Fructose-1,6-diphosphate : The new anti-aging material.

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Summary

Fructose-1, 6-diphosphate (FDP), a glycolytic metabolite is reported to ameliorate inflammation and inhibit the nitric oxide production in murine macrophages stimulated with endotoxin. It is also reported that FDP has cytoprotective effects against hypoxia or ischemia/reperfusion injury in brain and heart. In this study, we examined whether FDP has protective effects on UV-induced oxidative damage in skin cell culture system and human skin *in vivo*.

FDP had a protective role in UVB-induced LDH release and ROS accumulation in HaCaT although it did not show direct radical scavenging effect in the experiment using 1,1-diphenyl-2-picrylhydrazyl (DPPH). FDP also preserved cellular GSH content after UV irradiation in HaCaT and normal human fibroblast culture system.

Cellular oxidative stress induces multiple downstream signaling pathways that regulate expression of multiple gene including MMP-1 and collagen, we examined the effects of FDP on UV-induced alteration of these protein expression in fibroblast culture and human skin *in vivo*. The increased MMP-1 expression in fibroblast and human skin by UV irradiation was significantly decreased by FDP. FDP also prevented the UV-induced decrease of collagen expression in fibroblast and human skin. Moreover, the decreasing the intracellular levels of reducing equivalents in human fibroblast by glutathione (GSH) depletion lowered the UVA dose threshold for reduction of procollagen expression, indicating that the differences of glutathione contents define the susceptibility of fibroblasts towards UV-induced reduction of procollagen expression. FDP also preserved cellular GSH content after UV irradiation, indicating that FDP has protective effects on UV-induced reduction of procollagen expression, which are possibly through maintaining intracellular reducing equivalent.

Based on these premises, we examined the effect of daily use of a moisturizer containing FDP on facial wrinkle in comparison with vehicle moisturizer lacking FDP. In the clinical study, FDP significantly decreased facial wrinkle compared with vehicle alone after 6 months of use.

Our results suggest that FDP has anti-aging effects in skin by increasing cellular antioxidant system and preventing oxidative signal and inflammatory reaction.

Therefore, FDP may be useful anti-aging agent for cosmetic purpose.

Introduction

Aging of skin is a complex biological phenomenon consisting of two components; intrinsic aging and photoaging caused by environmental exposure, primarily UV light. However, decreased metabolic function and accumulated oxidative damage induced by ROS (reactive oxygen species) are responsible for cutaneous inflammatory disorder and skin aging [1,2,3]. It is well known that UV irradiation induces the formation of ROS in cutaneous tissue.

Fructose-1, 6-diphosphate (FDP), a glycoltic metabolite is reported to have cytoprotective effects against ischemia and postischemic reperfusion injury of brain and heart, presumably by augmenting anaerobic carbohydrate metabolism [4,5,6]. And also, it has been shown to mitigate the adverse effect of endotoxin by regulating the generation of nitric oxide [7]. In addition, it has been demonstrated that FDP completely inhibits generation of oxygen free radicals by stimulated neutrophils [8]. It is well documented that UV irradiation increases oxidative stress in irradiated tissue and oxidant components play an important role in the signaling events leading to gene activation after UV irradiation [9]. Recently, we showed that FDP reduced UVB-induced increase in cellular ROS level although it did not show direct radical scavenging effect in the experiment using 1,1-diphenyl-2-picrylhydrazyl (DPPH). And FDP could attenuate UVB-induced COX-2 expression, thereby reducing the prostaglandin production in HaCaT keratinocytes [10]. On these premises, we examined whether FDP could protect UV-induced cell damage and prevent UV-induced alteration of dermal matrix in fibroblast and human skin *in vivo*. FDP might be expected to benefit photodamaged skin, including the classical signs of fine wrinkles and roughness, we tested the efficacy and safety of FDP 0.5% cream in the facial skin for 6 months.

Materials and Methods

Cell cultures

HaCaT keratinocytes and normal human fibroblast (NHF) were grown in DMEM medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum and 1% antibiotic and antimycotic solution. For experiments, cells were maintained in DMEM supplemented with 1% fetal bovine serum (FBS) for indicating time.

In vitro ultraviolet irradiation

UVB irradiation was provided by a bank of Sankyo Denki G15T8E, a fluorescent bulb emitting 280~320 nm wave with a peak at 313 nm. UVB irradiation was delivered with the dose of 30 mJ/cm^2 and the irradiance was monitored with an IL1700 radiometer (International Light Inc., Newburyport, MA, USA). UVA irradiation was provide by high-intensity UVA source (Dermlight cube 401 equipped with UVA filters) emitting wavelengths of the 320-400 nm range at a distance of 40 cm. The amount of UVA administered was assessed with UVA meter (UVATEC, Inc.Sherman Oaks, CA). Before UV exposure the cells were washed twice in phosphate-buffered saline (PBS), and the cells were covered with PBS during UV irradiation. After the UV exposure, fresh culture medium was added and the cells and media were harvested at the indicated time points for further experiments.

Determination of LDH release

UV irradiation induced-cytotoxicity was determined by the lactate dehydrogenase (LDH) leakage assay (Sigma Co.). Cell was irradiated in 96-well plates with 30 mJ/cm^2 UVB for HaCaT and 15 J/cm^2 UVA for NHF. After exposure, the PBS solution was removed and replaced with medium and

the cells were kept in the incubator for 24 h. LDH release into the medium was assayed using the Cytotoxic 96 kit (Promega Corp., Madison, WI, USA), according to the manufacturer's directions.

MMP-1 and procollagen determination

UV irradiation induced-MMP-1 and procollagen expression were determined by western blotting in the conditioned medium. Procollagen levels in the conditioned media were determined with a monoclonal antibody type I amino terminal extension peptide (SP1.D8) antibody (developmental Studies Hybridoma Bank). Anti-MMP-1 antibody was purched from Oncogene.

ROS determination

Cells grown on a glass-bottom dish were loaded with 5 μM 2,7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) in PBS. Cultures were incubated for 30 min at 37°C, washed three times with PBS, and the fluorescence signal of DCFH (Ex = 490 nm; Em = 510 nm), the oxidation product of DCFH-DA by free radicals, was analyzed on the stage of a Nikon Diaphot inverted microscope equipped with 1 100W Xenon lamp. To minimize background signal caused by direct oxidation of DCFH-DA by illumination at 490 nm, intracellular levels of ROS were analyzed within 3 sec after illumination using a Quanticell 700 system (Applied Imaging).

Glutathione (GSH) measurement

Total glutathione (GSH plus GSSG) was determined by photometric determination of 5-thio-2-nitrobenzoate, formed from 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at a 405 nm, according to Akerboom and Sies [11] (1981). The assay mixtures were 18 mM Tris-HCl buffer (pH 7.4) contained 0.47 mg ml^{-1} BSA, 0.007% Tween 20, 0.7 mM glucose 6-phosphate, 21.5mM NADP, 1.4 U ml^{-1} yeast glucose 6-phosphate dehydrogenase, 0.61 mM DTNB, 0.26 U ml^{-1} glutathione reductase and 25 μl cell lysates.

Determination of catalase and superoxide dismutase (SOD) activities

Catalase activity was measured according to a modified method of Beer *et al.*[12]. The assay mixtures contained 0.01M phosphate buffer , pH 7.0 and 0.015 M hydrogen peroxide in a final volume of 1ml and 50 μg protein of cell lysates. Changes in the optical density at 240 nm were spectrophotoetrically monitored. Catalase activity was expressed as units mg^{-1} protein. One unit of enzyme activity was defined as the amount of the enzyme which decreases one micromole of the hydrogen peroxide per min under defined conditions. SOD activity was measured using the xanthine/xanthine oxidase and cytochrome c reduction assay described by McCord and Fridovich [13]. The assay mixtures contained 5 μmol xanthine in 0.001N NaOH, 2 μmol cytochrome c in 50mM phosphate buffer, pH 7.8 and 0.1mM EDTA, 0.2U xanthine oxidase in 0.1mM EDTA and 20 μl of cell lysates. SOD activity was expressed as units mg^{-1} protein. One unit of enzyme activity was defined as the amount of the enzyme which reduces 50% of cytochrome c reduction by superoxide induced by xanthine/xanthine oxidase system under defined conditions.

UV irradiation and skin sample

Korean adults, volunteers without current or prior skin disease, were studied in this report. A Waldmann UV-800 (Waldmann, Villingen-Schwenningen, Germany) phototherapy device, including F75/85W/UV21 fluorescent sunlamps, served as the UV source, having an emission spectrum between 275 and 380 nm (peak at 310±315 nm). Irradiation at the skin surface was measured with a Waldmann UV meter (Model 585100; Waldmann). The total irradiation 30 cm from the light source was 1.0 mW per cm2. The distribution of power output was 0.5% UVC (below 280 nm), 56.7% UVB (280±320 nm), and 42.8% UVA (320±400 nm). The skin of the buttocks was irradiated with unfiltered UV and the dose that caused minimal erythema (MED) was determined 24 h after irradiation. Usually the MED measured with unfiltered UV was around 70±90 mJ per cm2 for the brown skin of Koreans. Fructose 1,6-diphosphate 1% and its vehicle (70% distilled water, 30%

propylene glycol) were applied to skin under occlusion for 24h before UV treatment. The phototypes of Koreans include types III, IV, and V. Irradiated and nonirradiated buttock skin samples were obtained from each subject by punch biopsy. The Institutional Review Board at the Seoul National University Hospital approved this study, and all subjects gave written informed consent.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The total RNA was isolated from the whole punch-biopsied skin samples using a Trizol reagent (Gibco BRL, Gaithersburg, MD) following the manufacturer's instruction. RT-PCR was performed using One Step RNA PCR kit (Takara, Shiga, Japan). PCR primers were produced by custom oligonucleotide synthesis service (Bioneer, Korea). Target gene mRNA levels were quantified based on standard and normalized to GAPDH (control) mRNA level.

Clinical study of moisturizer containing FDP on facial wrinkle.

A total 50 healthy female (25 in the 0.5% FDP treatment group and 25 in the vehicle group) was studied. Patients were asked to apply the cream to the entire face twice a day for 6 months. A silicone replica of the crow's feet area was taken at baseline and at weeks 24. Skin replicas were then analyzed by Skin visiometer SV 600. Standard wrinkle and roughness were calculated on these profiles, as follows- R1 (Skin roughness), R2 (average roughness), R3 (maximum roughness) R4 (smoothness depth)- R5 (arithmetic average roughness)- the features were then calculated and statistically analyzed [14].

Statistical analysis

Statistical analysis was performed with Student's t-test. A P value of 0.05 was selected as the limit of statistical significance.

Results and discussion

FDP attenuates UV-induced cell cytotoxicity.

HaCaT keratinocytes exposed to UVB irradiation dose of 30 mJ $cm^{2\ -1}$ showed a significant increase of LDH release into the medium (245±9.28% of non irradiated control), and FDP reduced the accumulated levels of LDH in the media in a dose dependent manner when added to the culture media immediately after irradiation (Fig. 1). But Fructose-1-phosphate, Fructose-6-phosphate, Fructose-2,6-diphosphate did not reduce the UV-induced LDH release into the media (data not shown).

Theses data showed that only Fructose-1,6-diposphate(FDP) has protective effect of UV-induced cell damage.

FDP attenuates accumulation of ROS and preserves cellular total glutathione level and catalase activity after UVB irradiation.

In order to explore the relevant cellular events that may be involved in the cytoprotective effects by FDP, intracellular levels of reactive oxygen species, [ROS]i, were analyzed using DCFH-DA, a redox sensitive dye. The time course for the levels of ROS in HaCaT keratinocytes after UVB irradiation showed that increase of cellular ROS level was observed from 1 h post irradiation and maximum level was reached in 3 h (Fig. 2a). UVB-induced ROS accumulation was attenuated to control level by FDP (10 mM) at all time points of measurement (Fig 2a). The increase in [ROS]i was significantly reduced in the presence of FDP in a dose dependent manner measured at 3 h after irradiation. (Fig. 2b), although FDP did not show direct radical scavenging effect in the experiment using 1,1-diphenyl-2-picrylhydrazil (DPPH) (data not shown). On the other hand, FDP preserved the cellular antioxidant capacity such as catalase and glutathione that were significantly reduced after UVB irradiation (Fig. 3). UVB-irradiation reduced catalase activity to 86±4% of control, which was reversed by FDP. Cellular glutathione content was also reduced to 83±7% of control

after irradiation, and FDP restored it to 101±6%. The baseline levels of catalase and glutathione were significantly increased in HaCaT cells treated with FDP (125±4%, $P<0.05$ vs. control; 138±11%, $P<0.05$ vs. control, respectively). FDP failed to alter the cellular levels of superoxide dismutase (SOD) whether or not cells were irradiated

In this study it was observed that FDP significantly reduced the generation of ROS after UVB irradiation and this was not due to direct radical scavenging activity. It has been suggested that FDP could be a substrate for or stimulator of anaerobic ATP production [15]. It was also shown that FDP might upregulate pentose phosphate pathway (PPP), possibly by inhibiting phosphofructokinase [16,17,18]. It is well documented that pentose phosphate pathway plays an important role in the cellular redox regulation, by providing NADPH which is the principal intracellular reductant and a critical modulator of the redox potential in all cell types [19]. NADPH is used for the regeneration of glutathione and also required for the formation of active catalase tetramers [20]. Our data showed that FDP preserved cellular catalase activity and glutathione level (Fig 3) and increased the activity of glucose-6-phosphate dehydrogenase, the rate-limiting enzyme of PPP, in keratinocytes exposed to UVB (data not shown). Thus, it is highly plausible that FDP reduces ROS generation via activation of PPP.

FDP attenuates UV-induced MMP-1 expression and procollagen depression via cellular GSH preservation.

Ultraviolet (UV) irradiation from the sun increases hydrogen peroxides and other reactive oxygen specie (ROS), and that may be a major contributor to premature skin aging, referred to as photoaging [21]. And antioxidants are also decreased in photoaged and chronologically aged human skin [22].

GSH is a major endogenous antioxidant, the levels of which decline during aging in many tissues and also skin [23]. First of all, we investigated whether the GSH depletion may influence alteration

of collagen, the major structural component of skin, induced by UV irradiation in fibroblasts. GSH in fibroblast was depleted by addition of L-buthionine- [S, R]-sulfoxinine (BSO), a γ-glutamylcysteine synthetase inhibitor, to the culture medium in a dose dependent manner (Fig.4). GSH depression by BSO for 48h led to a slight decrease of procollagen production in fibroblast (Fig.5). And fibroblast exposed to UVA irradiation dose of 5 J cm^{2-1} failed to reduce the procollagen reduction. However, GSH depletion markedly enhanced UV-induced procollagen reduction compared with the levels induced by UVA alone. The results demonstrate the GSH depletion increased the susceptibility of fibroblast to oxidative stress associated collagen alteration.

It has been suggested that FDP directly affects GSH concentrations in hypoxic neurons and that preservation of the anti-oxidant environment may play a critical role in neuroprotection [24]. In previous studies FDP preserved UV-induced GSH reduction in HaCaT keratinocytes, for that reason we tested the effect of FDP on UVA-induced GSH reduction in fibroblast. Fibroblast exposed to UVA irradiation dose of 15J cm^{2-1} showed a significant decrease of GSH. The reduction in GSH induced by UVA was markedly prevented by FDP (Fig.6).

Cellular glutathione level preservation may prevent UV-induced collagen deprivation, then we investigated FDP effects on UV-induced alteration of procollagen and MMP-1, which is the major metalloproteinase degrade the collagen results skin collagen deficiency. Fibroblast exposed to UVA irradiation dose of 15J cm^{2-1} showed a significant decrease of procollagen expression and increase of MMP-1 expression detected by immunoblotting. The increase in MMP-1 expression stimulated by UVA was markedly reduced by FDP (Fig.7). The antioxidant, NAC (20 mM) suppressed UVA-induced MMP-1 expression significantly. FDP also prevented UV-induced procollagen decrease in fibroblast (Fig.7).

These data suggest that the depletion of GSH renders the fibroblast more susceptible to external stress, such as UV irradiation. And UV-induced GSH depletion may contribute to alteration of matrix protein expression. So, functional compensation of GSH depletion by FDP may play a role in

preventing UV-induced matrix protein alteration.

FDP attenuates UV-induced MMP-1 expression and procollagen depression in human skin *in vivo*.

UV irradiation induces the synthesis of matrix metalloproteinases (MMP) in human skin and MMP-mediated collagen destruction accounts, in large part, for the connective tissue damage that occurs in photoaging [25]. In order to whether FDP has protective role in UV-induced photoaging *in vivo*, we investigated the topically applied FDP effects on alteration of UV-induced MMP-1 and procollagen expression in human buttock skin. FDP 1% and its vehicle (70% distilled water, 30% propylene glycol) were applied to skin under occlusion for 24h before UV treatment. And then skin were irradiated with 2 MED of UV. Irradiated and nonirradiated buttock skin samples were obtained from each subject by 48h after irradiation (n=4).

UV-irradiation increased the MMP-1 expression and decreased the procollagen expression in skin, and topically applied FDP prevented this alterations (Fig. 8) although FDP did not absorb the UV range using this experiment(Data not shown). These data suggest that topically applied FDP may have protective role for UV-induced skin aging and could be useful for antiaging material.

FDP shows beneficial effects for improving the signs of skin aging.

Topically applied FDP prevented UV-induced MMP-mediated collagen destruction, therefore we investigated 6 months pilot study to determine the efficacy and safety of FDP on facial wrinkle in comparison with vehicle moisturizer lacking FDP. A total 50 healthy female (25 in the 0.5% FDP treatment group and 25 in the vehicle group) was studied. Volunteers were asked to apply the cream to the entire face twice a day for 6 months. A silicone replica of the crow's feet area was taken at baseline and at weeks 24. Skin replicas were then analyzed by Skin visiometer SV 600. Standard wrinkle and roughness features were then calculated and statistically analyzed [12].

FDP caused significant decrements of all parameters (R1-R5) compared with baseline. But vehicle treatment group, only 3 parameters (R1, R2, R3) decreased significantly (Fig. 9a). And FDP treatment group compared to vehicle treatment at 6 months, 3 parameters (R1, R3, R5) were significantly decreased although the baseline was not different between the two groups (Fig. 9b). No subjects dropped out of the study because of adverse events. These data suggest that FDP significantly decreased facial winkle compared with vehicle alone after 6 months of use.

Conclusion

FDP has a protective role in photoaging by decreasing MMP-1 expression and preservation of procollagne expression, which are possibly through maintaining cellular reducing equivalent. And topically applied FDP also has the effects of reducing the facial wrinkles and roughness, which are the feature of photoaged skin. Therefore, we proposed that the preservation of intracellular reducing state is the target for anti-aging strategy and FDP has anti-aging effects in skin by preservation of reducing equivalents and inhibition of UV-induced gene expression.

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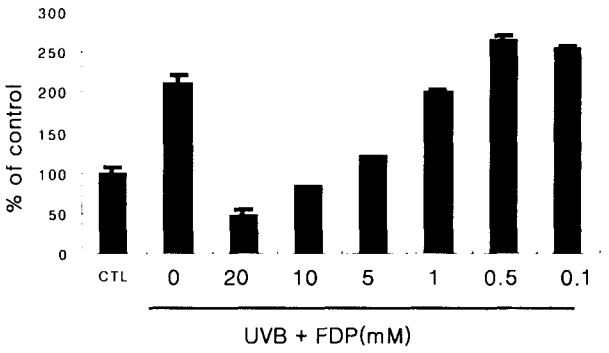


Figure 1. Effects of FDP on UV-induced cell death. Cells were UVB-irradiated (30mJ/cm^2) and further incubated with FDP for 24h in DMEM containing serum 1%. The activity of LDH released in media was measured. The results are expressed as mean±s.e. in four different experiments.

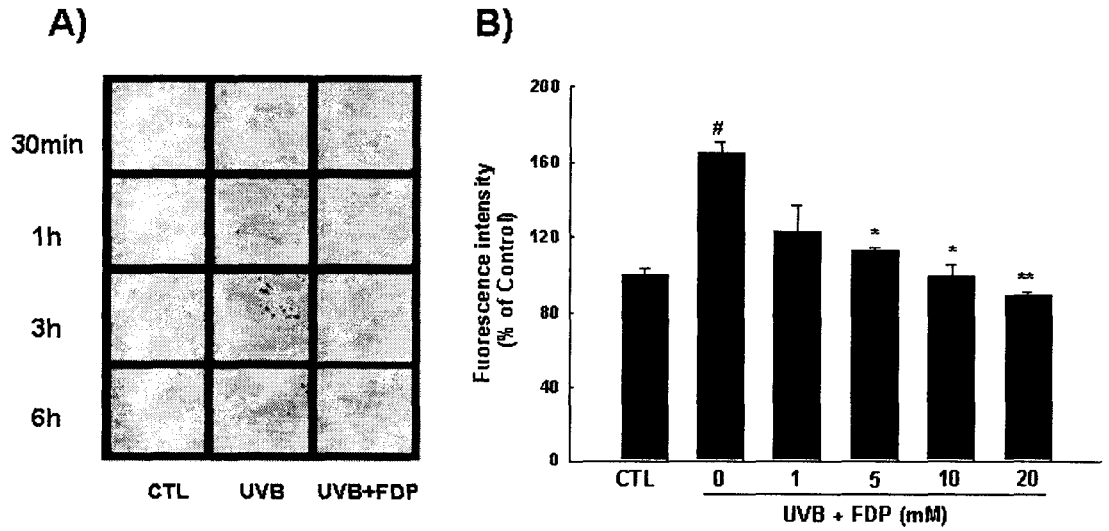


Figure 2. FDP attenuates the intracellular levels of UVB-induced reactive oxygen species (ROS). HaCaT keratinocytes that had been exposed to UV irradiation (3030mJ/cm^2) were incubated with FDP in fresh culture media containing 10% FBS. At indicated time, cells were loaded with 5 μM 2,7'-dichlorofluorescein diacetate (DCFH-DA) for 30 min in PBS. Cultures were incubated for another 30 min, washed three times with PBS, and the fluorescence signal was analyzed on the stage of a Nikon Diaphot inverted microscope equipped with 1 100 Xenon lamp. To minimize background signal caused by direct oxidation of DCFH-DA by illumination at 490 nm, intracellular levels of ROS were analyzed within 3 sec after illumination using a Quanticell 700 system. (A) Maximum level of cellular ROS was reached in 3 h after UVB-irradiation. UVB-induced ROS accumulation was reduced to control level by FDP (10 mM). (B) FDP dose dependently attenuates the cellular accumulation of ROS measured at 3 h after irradiation. The results are expressed as mean±s.e. in four different experiments. UVB-irradiation significantly increased ROS production compared with unirradiated control (# $P<0.01$). A significant difference relative to UVB control is indicated with * $P<0.05$ or ** $P<0.01$.

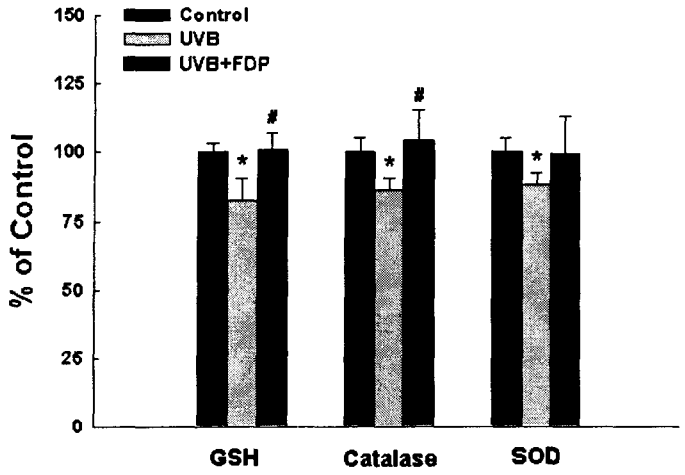


Figure 3. FDP preserves the cellular antioxidant capacity. HaCaT keratinocytes that had been exposed to UV irradiation (30mJ/cm^2) were incubated with FDP (10 mM) in fresh culture media containing 10% FBS for 24 h. Cells were harvested by scraping and antioxidative parameters were measured. The results are expressed as mean±s.e. in four different experiments. UVB-irradiation significantly decreased the total glutathione level and activities of catalase and SOD compared with unirradiated control (* $P<0.05$), but GR and GPx were not changed(data not shown). A significant difference relative to UVB control is indicated with # $P<0.05$.

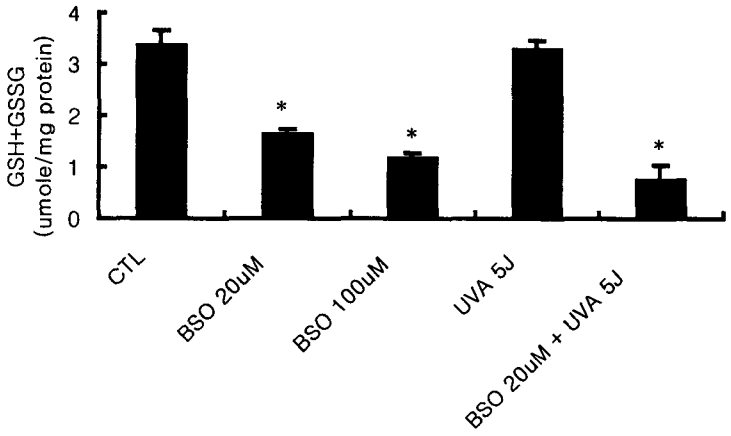


Figure 4. Glutathione content in normal human fibroblast following UV irradiation and BSO treatment were markedly decreased. Normal human fibroblast was irradiated UVA or BSO treatment containing 10% FBS for 48 h. Cells were harvested by scraping and total glutathione were measured. The results are expressed as mean±s.e. in three different experiments. BSO treatment significantly decreased the total glutathione level. A significant difference relative to control is indicated with # $P<0.05$. The total glutathione level of UV irradiated NHF that had been pretreated with BSO (20 μM) for 24h was almost deprived compared with control group. But, the glutathione level of UV irradiation alone (5J /cm^2) was not changed.

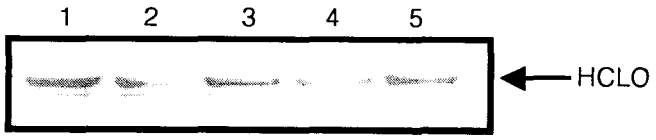
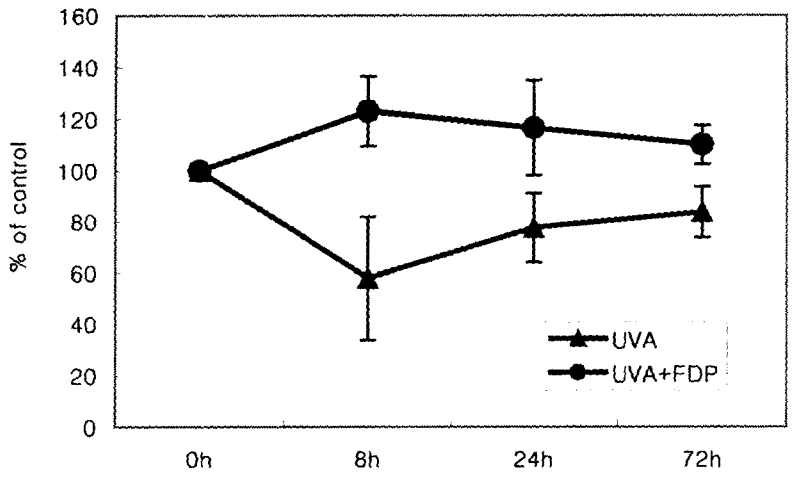


Figure 5. Cellular glutathione depletion lowered the UVA dose threshold for reduction of procollagen synthesis. Normal human fibroblasts were exposed to UVA and/or BSO treatment. After the treatment, cells were incubated fresh media without FBS and phenol red for 48h. The procollagen espression was determined in conditioned media using SP1.D8 antibody. Lane 1: unirradiated control; Lane 2 : UVA 15J/cm^2 alone; Lane 3: UVA 5J/cm^2 alone; Lane 4: BSO(20 μM) + UVA 5J/cm^2 ; Lane 5: BSO(20 μM) alone.



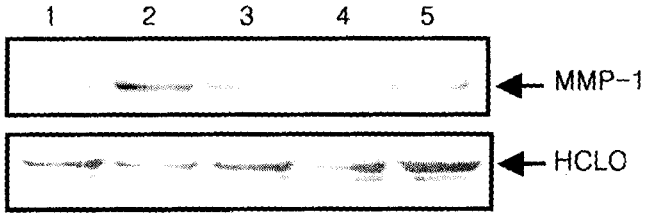


Figure 6. FDP preserves the GSH in cultured skin fibroblast after UVA irradiation. Normal human fibroblast that had been pretreated with FDP(1mM) for 3h were exposed to UVA(15J/cm^2). After UVA irradiation, cell were incubation with FDP(imM) in fresh culture media. UVA-irradiation significantly decreased total glutathione level compared with unirradiated control.

Figure 7. FDP prevent the UV-induced MMP-1 expression and procollagen depression in cultured skin fibroblast. Normal human fibroblast that had been pretreated with FDP(1,5 mM) and NAC(5mM) for 3h were exposed to UVA(15J/cm^2). After UVA irradiation, cell were incubation with FDP and NAC in fresh culture media without phenol-red and FBS for 48h and the level of MMP-1 and procollagen in the media were determined. The media containing protein were subjected to SDS-acrylamide gel electrophoresis, and analyzed by western blotting using specific antibody. Lane 1: unirradiated control; Lane 2 : UVA 15J/cm^2 alone; Lane 3: UVA 15J/cm^2 + FDP 1mM ; Lane 4: UVA 15J/cm^2 + FDP; Lane 5: UVA 15J/cm^2 + NAC 5mM.

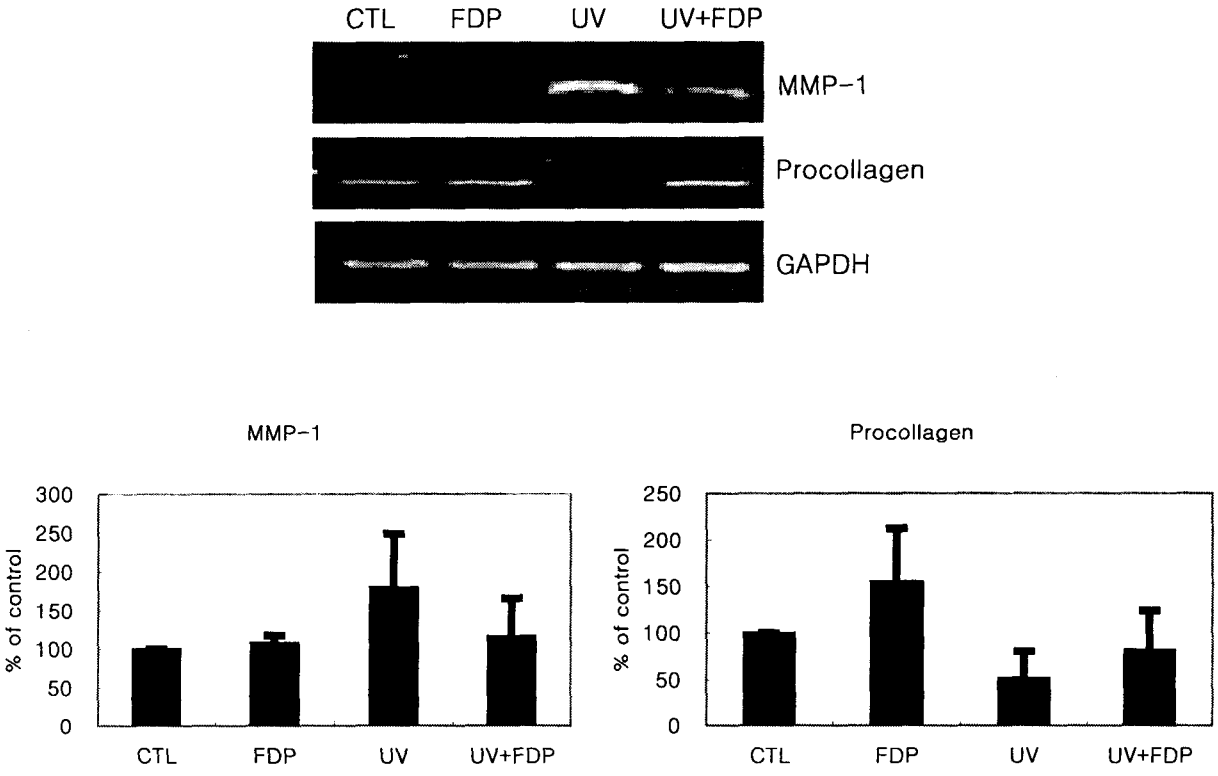
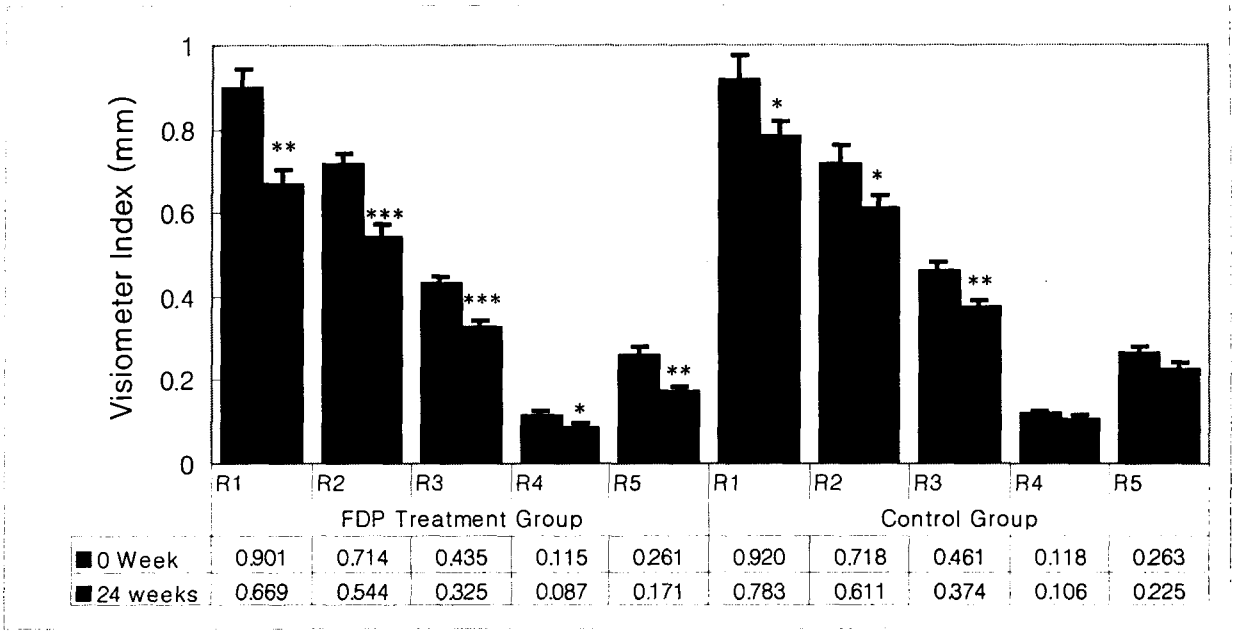


Figure 8. FDP prevent the UV-induced MMP-1 expression and procollagen depression in human skin *in vivo*. The buttock skin was treated with vehicle and FDP(1%) for 24h prior to UV exposure. Skin was obtained 48h after UV irradiation(2 MED UV). Total RNA was extracted from the skin, the level of MMP-1 and procollagen mRNA were determined by RT-PCR. These figures are representative of the findings from four subjects.

A)



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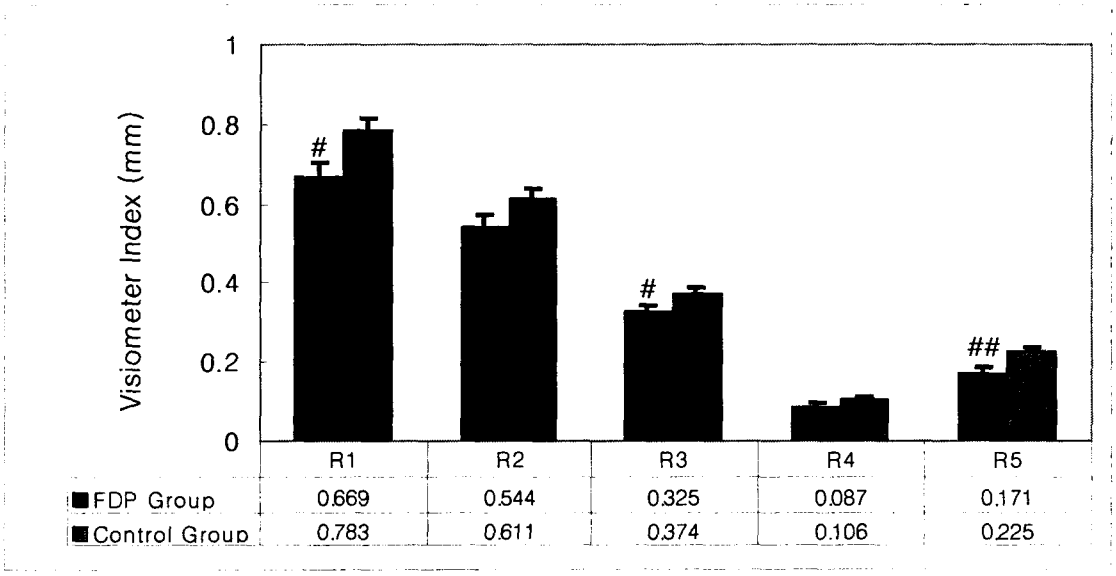

Figure 9. FDP decreased facial wrinkle compared with vehicle alone. A total 50 healthy females were asked to apply the cream to the entire face twice a day for 6 months. A silicone replica of the crow's feet area was taken at baseline and at weeks 24. Skin replicas were then analyzed by Skin visiometer SV 600. Standard wrinkle and roughness features were then calculated and statistically analyzed. (A) Mean changes of profilometric parameters from baseline to 24 weeks in efficacy. . *P<0.05, **P<0.01, ***P<0.001 (B) Profilometric parameters change between FDP treatment group and vehicle treatment group at 24 weeks. #P<0.05, ##P<0.01.