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Generation of free radicals by medical phototherapy and suppression by NanoSphere™ (Amphiphilic vitamin C capsule)

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In recent years, photo-rejuvenation has often been performed using laser devices such as YAG laser, CO2 laser and intense pulsed light in dermatology outpatient clinics. In addition, UV light therapy has been used for a treatment conventionally at a dermatology clinic. Indication of an UV light therapy in Japan is psoriasis, atopic dermatitis, pustulosis palmoplantaris, vitiligo. As the output of these devices is low, photo aging is generally not caused.However, the pigmentation and erythema often occur after these photo device use and become a problem in a dermatology outpatient. External preparations containing ascorbic acid derivatives (APS,APPS) are prescribed in order to restrain development of pigmentation and erythema after the photo device treatment by Japanese dermatologists . However, the action mechanism of these ascorbic acid derivatives is not yet clear. The present study measured levels free radicals generated by ESR (Electron spin resonance) assay after irradiation with both laser that is frequently used to remove stratum corneum and a medical laser from a home-use photo device that is commercially available in Japan.

1. Introduction

Under normal conditions, L-Ascorbic acid (vitamin C) Ascorbic acid strong reducing effect and undergoes oxidization to become discolored when used with in drugs. Synthesis of ester derivatives with modification of carbon 2, the most reactive site of Ascorbic acid, has been performed in Japan since the 1960s, and many esters of Ascorbic acid are now used as food additives, stock feed additives, and cosmetic ingredients 1). Among the C2 ester derivatives of Ascorbic acid, the most commonly used worldwide is L-Ascorbic acid-2-phosphate ester (AP). AP were first developed in the 1970s as ingredients of medical cosmetics that prevent pigmentation, and their tyrosinase inhibitory effect prevents melanin production by the pigment cells of the skin. AP activates collagen production 2), and it has an effect to promote cell specialization 3). In addition, AP erases reactive oxygen species(ROS) 4), it restrains lipids peroxidation 5). In this way AP protects a cell from ROS 6), and AP extends cell life time 7).

On the other hand, photo-rejuvenation has often been performed using laser devices such as YAG laser, CO2 laser and intense pulsed light in dermatology outpatient clinics. In addition, UV light therapy has been used for a treatment conventionally at a dermatology clinic. Ultraviolet rays lamp of 310nm is used for these treatments mainly. Indication of an UV light therapy in Japan is psoriasis, atopic dermatitis, pustulosis palmoplantaris, vitiligo. As the output of these devices is low, photo aging is generally not caused. However, the pigmentation and erythema often occur after these photo device use and become a problem in a dermatology outpatient. Industrial production of Na L-ascorbyl-2-phosphate-6-palmitate (APP: Appreciер™: Showa Denko K.K, Tokyo, Japan.), an APP with ester modification of a long-chain fatty acid of AP, started in 2005. AP are water soluble, but the lipid solubility of APP was increased by modification of palmitate, a long chain fatty acid, to carbon 6, markedly improving uptake by skin tissue and cells. The structural formulae of Ascorbic acid, AP, and APP are shown in Fig.1.

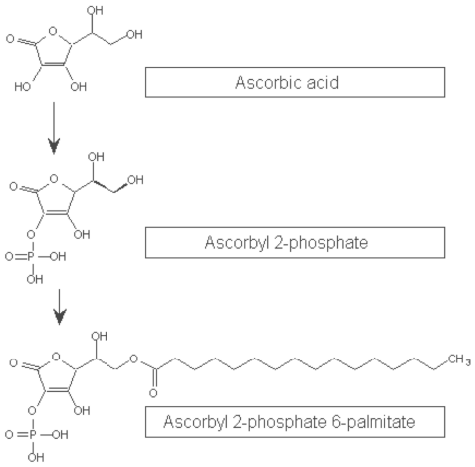

Fig.1 The structural formulae of Ascorbic acid, AP, and APP.

Ascorbyl 2-Phosphate 6-Palmitate, Appreciер TM (Showa Denko k.k.) is a novel vitamin C derivative, trisodium salt of ascorbyl 2-phosphate 6-palmitate(APP), newly designed Ascorbic acid amphiphilic derivative of ascorbyl 2-phosphate (AP), conjugated with a long acyl chain (C16:palmitoyl residue). AP has been proved very efficacious for skincare because of its capability of scavenging reactive oxygen species and promoting collagen synthesis. Thanks to its moderate hydro- phobicity, APP penetrates effectively into dermis and is enzymatically converted to ascorbic acid quickly during permeation. APP retain the stability of AP and in vivo Ascorbic acid activity, along with improved tissue permeability and intracellular delivery of Ascorbic acid due to their

reduced affinity for water. APP have a strong surfactant effect because of being water soluble, a required property, as well as being lipophilic. APP has an effect to restrain melanoma metastasis 8). Because APP are strong antioxidants that are equivalent to Ascorbic acid and can be used Ascorbic acid film coating for microcapsules to allow the production of microcapsules with a free radical barrier, it has become increasing clear that their characteristics have various applications. In the present article, some of the interesting properties of APP are discussed. The main effect of APP was examined in detail by Kato E., et al. (Showa Denko k.k., Tokyo, Japan) and Miwa N.(Hiroshima Prefectural University, Hiroshima, Japan). They are experimented on in order to evaluate APP, and reported some effects of APP.

2, Production of microcapsules using APP

APP are amphiphilic agents that form liquid crystals when combined with lipids as surfactants. Ascorbic acid result, lipids encapsulated by an APP film can be produced. The liquid crystal structure of APP is formed by multiple layers of water and lipid (i.e., water-lipid-water-lipid, etc.)(Fig.2.), so water-soluble materials can be mixed in the aqueous layers. Microcapsules made in this fashion become self-emulsifying (Picture) and addition of water leads to emulsification as dispersion (Picture). When the self-emulsifying process occurs, APP in the form of microcapsules become dispersed in water Ascorbic anionic dispersion, so electrophoresis easily performed (Picture). Using this phenomenon, water-soluble materials with no electric charge, such as lipids, peptides, sugar chains, and antibodies, can be introduced as ions by iontophoresis.

We have already succeeded in producing an APP-based self-emulsifying encapsulated lipid preparation (NanoSphere), in which CoQ10, astaxanthin, VCIP, and fullerene are encapsulated by APP. With these APP microcapsules, the stability of both the APP themselves and the encapsulated lipids is improved. Particularly,

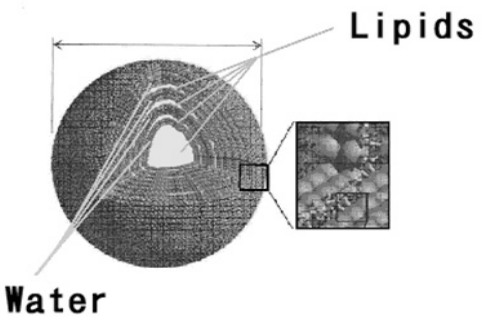

Fig.2. Multi-layer liquid crystal structure of self-emulsifying APP/lipid microcapsules (NanoSphere™) .

when encapsulated APP/lipids which contain various water-soluble and fat-soluble antioxidant molecules, the stability of both APP in the coating and also the molecules inside the microcapsules is greatly improved. This concurrent stabilization of the APP coating and encapsulated antioxidant molecules is thought to depend on reproducing the redox balance between the various antioxidant molecules in cells. The stability of a 1% APP solution and of APP microcapsules is shown. Also, the stability of VCIP in emulsifying dispersions (VCIP encapsulated with APP) is shown. Carotinoids with strong singlet-oxygen scavenging ability, such a astaxanthin, are unstable when added to drugs, and undergo oxidative decomposition to become discolored relatively easily even when antioxidants are also added, but their stability is improved by encapsulation with APP.

3, Effect of NanoSphere on laser light radiation

Dorsal skin of hairless mice were anaesthetized with 50 mg /kg of Nembutal was irradiated using YAG laser or home-use hair removal laser. Using skin samples including epidermis and dermis (weight, 20-100mg), 20-200 μL of DMPO (5,5-Dimethyl-1-pyrroline N-oxide: as spin trap agent) was added and mounted on the flat cell for ESR spectroscopy (JES-FA200), then ESR was used to detect and quantify levels of various free radicals. Peak strength of various free radicals was measured using Mn as the internal standard. Next, to ascertain the suppressive effects of various ascorbates on free radicals, 4% (w/w) gel of Na L-ascorbic acid(AS), Na L-ascorbyl-2-phosphate(APS), Na L-ascorbyl-2-phosphate-6-palmitate(APPS), and it's microcapsules (NanoSphere) were applied to skin before or after laser irradiation, and levels of free radicals were measured directly by same ESR spin trap method. Irradiation with the medical laser led to levels of hydroxyl radicals almost 2 times and levels of superoxides increasing almost 5 times. Levels of ascorbyl radical tended to increase. Application of APPS and NanoSphere before laser irradiation almost 1/2 levels of superoxides, and levels of hydroxyl radicals tended to decrease (Fig.3). The degree of decrease in superoxides was highest for APPS and NanoSphere. When the medical laser was used after AS, APS, APPS and NanoSphere application, the increase in superoxides was limited to just 1.1-1.5 times(LSD-test, $P < 0.05$). With the commercially available home-use laser, increases in free radicals were not seen under the maker recommended condition (single irradiation for 3 s). However, when the home-use laser was used 10 times for 3 s each, levels of superoxides almost doubled , although no marked changes in other radicals were noted. APS, APPS and NanoSphere application decreased superoxides to pre-irradiation levels. Levels of free radicals were not increased when AS, APS, APPS and NanoSphere was applied before home-use laser irradiation.

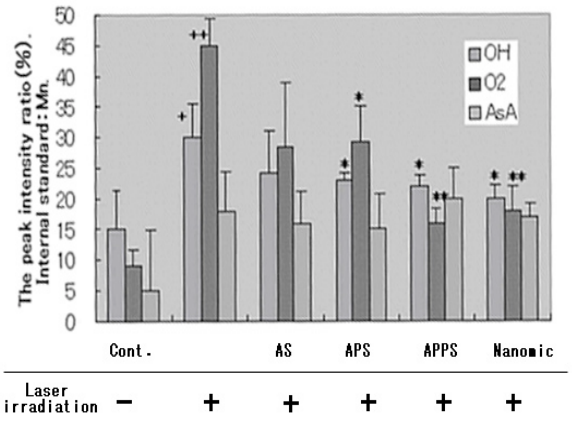

Fig.3: Generated level of Hydroxyl radicals(OH), superoxides (O2) and ascorbyl radicals(AsA) in hairless mice skin by YAG laser photo-rejuvenation and suppression by various ascorbates application. v.s.Cont :+ p<0.05,++ p<0.01 , v.s. irradiation cont.: * p<0.05,** p<0.01.

4, Effect of NanoSphere on UV radiation

We developed various encapsulated APP/lipid samples and assessed their free-radical scavenging ability by the ESR-ST method (spin trap agent: DMPO). We confirmed that NanoSphere C scavenged hydroxyl radicals and methyl radicals generated in this reaction system.

An experiment using the ESR-ST method showed that NanoSphere E,Q or A (Table.1) could scavenge super oxide generated by the xanthine-xanthine oxidase system . We plan to develop a NanoSphere series that is able to scavenge many kinds of free radicals and mix these agents to meet the needs of cosmetic manufacturers and doctors. The applications and effects of members of the NanoSphere series that are already available are shown. It is known that ascorbyl As radicals are generated when concentrated NanoSphere C (5%) is mixed with skin extract and irradiated with UV light at 310 nm. UV light therapy (310nm) has been used for a treatment for psoriasis, atopic dermatitis, pustulosis palmoplantaris or vitiligo conventionally at a dermatology clinic in Japan. It was recently confirmed that generation of As radicals is suppressed when fullerene (Radical sponge™ ,VitaminC60Bio research Co.,Tokyo, Japan) is added to concentrated NanoSphere C at a final concentration of 1% (Fig.4.). Fullerene is known to persistently suppress the generation of a variety of radicals, such as hydroxyl radicals, superoxides, and hydroxyperoxides. In the present study, we confirmed that fullerene also effectively scavenges UV-induced As radicals. By mixing NanoSphere C with fullerene, we developed NanoSphere CF, which had a wider radical-scavenging spectrum than NanoSphere C. It is thought that NanoSphere CF can be used to produce superior antioxidant capsules.

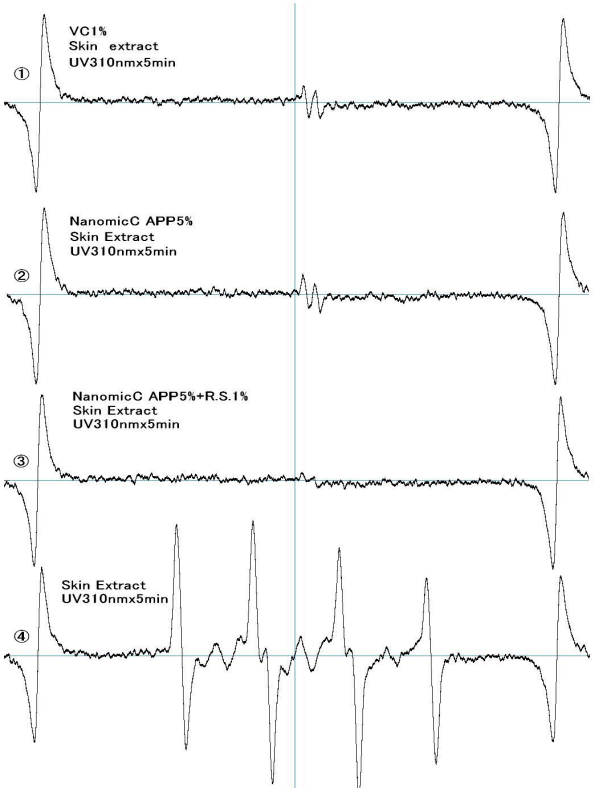

Fig4. ① Free radical signals were detected by electron spin resonance when 1% ascorbic acid was added to mouse skin extract and irradiated with UV light at 310 nm, confirming the peak of As radicals. ② As radical signals were confirmed when 5% NanoSphere C was added instead of ascorbic acid under the same conditions as ①. ③ As radicals were scavenged when 5% NanoSphere C was added together with fullerene. ④ A high hydroxy radical peak was obtained when skin extract was irradiated with UV light.

As described above, we showed that APP/lipid microcapsules could scavenge hydroxyl radicals, super oxide, and methyl radicals. Ascorbic acid radicals were not generated when APP/lipid microcapsules were added, indicating the strong free-radical scavenging ability of the microcapsules. APP are Ascorbic acid derivatives with surfactant properties that show efficient tissue uptake, and thus are new functional materials that possess strong active-oxygen scavenging activity and also promote collagen synthesis.In the field of cosmetic dermatology, the number of clinics that recommend APP lotions or creams as home-care products after laser treatment is increasing, and these clinics have received favorable feedback from their patients. APP contain lipid groups, and therefore provide a stronger skin

Table 1 Nanomic series and application.

Product name	Main ingredient	Scavenge active oxygen	Object
Nanomic C	APP	Hydroxyl radical	Whitening, Acne
Nanomic E	APP、Water soluble vitamin E	Hydroxyl radical Super oxide	Anti-aging, Removing hair Climacteric disturbance
Nanomic Q	CoenzymeQ10 Biotin, APP	Super oxide	Dry skin、Atopic dermatitis
Nanomic A	Astaxanthin, APP	Super oxide Singlet oxygen	Wrinkle
Nanomic F	Fullerene, APP	Super oxide Hydroxyl radical Methyl radicals	Total Anti-aging

barrier compared with ordinary water-soluble Ascorbic acid derivatives and also cause less drying of the skin than other vitamin C containing lotions. APP are the latest Ascorbic acid derivatives with the properties of vitamin C. We hope to establish an anti-aging drug with an effective redox balance, in which water-soluble and fat-soluble redox molecules resonate with each other due to the amphiphilic properties of APP, and we plan to expand our research into various industrial fields such as cosmetics and drugs.

Acknowledgement

The sample of APPS (Ascorbyl-2-phosphate-6-palmitate, Apprecier™) was offered from Showa Denko k.k. (Tokyo, Japan), and the sample of Fullerens (Radical sponge™) was offered from VitaminC60Bio research Co. (Tokyo, Japan). We would like to express our deep thanks to Director Kitaguni k.(Showa Denko), Director Tuduki T.(Showa Denko), President Shishido K. (VitaminC60Bio research) and Director Matsubayashi K. (VitaminC60Bio research).

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