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The water-soluble fullerene derivative 'Radical Sponge®' exerts cytoprotective action against UVA irradiation but not visible-light-catalyzed cytotoxicity in human skin keratinocytes

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Abstract—Fullerene was entrapped in polyvinylpyrrolidone of 60-80 kDa at a molar ratio range of 0.42–0.67:1, resulting in a water-soluble derivative with a mean particle diameter of about 688 nm, named 'Radical Sponge®' because of its ROS-scavenging ability as previously demonstrated, and examined in the present study for its photo-biological actions toward human skin keratinocytes HaCaT. The keratinocytes were repeatedly irradiated with a visible light of wavelengths of 400–2000 nm (approximately 19,800 lux) in the presence or absence of Radical Sponge® of 25–75 μM and did not exhibit any photo-cytotoxicity due to coexistent Radical Sponge® as compared with the sham-irradiation control. Radical Sponge® exerted a more marked cytoprotection at doses of 10–40 μM against UVA irradiation of 30 J/cm^2 when it was pre-irradiationally administered and rinsed out immediately before the irradiation, than when administered only during or after the irradiation, indicating the preventive rather than therapeutic or ray-sheltering effect of Radical Sponge® on UVA injuries. Cytoprotection by Radical Sponge® against UVA was achieved at the advisable range doses of 10–40 μM in contrast to no effect of polyvinylpyrrolidone alone; its dose-dependency was advantageous over that of VC-IP, a tetra-alkyl-esterized provitamin C, which became less cytoprotective above 20 μM. Thus, Radical Sponge® is expected as an anti-UVA-preventive agent without visible-light-catalyzed cytotoxicity toward human skin keratinocytes.

Ultraviolet A (320–400 nm) radiation generates reactive oxygen species (ROS), which can cause a series of biological effects in human skin cells, resulting in cell damage or cell death.[1] Antioxidants have been reported to inhibit the progression of ultraviolet (UV) radiation-induced skin cell death.[2] Fullerene and its derivatives, especially water-soluble fullerene derivatives, as a potent antioxidant protect human or mammalian cells against oxidative stress through stoichiometric 'scavenging' and catalytic dismutation of superoxide.[3] Several biological studies have demonstrated that water-soluble fullerenes such as tris-malonic acid derivative of fullerene (carboxyfullerene) and polyhydroxylated fullerene derivative (fullerenol) act as radical scavengers to protect neurocyte and other cell growth from various toxins that can induce apoptotic injuries in vitro.[4] There are also more evidences demonstrating the antioxidative effect of water-soluble fullerenes in vivo.[5]

Keywords: Water-soluble fullerene; Cytoprotective; UVA-cytotoxicity.
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Hydrophilic polymer-wrapped fullerene that was prepared through interaction of C60-fullerene with poly(vinylpyrrolidone) (PVP) was endowed with the innate antioxidant properties of C60.[6] In our previous study,[7] we had successfully prepared the similar hydrophilic polymer-wrapped fullerene, Radical Sponge®. And Radical Sponge® was shown to diminish the ROS amounts in terms of the molecular and cellular levels against either UVB or the hydroperoxide *t*-BuOOH. In the present study, we examined the effective concentration range of Radical Sponge® without any cytotoxicity and phototoxicity, and the optimal drug-administration schedule before or after UVA irradiation.

We first prepared Radical Sponge®. Radical Sponge® is composed of the purified fullerene BioFullerene® from which a trace of impurities were thoroughly eliminated by careful sublimation, and PVP(polyvinylpyrrolidone) of 60–80 kDa at a mixing molar ratio range of 0.42–0.67: 1. Preparation of PVP-entrapped BioFullerene® was conducted as follows: a stirred solution of sublimed BioFullerene® (0.8 mg) dissolved in toluene (1.0 ml) was

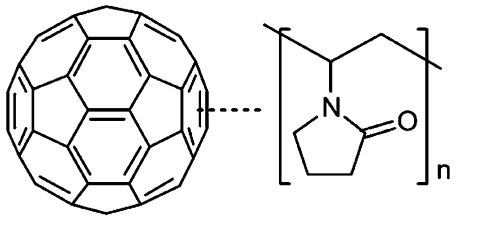
added to a chloroform solution of PVP (100 mg) at room temperature. The mixture was vigorously homogenized, and then the solvent was evaporated under reduced pressure. The resulting residual solid was dissolved again in 2.0 ml of Milli-Q reverse-osmosis water, and the obtained aqueous solution was sonicated to produce a lucid solution. By azeotropic distillation of the suspension, toluene as a solvent was thoroughly removed. The mixture was filtered with a pore of 0.1 μm and dried up under vacuum to give a dark-brownish solid (Scheme 1).

The particle size of Radical Sponge® was determined by the dynamic light scattering method at 23.7 °C at 532 nm with a Nikkiso DPSS laser apparatus MicroTrak UPA-150. And the morphology of the matrix particles was observed with a scanning electronic microscope (Hitachi S-2460N).

Human skin keratinocytes HaCaT were kindly provided by Prof. Norbert E. Fusenig[8] and grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1 mM L-arginine, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator of 95% air–5% CO_2.

The photo-cytotoxicity of Radical Sponge® was estimated with a 24-well plate at an initial density of 8000 cells/well. When cells were attached to the bottom of culture plates, the culture medium was replaced by phenol red (PR)-free DMEM containing 10% FBS and Radical Sponge® at diverse concentrations. Then the cultures were irradiated with a 300-W photo-reflector lamp located at a distance of 30 cm (approximately 19,800 lux) above the 24-well plate. To avoid UV irradiation damage to the cells, the UVB portion was sheltered by a piece of silica glass filter. Irradiation was carried out three times, each time for 0.5 h with a 24-h interval between two consecutive irradiation times. After the first, the second or the third irradiation, respectively, the cell viability was estimated by the WST-1 assay.

After the culture medium was removed, the cells were irradiated, in 200 μl of PR-free DMEM containing no fullerene, with fluorescent lamps. To avoid the possible filter effect of Radical Sponge on UVA light, the keratinocytes that were previously administered with Radical Sponge were rinsed three times by PR-free DMEM immediately before UVA irradiation. The emission maximum of the lamp was centered at 365 nm with shel-



Fullerene (C_{60}) Polyvinylpyrrolidone (PVP)

Scheme 1.

tering of UVB ray by a silica glass filter. The control samples were kept in the dark under the same conditions. For WST-1 assay and other analyses, fresh medium containing 10% FBS was added after exposure to UVA ray, and the cells were further incubated at 37 °C for 48 h.

Cell viability was evaluated by photometric assay using the formazan-forming redox indicator dye WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2-disulfophenyl)-2*H*-tetrazolium, monosodium salt). At different time points, the cells were rinsed twice with PR-free DMEM containing 10% WST-1 at 37 °C. Cell viability was determined based on mitochondrial conversion of WST-1 to yellowish formazan, being indicative of the number of viable cells.[9] The absorbance was read with an absorbance multiplate reader at 450 nm.

All data were processed statistically by the software of SPSS 11.5 for Windows and are expressed as means ± standard deviation of 3–5 independent experiments. Significant differences were determined by LSD or Tamhane's T2 test.

It has been reported that fullerenes can exert photodynamic activities under specified conditions;[10] in the presence of a non-physiologically excess amount of NADH, an in vivo ubiquitous reductant, C60-fullerene/PVP (140 μM C60) and C70-fullerene/PVP (70 μM C70) showed DNA-cleavage activity with visible light irradiation. However, in the absence of NADH, neither C60 nor C70 had any destructive effect on DNA even under irradiation with a 300 W photoreflector lamp for 4 h.[11] Several types of water-soluble fullerenes cause oxidative damage to cellular membranes resulting in cell death with or without visible light ray, although some derivatives of C60- and C70-fullerenes have been reported not to show any photocytotoxic effect even at higher doses.[12,13] Thus, it is a serious controversy for biomedical applications whether water-soluble fullerene derivatives may exert the photo-cytotoxicity. It seems to be necessary to determine whether fullerene derivatives show a double-edged effect and therefore our present aim is to find how to efficaciously apply their beneficial function concurrently with avoidance of their putative harmful function.

Particle size distribution of aqueous solution and desiccative powder of Radical Sponge®: To investigate the molecular state of C60-fullerene in liquid state, the particle size distribution and the nanosphere shape were observed. Figure 1a shows that mean particle size was about 688 nm that was measured for the aqueous solution of Radical Sponge®. The size is suitable for cosmetic application, because it is considered that the microparticles with a diameter exceeding 1000 nm barely penetrate into the human skin, whereas the harmful effect to skin cells could be occasionally exerted by particles smaller than 100 nm. The electronic microscopy showed that the individual particle of drying Radical Sponge® is as large as 150–250 nm in a diameter, which is smaller than the diameter (688 nm) that was determined by MicroTrak (Fig. 1b). The difference may be attributed to swelling and

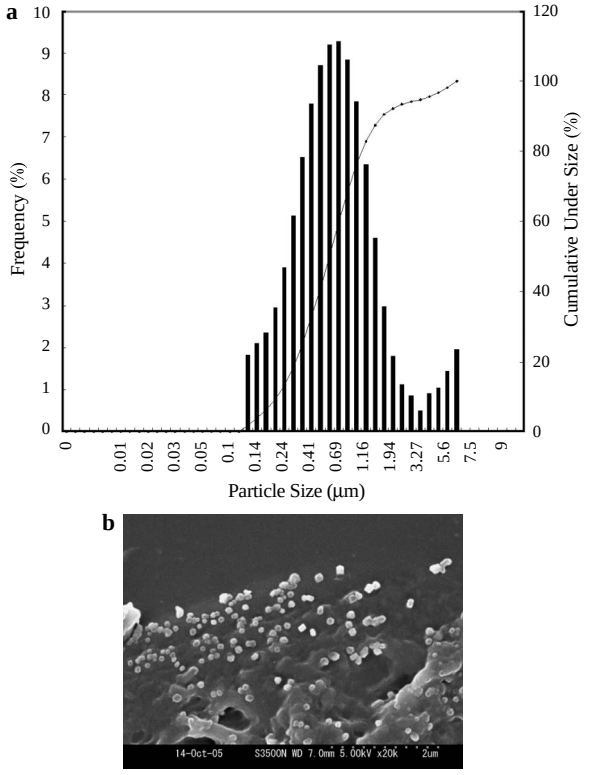


Figure 1. (a) Particle size distribution of aqueous solution of Radical Sponge® as assessed by light scattering method. (b) Particle shape and size of dried preparation of Radical Sponge® as observed by scanning electronic microscopy.

shrinkage of particle PVP-polymer during transition between two states of the particles after suspended in water and the subsequent drying.

Effect of irradiation with visible light on human skin keratinocytes HaCaT in the presence or absence of the water-soluble derivative of fullerene, Radical Sponge®: we evaluated the cell viability of the keratinocytes, to which Radical Sponge® was administered under visible light irradiation for four modes (1, 2 or 3 times irradiation and sham irradiation). As shown in Figure 2, after 1, 2 or 3 times of visible light irradiation, the survival rates of HaCaT cells to which Radical Sponge® was administered at the graded concentrations were not decreased as compared with the survival of cells under the dark condition. Thus, in the presence of 25–75 μM of Radical Sponge® exposure of visible light to keratinocytes for 1, 2 or 3 times for 30 min per each time did not produce any photo-cytotoxicity. In addition, at the sham-irradiation mode, the cell viability of HaCaT cells in the presence of Radical Sponge® was not lower than that of the control cells, indicating that, at the examined concentration range, Radical Sponge® by itself did not cause any cytotoxicity to the keratinocytes, either. Thus, the keratinocytes showed no cytotoxicity due to Radical Sponge® below 75 μM irrespective of the dark or visible light condition.

Cytoprotective effect of Radical Sponge® on UVA-irradiation in human keratinocytes HaCaT and the optimal drug-administration conditions: to examine the cytoprotective effects and the optimal dosages of Radical Sponge®, we designed a series of experiment modes concerning the drug-administration schedule (Fig. 3), including Mode I (3 h before UVA irradiation), Mode II (3 h before and during UVA irradiation), and Mode III (3 h before, during, and after UVA irradiation until assessment by the WST-1 assay). After UVA irradiation (30 J/cm^2), HaCaT cells underwent severe morphological damage (Fig. 5b), and the cell viability was markedly

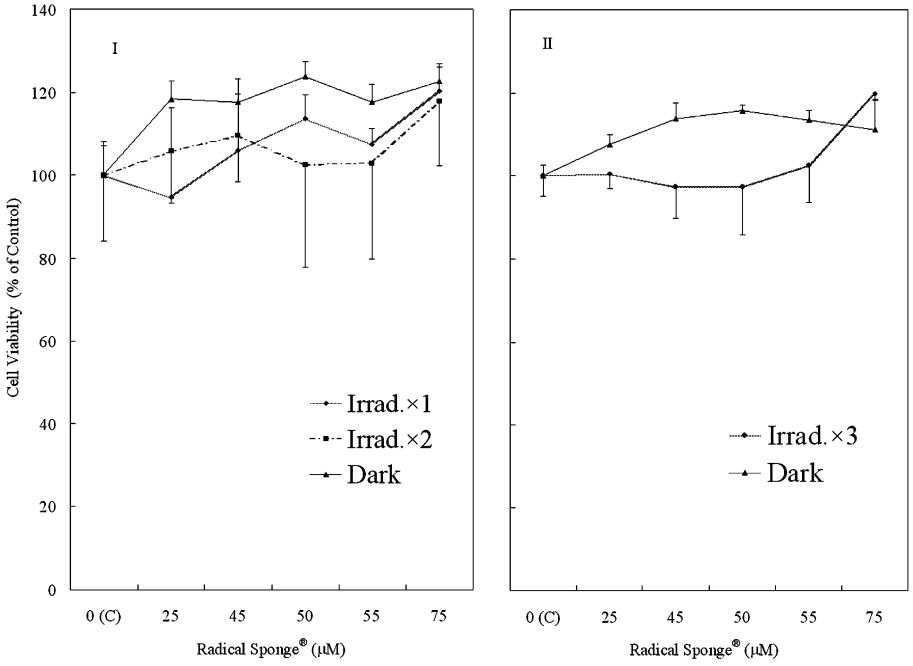


Figure 2. Effect of irradiation with visible light on cell viability of human skin keratinocytes HaCaT in the presence or absence of Radical Sponge® (control) = 0 (C); (dark) = sham-irradiation.

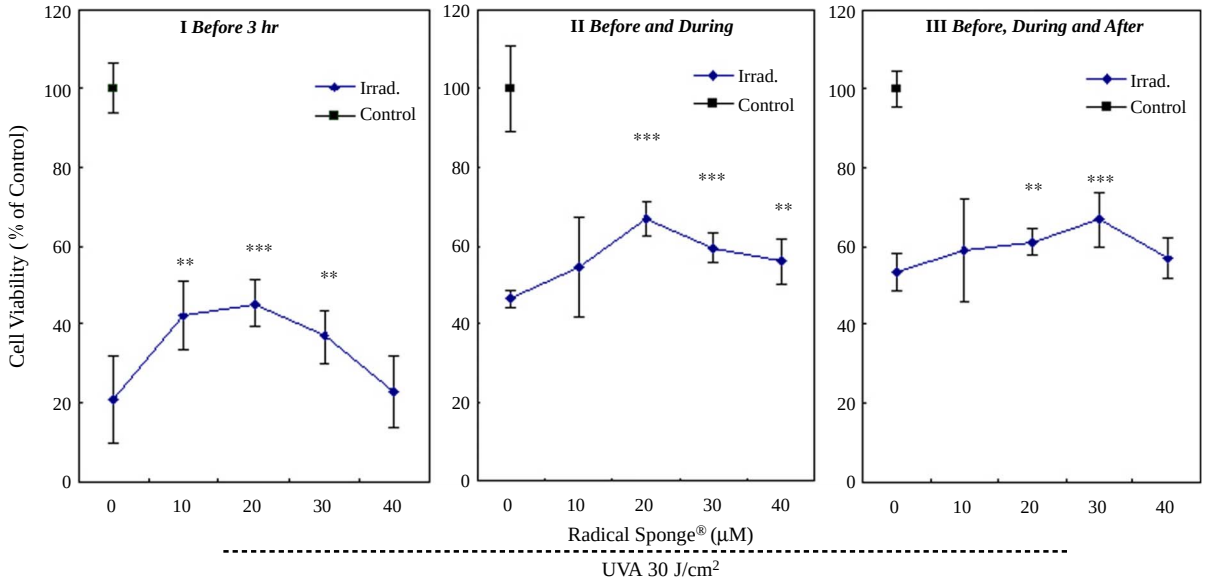


Figure 3. Cytoprotective effect of Radical Sponge® on UVA-induced proliferation–inhibition of HaCaT cells. (***$p < 0.001$, **$p < 0.01$, compare with '0'.)

decreased to 45–80% (Figs. 3 and 4). Figure 3 shows that Radical Sponge® significantly restored the cell viability of HaCaT cells that were treated with Modes I–III. Especially a cell viability of keratinocytes was increased to 2.18-fold by administration with 20 μM of Radical Sponge® at 3 h before UVA irradiation compared to that without administration (Fig. 3, I).

To determine the period substantial for cytoprotective action of Radical Sponge®, we further designed the schedules of Radical Sponge®-administration for specified definite periods (Fig. 4): Mode I, administration at 2 h before UVA irradiation and rinsing out immediately before the irradiation; Mode II, administration during UVA irradiation and rinsing out immediately after the irradiation; Mode III, administration immediately after UVA irradiation until the WST-1 assay. The experiment showed that Radical Sponge® significantly enhanced the cell viability of HaCaT cells for Mode I at concentrations of 10–40 μM (Fig. 4, I). Administration with Radical Sponge® at 10 μM or 30 μM during UVA irradiation (Mode II) showed a cytoprotective effect weaker as compared with that of Mode I, suggesting that Radical Sponge® was likely to slightly defilade UVA irradiation (Fig. 4, II). From Figure 4, III, post-irradiational administration with Radical Sponge® did not

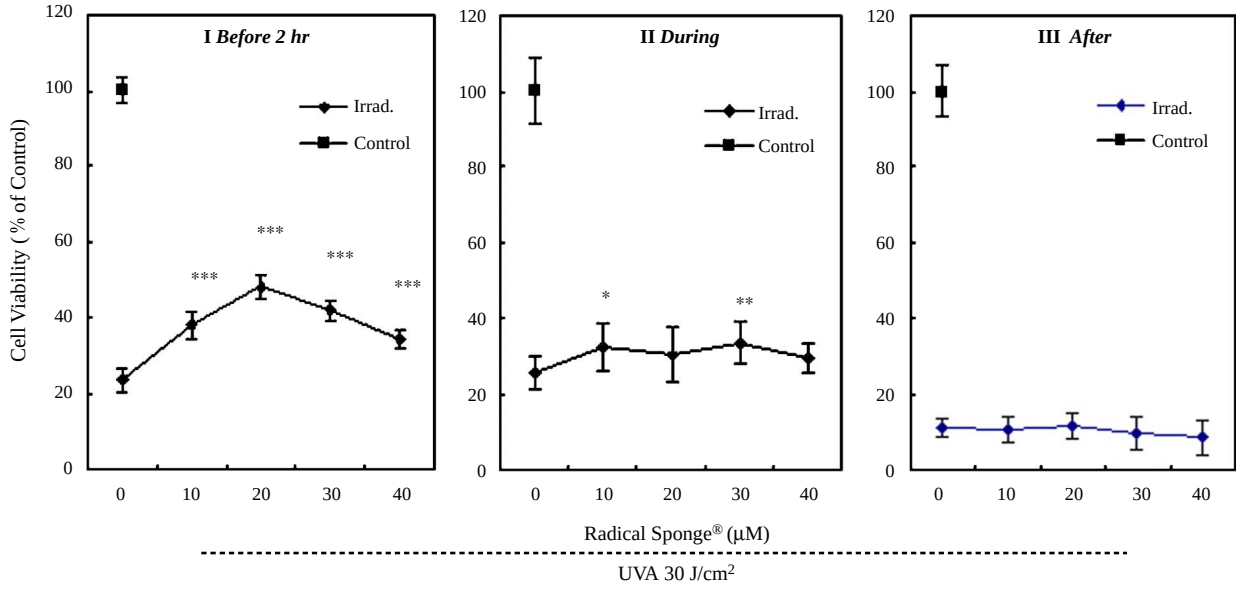


Figure 4. Administration timing of Radical Sponge® and its correlation with of the cytoprotective effects on UVA-induced proliferation–inhibition of HaCaT cells. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compare with '0'.)

recover the UVA-induced decrease in cell viability of HaCaT cells. These findings also demonstrated that the cytoprotective effect of Radical Sponge® on UVA injuries is via the preventive or ROS-scavenging action but is scarcely exerted by a putative UVA-sheltering or UVA-absorbing action, because less cytoprotection occurred for HaCaT cells that were administered with Radical Sponge during UVA irradiation.

The preventive effect of Radical Sponge® on UVA-irradiation-induced cell death on HaCaT cells and the optimal drug-pretreatment schedule: From the above-described results, we can conclude that the efficient cytoprotection is achieved by pre-administration with Radical Sponge® at doses of 20–30 μM. To further investigate the optimal drug-pretreatment schedule under the above-described conditions, we examined different pre-administration times of Radical Sponge® with 20–30 μM to HaCaT cells before UVA irradiation. We observed a significant reduction of UVA-induced cell death by administration with Radical Sponge® at 20 μM to HaCaT cells, and administration at 3 h before UVA irradiation was more effective than the other pre-administration periods including 2, 4, and 6 h (Fig. 5a). Although UVA irradiation induced a marked degeneration of HaCaT cells, which became unhealthy and detached from the plate bottom, Radical Sponge® at 20 μM could reduce the morphological abnormalities, which are shown as Figure 5b.

Cytoprotection of Radical Sponge® superior to that of VC-IP on UVA injury and its attribution not to PVP: VC-IP (ascorbic acid-2,3,5,6-*O*-tetra-(2′-hexyl)decylester), a lipophilic derivative of vitamin C, is known to exert the excellent function of scavenging some free radicals and to protect skin cells from ROS injuries as a potent antioxidant.[14] To test the hypothesis that Radical Sponge® can independently protect HaCaT cells from UVA injuries as well as VC-IP without the accessorial effect of PVP, we determined the cell viability of HaCaT cells which were pre-administered with Radical Sponge®, VC-IP or PVP under the same condition. Figure 6 shows that both Radical Sponge® and VC-IP significant increased the cell viability at the definite concentration, whereas PVP showed no cytoprotective effect, which suggested that the cytoprotection of Radical Sponge® was achieved substantially by fullerene and not by PVP. Radical Sponge® was shown to be advantageous over VC-IP in terms of a stable plateau-like effect at doses with wide range of 10–40 μM on UVA-induced diminishment of cell viability, This may be attributed to a difference in the ROS-scavenging modes that extra-molecular release of intramolecular moieties could not be occurred from fullerene, but occurred as a result of esterolysis of four putative cytostatic fatty acid esters composing a VC-IP (vitamin C tetra-*iso*palmityl ester) molecule (see Fig. 6).

The present study demonstrated that Radical Sponge® has no visible-light catalysis or cytotoxicity to human keratinocytes HaCaT at concentrations of 25–75 μM, and at the similar concentration range (10–40 μM) it can protect HaCaT cells from UVA injures via the preventive action.

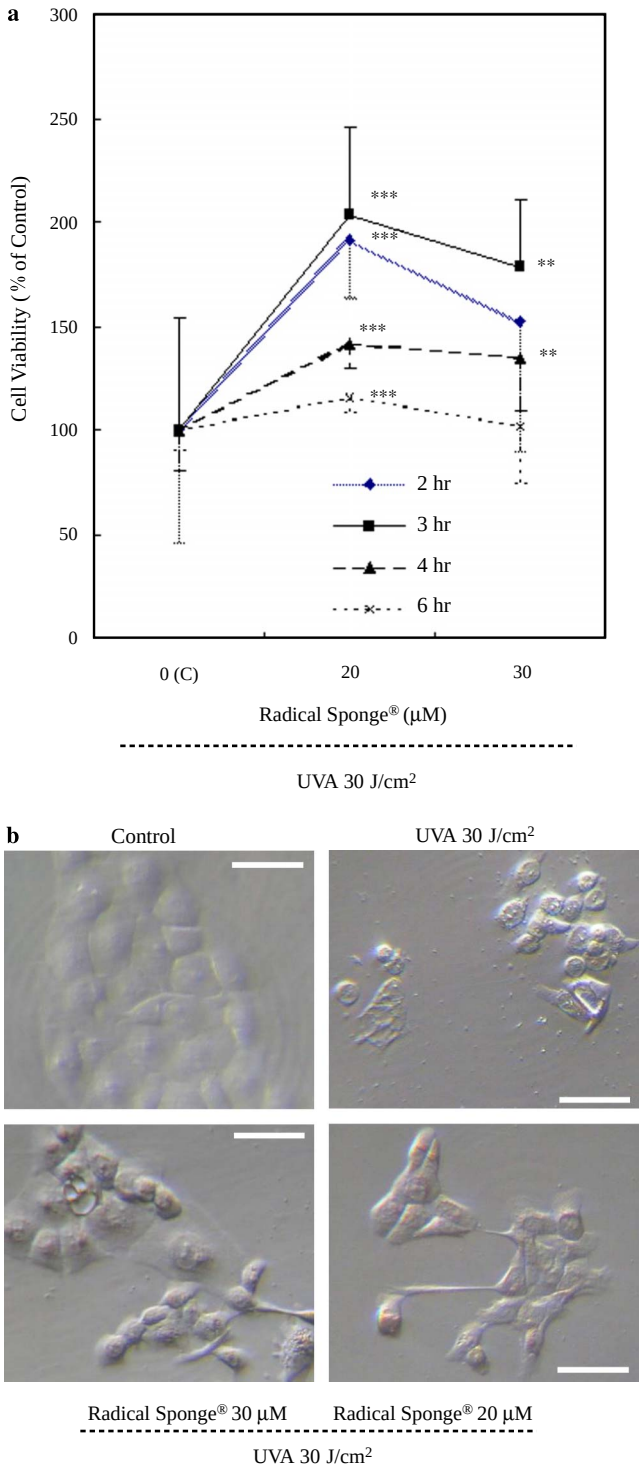


Figure 5. (a) Dependence of cytoprotective effects of Radical Sponge® on periods for administration before UVA irradiation. (Control) = 0 (C). (***$p < 0.001$, **$p < 0.01$, compare with '0 (C)'.) (b) Morphological aspects of HaCaT cells that were protected by Radical Sponge® from UVA-induced damage. Scale bar = 50 μm.

How does Radical Sponge® exert the preventive effect on UVA injuries? According to the previous study, C60-fullerene is able to cross the cell membrane and localize preferentially to mitochondria which generate the great mass of cellular oxygen-free radicals, suggesting a possibility that the cytoprotection is attributed to the ROS-scaveng-

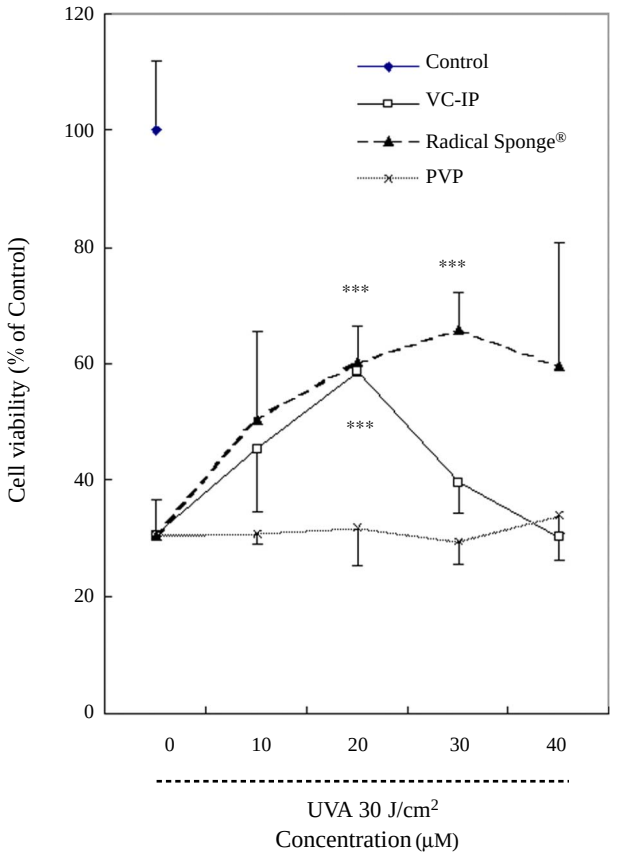


Figure 6. Comparison of Radical Sponge® with the Pro-vitamin-C VC-IP and PVP in terms of dose ranges for cytoprotective effects on UVA-induced proliferation–inhibition of HaCaT cells. (***$p < 0.001$, compare with '0'.)

ing ability of Radical Sponge® that is perhaps bound to the cell membrane or organelle.[15] As shown by our previous study, the peroxylipid-induced cell death is repressed by Radical Sponge® which scavenges ROS such as hydroxyl radicals and superoxide anion radicals, suggesting the cytoprotection against UVA injuries by Radical Sponge® through its ability to scavenge the UVA-derived ROS. To elucidate the mechanism of inhibition of UVA-induced cell death by Radical Sponge®, it needs to explore the cellular local binding site of Radical Sponge® which next we will investigate.

Fullerene as the nano-technological material has showed some pharmacodynamic advantages such as its efficient permeability into the depth of the human skin epidermis owing to the stability being more reliable than vitamin C that is so labile to be oxidatively decomposed in the aqueous solution. Thus, the Radical Sponge® molecule would be a new therapeutic material in the definite concentration range for the prevention of both UV skin-injuries and skin aging without photosensitization and cytotoxicity.

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