Effect of Squalene Monohydroperoxide on Cytotoxicity and Cytokine Release in a Three-Dimensional Human Skin Model and Human Epidermal Keratinocytes

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In order to clarify that squalene monohydroperoxide (SQOOH) correlates with changes in morphology through cytotoxicity and establish *in vitro* evaluation of the cytotoxicity of lipid hydroperoxide, the effect of SQOOH on cytotoxicity and morphology in normal human epidermal keratinocytes (NHEK(B)) and the Gunze three-dimensional cultured human skin model (Vitrolife-skin) was investigated. Additionally, the effect of radical scavengers (mannitol, vitamin C and (+)-catechin) on the cytotoxicity in Vitrolife-skin was studied. The level of lipid hydroperoxide (phoshatidylcholine hydroperoxide: PCOOH) in the cellular membrane was increased with the concentration of SQOOH, and the rise in cytotoxicity in NHEK(B) was associated with changes to the cellular membrane. A concentration-dependent and protective effect on the increase in cytotoxicity and PCOOH content was observed. To clarify the effect of SQOOH on the release of cytokine from cells, IL-2 level from NHEK(B) and Vitrolife-skin were investigated. IL-2 release from the cells was enhanced by SQOOH and increased at a non-cytotoxic dose.

These results suggest that the increase in lipid hydroperoxides resulting from the auto-oxidation of lipids within cellular membranes in the presence of SQOOH correlates with changes in morphology due to cytotoxicity. SQOOH enhanced the release from cells at a non-cytotoxic dose. A method for assessing the protective effect on the cytotoxicity of lipid hydroperoxides using cells would be useful for *in vitro* evaluation of the cytotoxicity.

Key words squalene monohydroperoxide; cytotoxicity; IL-2; normal human epidermal keratinocytes (NHEK), Vitrolife-skin

Squalene monohydroperoxide (SQOOH) is a primary oxidized lipid produced from squalene by solar UV.[1,2] We have already reported on the cytotoxicity and PGE_2 release due to the production of SQOOH after UV irradiation in the presence of a photosensitizer in a three-dimensional human skin model.[3,4] Others have reported that SQOOH is a product of skin aging and atopic dermatitis.[2,5] Many reports about the effects of hydroperoxides on cytotoxicity in cultured cells have concentrated on the cytotoxicity of *tert*-butyl hydroperoxide[6—9] (a type of peroxidant of cellular membrane lipids which is water soluble). Only a few papers have described the cytotoxicity of SQOOH. And it was not yet clear whether the mechanism of cytotoxicity involves lipid hydroperoxides. Yoshikawa *et al.*,[10] Ohrmori *et al.*[11] and Henderson *et al.*[12] reported that active oxigen induced inflammation and histamine release. Shimura *et al.*[13] reported that cumene hydroperoxide inhibited the immunoreaction of rat splenocytes. But few papers have described the effect of SQOOH on cytokines release.

To clarify whether the SQOOH concentration correlates with changes in morphology through cytotoxicity and evaluate *in vitro* the cytotoxicity of lipid hydroperoxides, SQOOH was added to keratinocytes (easy to culture and a simple model for evaluation) and a three-dimensional human skin model (with the barrier effect of skin: a more useful model for evaluation). In addition, to elucidate the effect of SQOOH on cytokine release, a non-cytotoxic dose was added to keratinocytes and a three-dimensional human skin model and cytokine release evaluated. This report describes the harmful effects of SQOOH and the mechanism of its cytotoxicity in human keratinocytes and the three-dimensional human skin model.

MATERIALS AND METHODS

Cells Normal human epidermis keratinocytes (NHEK(B)) and the Gunze three-dimensional cultured human skin model (Vitrolife-skin; a new skin model (sizes, 8 mm) composed of two types of collagen sponge and two types of human skin cells, fibroblasts and keratinocytes, and has two layers dermis and epidermis, like real skin, and keratinocytes which were cultured with an air interface)[14,15] were purchased from Kurabo Co. (Osaka, Japan) and Gunze Co. (Kyoto, Japan), respectively.

Reagents SQOOH was a gift from Dr. Yoshiyuki Kohno (Shiseido Co.). IL-2 kits were purchased from Funakoshi Co. (Tokyo, Japan). Medium for NHEK(B) (Humedia-KG2) and medium for Vitrolife-skin (DMEM+5% fetal bovine serum (FBS)) were purchased from Kurabo Co. (Osaka, Japan) and Gunze Co. (Kyoto, Japan), respectively. Mannitol (MA) was purchased from Kanto Chemicals (Tokyo, Japan). Other reagents were from Wako Chemicals (Osaka, Japan).

Cytotoxicity NHEK(B) cells at 5×10^3/well in 0.1 ml of medium were placed into 96-well plates and incubated at 37 °C, 5% CO_2, and a humidity above 90% for 48 h. After the incubation, hydroperoxides (0—1000 μM SQOOH dissolved in 0.2 ml of medium containing 1% EtOH) was added and the plates were incubated for 24 h. The viability of NHEK(B) was then determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay-1[3,4,16] and concentrations of IL-2 released as an indicator of the immunoreaction were determined by enzyme immuno assay (EIA).[17]

Vitrolife-skin was placed into 24-well plates and pre-incubated at 37 °C, 5% CO_2, and a humidity above 90% for 1 h. Then hydroperoxide (0—100 mM SQOOH dissolved in 0.1 ml of medium containing 1% EtOH) was added to the surface of the cells and the incubation continued for 24 h. Fi-

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nally, the viability of Vitrolife-skin was determined by an MTT assay-2[14] and lactate dehydrogenase (LDH) assay,[18] and the concentration of IL-2 released was determined by EIA.[17]

After 1 h pre-incubation, anti-oxidants were added to 0.25 ml of medium of another set of Vitrolife-skin samples (in 24-well plate) and incubated at 37 °C, 5% CO_2, and a humidity above 90% for 2 h. After the incubation, 100 mM SQOOH diluted with 0.2 ml of squalene (SQ) was added to the surface of the cells. The cells were again incubated at 37 °C, 5% CO_2, and a humidity above 90% for 24 h. The viability of the Vitrolife-skin was determined by MTT assay-2, the concentration of IL-2 release was determined by EIA and the levels of phoshatidylcholine hydroperoxide (PCOOH) and phoshatidylethanolamine hydroperoxide (PEOOH) (lipid hydroperoxides of the cellular membrane) were determined by chemiluminescence high-performance liquid chromatography (CL-HPLC).[3,19]

MTT Assay-1 One hundred microliters of MTT solution (MTT diluted with medium: 2 mg/ml) was added to each well and NHEK(B) cells were incubated at 37 °C, 5% CO_2, and a humidity above 90% for 2 h. After the incubation, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and then 200 μl of MTT extracting solution (isopropanol containing 0.05 M HCl) was added to each well and the plate shaked continously at 60 rpm and room temperature for 1 h. The absorbance at 540 nm (A_{540}) was determined spectrophotometrically by a microplate reader (Model 450, Bio-Rad, Hercules, CA, U.S.A.).

Viability was calculated according to the following equation:

$$\text{viability (\%)} = (A_{S540} - A_{b540})/(A_{c540} - A_{b540}) \times 100$$

A_{S540}=absorbance of the extract when SQOOH was applied to NHEK(B) at 540 nm

A_{c540}=absorbance of the extract when only medium (without SQOOH) was applied to NHEK(B) (control) at 540 nm

A_{b540}=absorbance of the extract when medium (without cells) was used as a blank at 540 nm

MTT Assay-2 One milliliter of MTT solution (MTT diluted with medium: 2 mg/ml) was added to each well and the Vitrolife-skin incubated at 37 °C, 5% CO_2, and a humidity above 90% for 3 h. After the incubation, the tissus were washed twice with PBS and holed out with a punch. Then 2 ml of MTT extracting solution and 40 μl of sodium dodecyl sulfate (SDS; helpful for extracting) were added to each well. The rest of the procedure was as for the MTT assay-1.

Viability was calculated according to the following equation:

$$\text{viability (\%)} = (A_{S540} - A_{b540})/(A_{c540} - A_{b540}) \times 100$$

A_{S540}=absorbance of the extract when SQOOH was applied to Vitrolife-skin at 540 nm

A_{c540}=absorbance of the extract when only medium containing 1% EtOH was applied to Vitrolife-skin (control) or SQ was applied to Vitrolife-skin (negative control) at 540 nm

A_{b540}=absorbance of the extract when collagen sponge without cells was used as a blank at 540 nm

LDH Assay Fifty microliters of medium and reagent (nitro blue tetrazolium at 0.74 mg/ml, diaphorase and NAD^+ dissolved with Lithium DL-lactate buffer at 50 mg/ml) was added to the wells and then the Vitrolife-skin was incubated at room temperature for 45 min. After the incubation, 100 μl of stop reagent (0.5 M HCl) was added and then the absorbance at 570 nm was determined. The absorbance of the sample was calculated according to the following equation:

$$\Delta OD570 = A_{s570} - A_{c570}$$

A_{s570}=absorbance of the medium when SQOOH was applied to Vitrolife-skin at 570 nm

A_{c570}=absorbance of the medium when only medium containing 1% EtOH was applied to Vitrolife-skin at 570 nm

IL-2 Assay Each well in a 96-well assay plate was rinsed immediately with 0.5 ml/l of Tween 20 in PBS (the Wash Buffer) and then 50 μl of culture medium, with either 100 μl of acetylcholinesterase: IL-2 Fab'conjugate solution, was added to each well. After incubatition at 4 °C for 18 h, the wells were rinsed five times with the Wash Buffer, and 200 μl of Ellman's Reagent was added. The absorbance at 405 nm was then determined and the concentrations of IL-2 was calculated by comparison with the absorbance of standards.

Determination of PCOOH and PEOOH Lipids in the Vitrolife-skin were extracted twice with a cooled mixture of chloroform/2-propanol (1 : 1, v/v) containing 10 ppm butylhydroxytoluene. The lower layer was collected and concentrated with a rotary evaporator under reduced pressure and the residue was redissolved in 100 μl of a mixture of chloroform/methanol (1 : 1, v/v) containing 100 μg/ml ρ-nitrophenol as an internal standard. Ten microliters of sample solution was injected into the CL-HPLC system.

Histochemical Studies Vitrolife-skin for light microscopic observation was fixed in 10% formalin (for over night), embedded in paraffin, and stained with hematoxylin and eosin.

Statical Analysis Significant differences from the control were evaluated by Student's *t*-test.

RESULTS

Effect of Lipid Hydroperoxides on Cytotoxicity and Cytokine Release from the Cells After SQOOH was added to NHEK(B), and the cells incubated for 24 h at 37 °C, a significant decrease in viability as determined by the MTT assay was observed depending on the concentration of SQOOH. The IC_{50} of SQOOH against NHEK(B) was 630 μM (Fig. 1).

The time course of the release of IL-2 from NHEK(B) was investigated. After 1 h incubation, the IL-2 release significantly increased depending on the concentration of SQOOH and after 4 h, the increase in IL-2 reached a plateau (Fig. 2). In the presence of 100 μM SQOOH, a significant decrease in viability was not observed, but a significant increase in IL-2 was (Fig. 2).

SQOOH was added to the Vitrolife-skin (considered more useful for estimating the harmful effects of lipid hydroperoxide on the human body than NHEK(B)), the system incubated for 24 h at 37 °C, and the viability assessed (Fig. 3). The viability of Vitrolife-skin decreased significantly depending on the concentration of SQOOH. Therefore, the re-

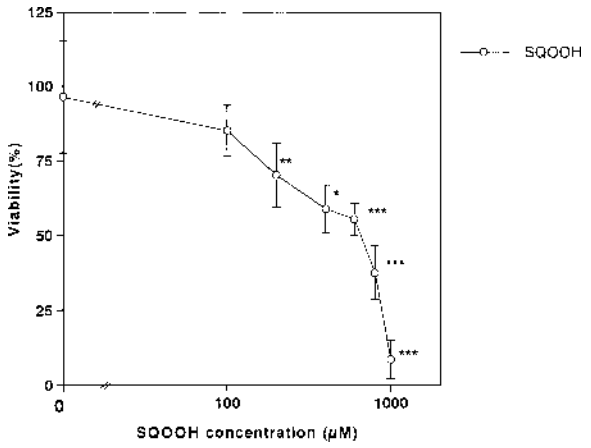

Fig. 1. Effect of SQOOH on Cytotoxicity in NHEK(B)

NHEK(B) cells were incubated for 24 h after the addition of SQOOH. Data are means±S.D. (n=3—4). Significantly different from control (only medium was applied to NHEK(B)), $*p<0.05$, $**p<0.01$, $***p<0.001$.

lease of LDH from Vitrolife-skin in the presence of 100 mM SQOOH (the viability of the cells at 24 h after addition of SQOOH was reduce more than 50%) was investigated. The LDH release from Vitrolife-skin increased after 4 h and reached a peak after 8 h (Fig. 4). In addition, Vitrolife-skin stained with HE in the presence of SQOOH was investigated for 24 h. First, swelling of the stratum corneum was observed after 2 h. After 4 h, the cells in the epidermis shrunk and curled, and then shrinking, curling and vacualization of the cells in the dermis was observed after 8 and 24 h (Fig. 5). The control after 24 h was same as in the presence of SQOOH after 0 h (Fig. 5a). The amount of IL-2 release at 24 h after the addition of SQOOH was investigated. A significant increase in the amount released was observed depending on the concentration of SQOOH (Fig. 6).

Effect of Anti-oxidants on Cytotoxicity, Cytokine Release and PCOOH and PEOOH Contents To clarify the cytotoxic contribution of SQOOH to auto-oxidation, the effect of anti-oxidants on the cytotoxicity of SQOOH in Vitrolife-skin was investigated at 24 h. In the presence of MA (OH

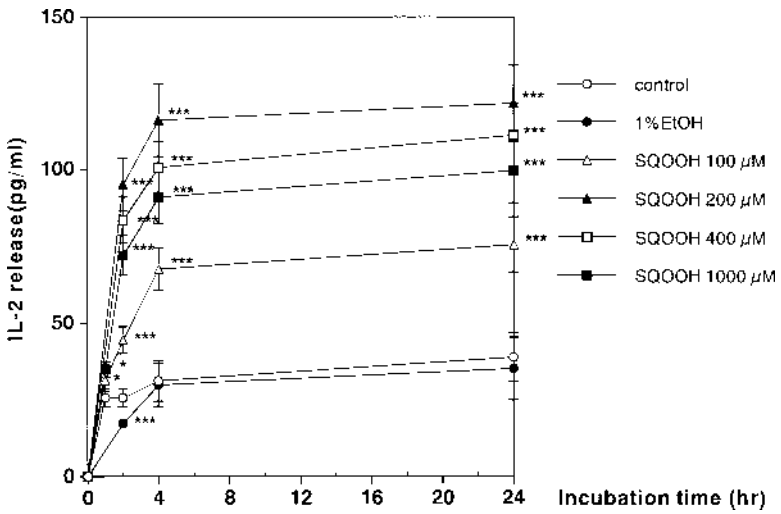

Fig. 2. Effect of SQOOH Concentration on IL-2 Release from NHEK(B)

Data are means±S.D. (n=3—5). Significantly different from control (only medium was applied to NHEK(B)), $*p<0.05$, $***p<0.001$.

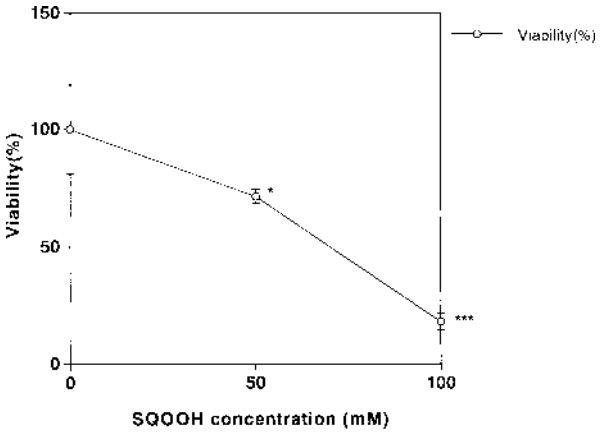

Fig. 3. Effect of SQOOH on Cytotoxicity in Vitrolife-Skin

Vitrolife-skin was incubated for 24 h after the addition of SQOOH. Data are means±S.D. (n=3—4). Significantly different from control (only medium containing 1% EtOH was applied to Vitrolife-skin), $*p<0.05$, $***p<0.001$.

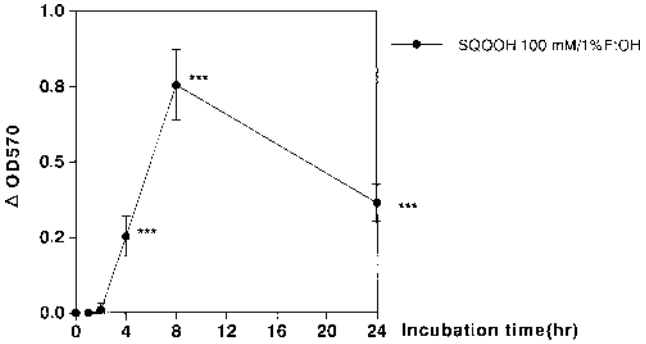

Fig. 4. Effect of SQOOH on LDH Release from Vitrolife-Skin

Data are means±S.D. (n=3—4). Significantly different from control (incubation time 0 h), $***p<0.001$.

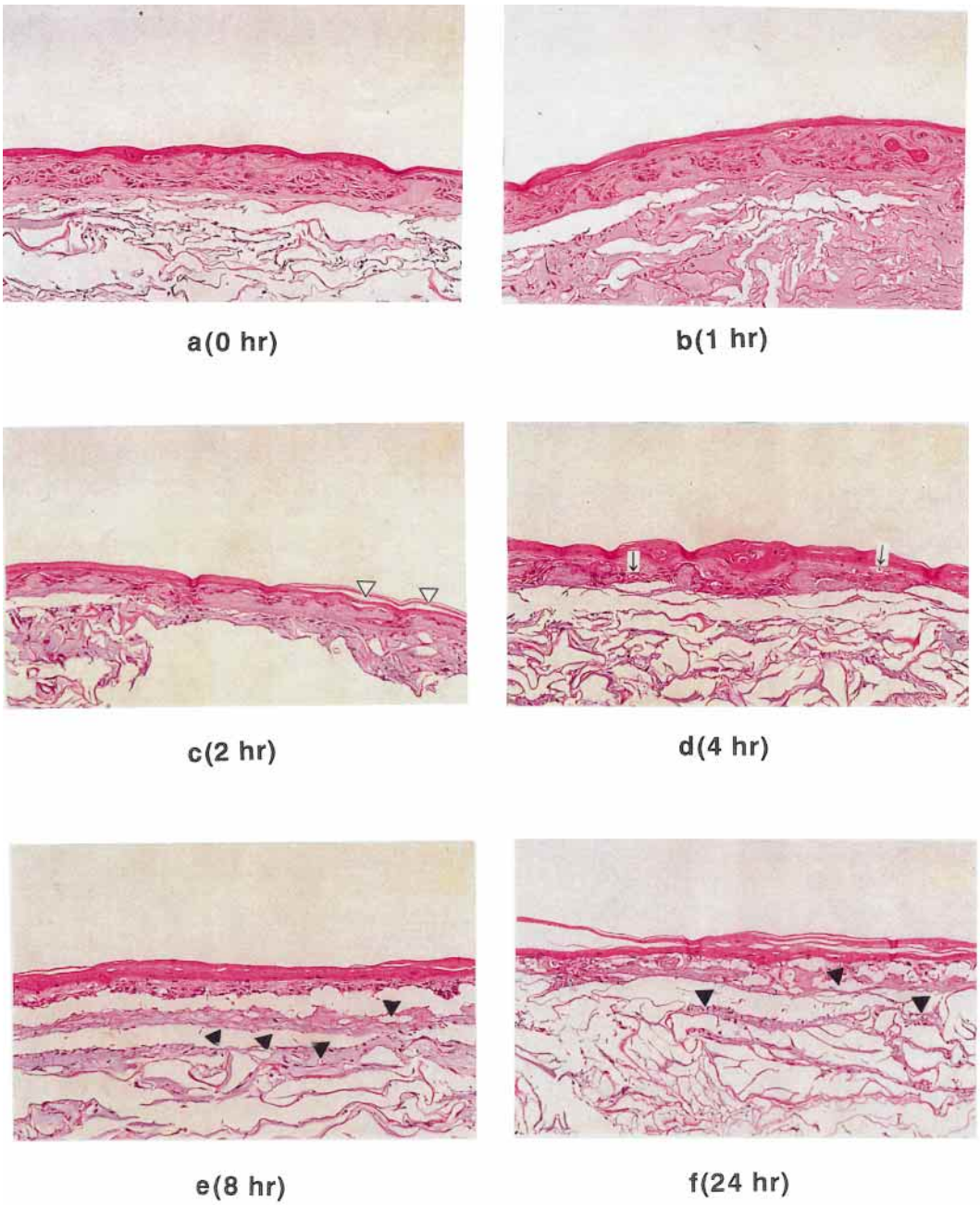

Fig. 5. Effect of SQOOH on Vitrolife-Skin

Vitrolife-skin was incubated for 0 (a), 1 (b), 2 (c), 4 (d), 8 (e) or 24 h (f) with 100 mM SQOOH and stained with hematoxylin and eosin. ×200. ▽, swelling of the stratum corneum (Fig. 5c); ↓, shrinking and curling of the cells in the epidermis (Fig. 5d); ▼, shrinking, curling and vacualization of the cells in the dermis (Figs. 5e, f).

radical scavenger), (+)-catechin (CATC, radical scavenger) or ascorbic acid (VC, radical scavenger and reducer), the cytotoxicity decreased significantly compared to that in the SQOOH-treated group (Fig. 7-1). CATC, VC and MA also significantly inhibited the production of IL-2 at 24 h (Fig. 7-2). In the absence of SQOOH, there was no significant effect of CATC, VC and MA on viability and IL-2 release (data not shown).

To clarify the contribution of the oxidation of membrane lipids caused by the auto-oxidation of SQOOH to the cytotoxicity, the effect of CATC on the amount of PCOOH and PEOOH (hydroperoxides of cell membrane lipids) in Vitrolife-skin 24 h after addition of 100 mM SQOOH was investigated. The PCOOH content of Vitrolife-skin increased significantly on addition of SQOOH. But in the presence of CATC, this increase was significantly inhibited and the PCOOH content was no different than in the control (without SQOOH) group (Fig. 8). PEOOH was not detected.

DISCUSSION

Kohno and Takahashi[2] reported that when Chinese hamster lung fibroblasts were incubated in DMSO with SQOOH for 48 h, the IC_{50} of SQOOH was about 230 μM. We obtained a slightly higher value than theirs in the case of NHEK(B), but in the case of Vitrolife-skin, the IC_{50} (about 70 mM) was over 100 times higher. So we considered that the stratum corneum in Vitrolife-skin might protect cells from damage

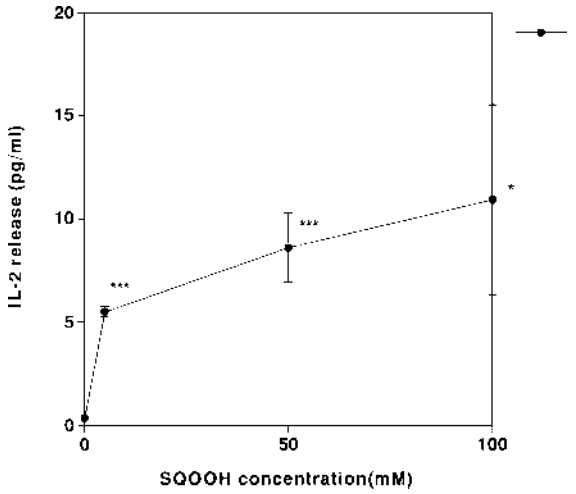

Fig. 6. Effect of SQOOH Concentration on IL-2 Release from Vitrolife-Skin

Data are means±S.D. (n=3). Significantly different from control (only medium containing 1% EtOH was applied to Vitrolife-skin), $*p<0.05$, $***p<0.001$.

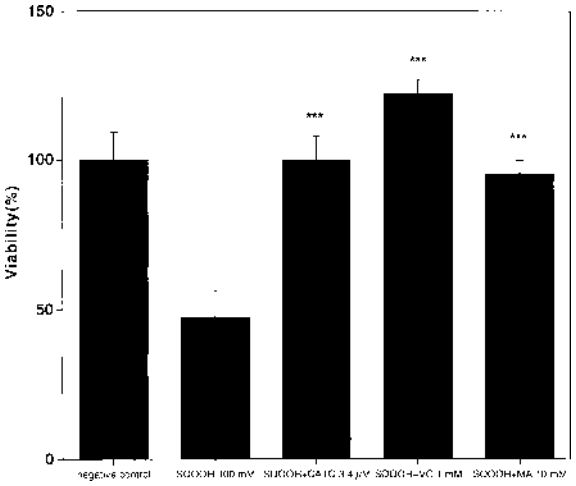

Fig. 7-1. Effect of Anti-oxidants on SQOOH Induced-Cytotoxicity against Vitrolife-Skin

Vitrolife-skin was incubated for 2 h before SQOOH and then for 24 h after the addition of SQOOH. Data are means±S.D. (n=3—5). Significantly different from the SQOOH 100 mM group, $***p<0.001$. Negative control=tissues which were applied with SQ only.

due to SQOOH.

Kohno and Takahashi[20] reported that no cytotoxicity for TESTSKIN™ (three-dimensional cultured human skin model) on addition of 2100 mM SQOOH was observed after 4 h, but that cytotoxicity was strong after 24 h. So they considered that the mechanisms of cytotoxicity result from the penetration by SQOOH of the epidermis and dermis of TESTSKIN™ and proposed that SQOOH damaged the membrane lipids in cells through auto-oxidation. Chiba *et al.*[21] reported that repeated topical application of 10 mM SQOOH to hairless mice for 3 weeks induced slight hyperkeratosis, moderate epidermal thickening and slight hyperplasia of sebaceous glands. In the present study, cytotoxicity against cells in the epidermis was induced 4 h after the addition of SQOOH, and at 8 h strong cytotoxicity was observed. CATC, VC and MA as radical scavengers significantly protected cells from the cytotoxicity by attenuating the activity

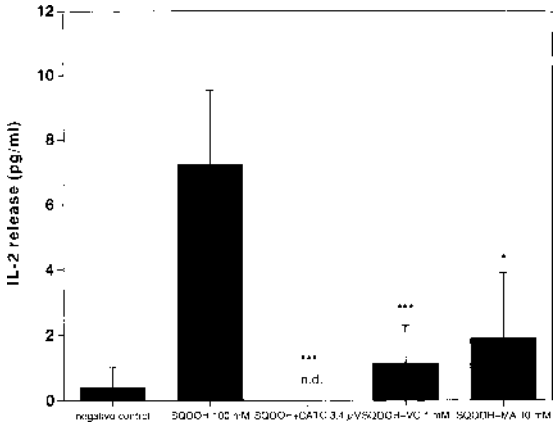

Fig. 7-2. Effect of Anti-oxidants on SQOOH-Induced IL-2 Release from Vitrolife-Skin

Vitrolife-skin was incubated for 2 h before adding SQOOH and then incubated for 24 h after the addition of SQOOH. Data are means±S.D. (n=3). Significantly different from the SQOOH 100 mM group, $*p<0.05$, $***p<0.001$. Negative control=tissues which were applied with SQ only. n.d.=not detected.

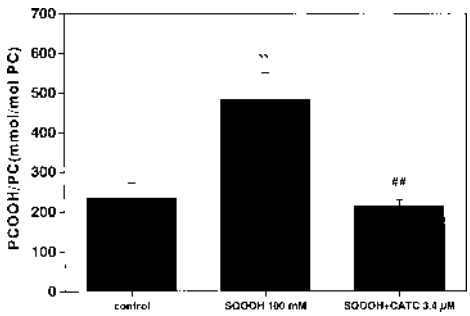

Fig. 8. Effect of SQOOH on PCOOH Content in Vitrolife-Skin

Vitrolife-skin was incubated for 24 h after the addition of SQOOH. Data are means±S.D. (n=3). Significantly different from control (tissues without SQOOH), $**p<0.01$. Significantly different from the SQOOH 100 mM group, $\#\#p<0.01$.

of SQOOH. SQOOH increased the amount of PCOOH in cellular membrane but CATC significantly protected the cells from this increase by preventing the peroxidation of cellular membrane lipids due to SQOOH. It was not clear whether PEOOH was present, but it is conceivable that a lack of detection of PEOOH may have caused the difference between PE (inner-celluar membrane) and PC (surface celluar membrane). As reported by Kohno and Takahashi, it is conceivable that SQOOH caused the peroxidation of cellular membrane lipids within the dermis and epidermis of Vitrolife-skin due to auto-oxidation and consequently the cytotoxic effects appeared after 4 h.

These results suggest that the increase in lipid hydroperoxides resulting from the auto-oxidation of lipids within cellular membranes in the presence of SQOOH correlates with changes in morphology through cytotoxicity. And SQOOH enhanced IL-2 release from the cells at a non-cytotoxic dose. A method for assessing the protective effect on cytotoxicity and cytokine release of lipid hydroperoxides using cells would be useful for the *in vitro* evaluation of the cytotoxicity of lipid hydroperoxides.

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