scavenging activity (Niwa and Akamatsu, 1991) and iron chelating activity (McBryde and Atkinson, 1961). Reactive oxygen species are thought to be associated with the wrinkling due to photodamages of the skin induced by exposure to UV irradiation (Black, 1987). Especially the hydroxyl radical is very damaging to a variety of biological substances. Exposure of the homogenate of the hairless mouse dorsal skin to solar-simulating UV irradiation facilitated lipid peroxidation through the formation of the hydroxyl radical. The availability of kojic acid as a radical scavenger should contribute to the suppression of lipid peroxidation in the skin exposed to the UV irradiation. So far, some investigators have evaluated the anti-wrinkling activity of radical scavengers (Bissett et

al., 1990). Based on these investigations, it appears that some radical scavengers are mildly protective against the skin photodamage, albeit not markedly. The efficacious amount of the radical scavenger for complete scavenging of the free radicals should be more than that of free radicals produced. On the contrary, in the presence of a catalytic amount of iron, the reactive oxygen species can be converted to highly damaging oxygen radicals such as the hydroxyl radical. Even though the content of iron in the skin is not significantly high but yet catalytic, iron still plays a role in photodamage. Therefore, iron chelators are effective agent for protecting against the photodamage including wrinkling.

In hairless mice, topical application of certain iron chelators including 1,10-phenanthroline and 2-furildioxime before UV irradiation has been observed to dramatically delay the visible and histological skin changes induced by chronic UVB irradiation (Bissett and McBride, 1996; Bissett et al., 1991, 1994). In the present study, the efficacy of kojic acid was evaluated by comparing it with that of these iron-chelators as positive controls. Our data indicate a strong photoprotective effect of kojic acid, which is comparable to that of typical iron chelators including 1,10-phenanthroline and 2-furildioxime in mice. Because the human skin contains iron, with higher levels present in sun-exposed human skin, there is a possibility of a role of iron in the photodamage and of iron chelators in photoprotection.

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