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Consumer Care

High Purity Hydroxytyrosol Protects Skin Cells from Environmental Stressors and Increases *in-vitro* Cell Viability

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Abstract

Olive oil and its various polyphenols have long been known to have internal and topical health benefits. Hydroxytyrosol is one of the main actives found in olives and has multiple benefits for protecting skin cells against environmental stresses. Our research has found that hydroxytyrosol is able to reduce reactive oxidative species and protect against photoaging. An *in vitro* study found that hydroxytyrosol increases cell viability of fibroblasts after UVA and UVB irradiation. Part of the mechanism of this increased cell viability may be through hydroxytyrosol reducing the generation of reactive oxygen species (ROS) under UV irradiation. This detected reduction in ROS may decrease the damage of cellular structures and processes within the cells. ROS and UV exposure trigger matrix metalloproteinase (MMP). Hydroxytyrosol was also found to reduce the level of MMPs. This highly purified polyphenol can help protect the skin from environmental stress.

Introduction

Aging is a complex phenomenon characterized by progressive decline in cellular functions. Although aging is almost universally conserved among all organisms, the underlying molecular mechanisms of aging remain largely obscure. Many theories of aging have been proposed, including the free-radical and mitochondrial theories of aging. Both theories hypothesize that cumulative damage to the cell, mitochondria and mitochondrial DNA (mtDNA) caused by reactive oxygen species (ROS) is one of the causes of aging.

ROS are highly reactive molecules that consist of a number of diverse chemical species including superoxide anion (O2 –), hydroxyl radical (·O H), and hydrogen peroxide (H2O2). Because of their potential to cause oxidative deterioration of DNA, protein, and lipids, ROS have been implicated as one of the causative factors of aging. ROS are generated mainly as by-products of mitochondrial respiration and exposure to UV [1, 2]. Details of the precise relationship between ROS-induced damage and aging remain to be elucidated.

Chronic exposure to sunlight is a significant extrinsic aging factor. Generation of ROS and modifications of DNA and other critical cellular macromolecules by UV are damaging to the skin [1]. UVA exposure of fibroblasts stimulates expression of matrix metalloprotease (MMP)-1, which degrades collagen [3]. Upon exposure to UV light, there is an increase of ROS in the skin, along with an increase of MMP-1 and a potential decrease in cell viability. These may eventually lead to hyperpigmentation, uneven skin texture and sagging skin.

There are extensive literature references for plant based compounds that are antioxidants and that may mitigate environmental stressors. Numerous journal articles have been written on the health benefits of olives. The health benefits of olives and olive oil have been known since Neolithic times. One of the major phenols in oils is hydroxytyrosol [4, 5]. Hydroxytyrosol has been noted as a skin antioxidant, to ameliorate oxidative stress, and to help protect against UV induced apoptosis [6, 7]. We synthesized a greater than 90% pure hydroxytyrosol (HTS) that was colorless, stable and easy to use in finished cosmetic formulations. Since HTS is known to be an antioxidant, we hypothesized that hydroxytyrosol would be able to protect against photoaging (Fig. 1). Along with the well-known antioxidant EGCG from green tea and Trolox, a hydrophobically-modified Vitamin E derivative used as a standard in ORAC antioxidant studies, we tested if hydroxytyrosol was able to reduce ROS, increase cell viability and decrease MMP-1, all of which are negatively affected upon UV exposure.

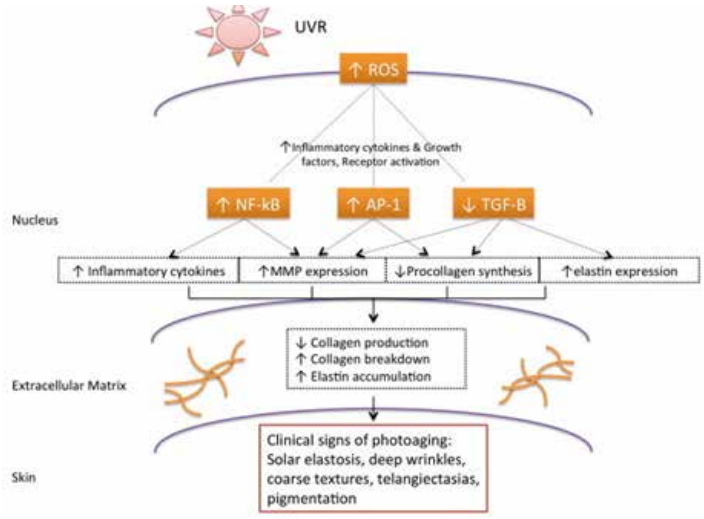

Fig. 1. Hydroxytyrosol is a known antioxidant. Our hypothesis was hydroxytyrosol would be able to reduce reactive oxygen species in cells exposed to UV light, which may increase cell viability and also prevent downstream undesired effects of exposure to UV light, such as the increase of MMP-1. Figure adapted from Chen et al (2012) [8].

Materials and Methods

Cells irradiated with UV light undergo oxidative stress which ultimately, if not counteracted, leads to cell death. UV light generates reactive oxygen species (ROS) which, when accumulating, lead to damage of cellular structures and processes. Matrix metallo-proteinases (MMP) are induced by ROS and are responsible for degrading extra-cellular matrix damaged through oxidative stress. Cells can take their own countermeasures against ROS by certain antioxidative metabolites and enzymes. Antioxidants can be added externally and reduce cellular ROS and the release of MMP-1 from cells. This will be employed here to determine antioxidative activity of substances.

Adult normal human epidermal keratinocytes (NHEK-ad, Lonza, cat. # 192627) and adult normal human dermal fibroblasts (NHDF-ad, Lonza, cat. # CC-2511) were provided by Lonza Clonetics™ and cultured according to provider's protocol. (-)-Epigallocatechin gallate (EGCG, Cayman Chemical, cat. # 70935), Trolox (Sigma-Aldrich, cat. # 238813), and Hydroxytyrosol (Lonza) were used for all three studies.

Cell Viability and ROS Study

Cell Viability experiment used ViaLight™ Plus Cell Proliferation and Cytotoxicity BioAssay Kit (Lonza, cat. #LT07-221), performance according to provider's protocol. ROS study used Dihydrorhodamine 123 (DHR, Sigma-Aldrich, cat. # D1054-2MG). 2x104 NHEK-adult and 5x104 NHDF-adult cells/ 96 well were seeded 24h before irradiation. 10 µM fluorescent indicator dye DHR was loaded 45 min right before UV irradiation. The Cells were washed twice with HBSS buffer. Medium with 20mM HEPES buffer was added to the cells before irradiation. UVA (365 nm) or UVB (312 nm) was used for irradiation. For NHEK-adult: 5 Joule/ cm2 UVA and 2 Joule/ cm2 UVB was used. For NHDF-adult: 10 Joule/ cm2 UVA and 1 Joule/ cm2 UVB was used. After irradiation, the cells were washed twice with HBSS. The cells were loaded with testing materials and reference compounds for 3 hours after irradiation. Hydroxytyrosol 0.003% (w/v), Epigallocatechin gallate 0,005% and Trolox 125µg/ml (NHEK-adult 24h 45min) were the antioxidants applied to the cells. For the cell lysis buffer, half of the cell lysate was partitioned for measuring DHR fluorescence (RFU, relative fluorescence units) in plate reader (BMG Labtech FLUOstar Omega). The other half of the cell lysis buffer was used for viability testing by ViaLight™ Plus Kit measuring luminescence (RLU, relative luminescence units) in plate reader (BMG Labtech FLUOstar Omega).

Each run was done in triplicates. From each run means of the triplicates were calculated. Means of fluorescence (ROS) data were divided by the means of luminescence (viability) data. Those results from antioxidant treated samples were related to results of samples treated with carrier only (solvent DMSO) in order to gain "% ROS". In figures 1 & 2 the decrease of ROS (100 % minus %ROS) is shown.

Matrix metallo-proteinases study

Human Total MMP-1 ELISA kit (R&D-Systems, cat. #DY901) was used according to provider's protocol. 1x105 cells per well were seeded in a 96 well plate with Lonza complete medium (with supplements) 28 hours before irradiation. Cells were washed twice with HEPES Buffered Salt Solution (HBSS). 30 µl of PBS was added per 96 well. Cells were irradiated with 10 J UVA (365 nm) or 100 mJ UVB (312 nm). The supernatants were discarded. The test materials, dissolved in Lonza basal medium (without supplements), were loaded into the cells immediately after irradiation. The following antioxidants were used, hydroxytyrosol (HTS) 0.001% (w/v), Epigallocatechin gallate (EGCG) 0.0005%, and Trolox 0.0125% (=125µg/ml). The cells were incubated for 24h under cell culture conditions. Supernatants were harvested for analysis of matrix metallo-proteinase 1 and used in the human Total MMP-1 ELISA kit measuring absorbance in a plate reader (Tecan Genius Spectra FLUOR Plus). Cells were lysed for viability testing using ViaLight™ Plus Kit measuring luminescence (RLU, relative luminescence units) in a plate reader (BMG Labtech FLUOstar Omega). Each run was done in triplicate. From each run means of the triplicates were calculated. For calculation of "fold induction" and "percent viability" data was compared to data of corresponding non-irradiated samples.

Results and Discussion

Hydroxytyrosol, trolox and EGCG are all well-known antioxidants. Because UV exposure to cells causes an increase of cellular ROS, a decrease in cell viability and an increase of MMPs, the three antioxidants were tested to see if they could mitigate these deleterious effects of UV exposure. Hydroxytyrosol, Trolox and EGCG were applied to fibroblasts and keratinocytes 24 hours before exposure to either UVA or UVB. Cell viability was then determined. Hydroxytyrosol and EGCG both were able to substantially increase fibroblast cell viability upon exposure to both UVA and UVB (Fig. 1). Trolox caused a lower increase in viability. For keratinocytes, hydroxytyrosol was the antioxidant able to cause the highest increase of cell viability in both UVA exposed cells and cells without UV exposure (Fig. 2). Since hydroxytyrosol and EGCG increased cell viability, it was hypothesized that they may be decreasing cellular ROS production when the cells were exposed to UV irradiation.

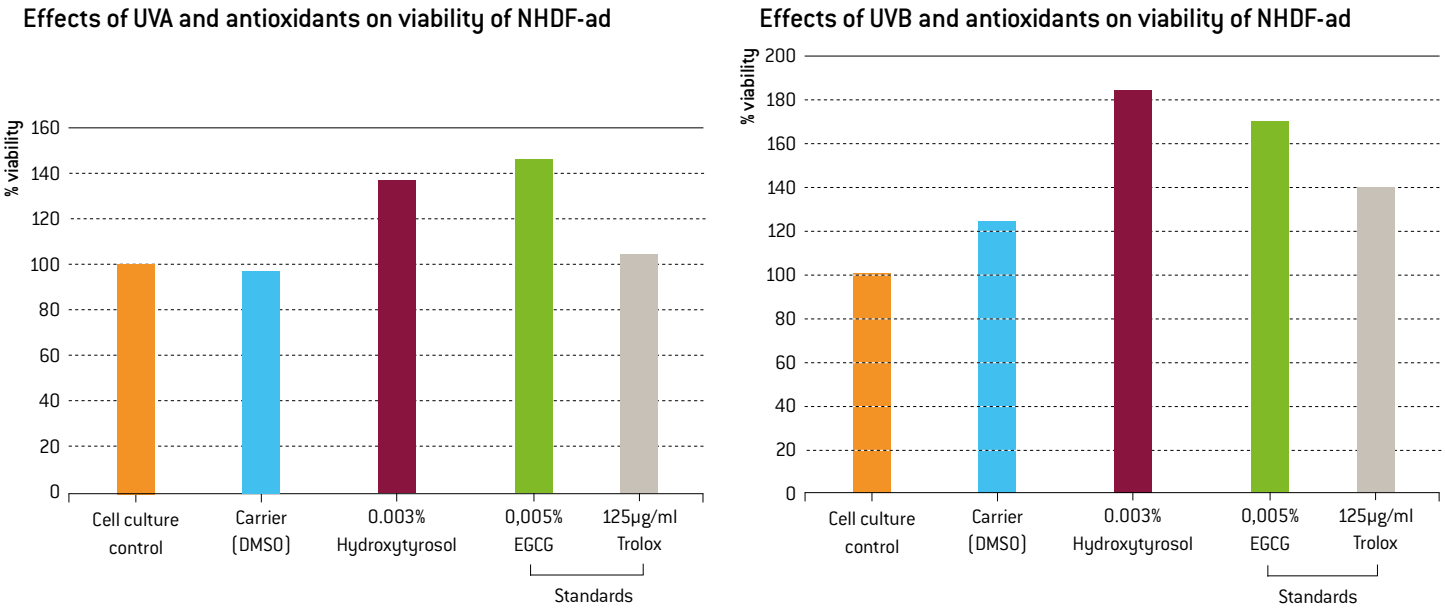

Fig. 1

Hydroxytyrosol increased viability of fibroblasts exposed to UVA and UVB. The cells were treated with the antioxidants for 24 hours before irradiation and measurement of viability assays were done immediately after irradiation. Hydroxytyrosol caused an increase in cell viability. Hydroxytyrosol caused a higher increase in cell viability than Trolox and a similar effect to EGCG.

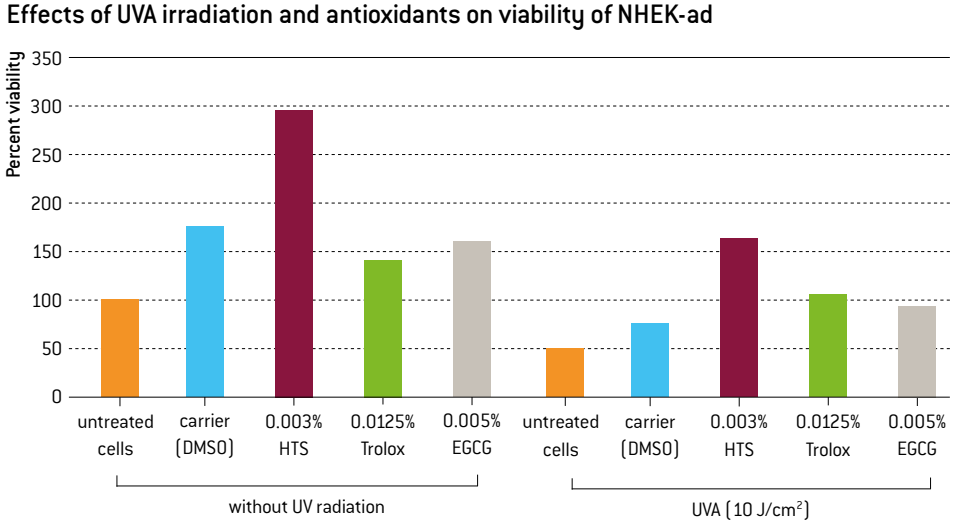

Fig. 2

Hydroxytyrosol increased viability of keratinocytes both exposed to UVA and with no UV exposure. Hydroxytyrosol demonstrates a 3-fold increase in viability of irradiated as well as in non-irradiated cells compared to untreated controls. Standard antioxidants Trolox and EGCG showed only a 1.5 to 2-fold increase. Application of hydroxytyrosol improves viability of keratinocytes after UVA-exposure.

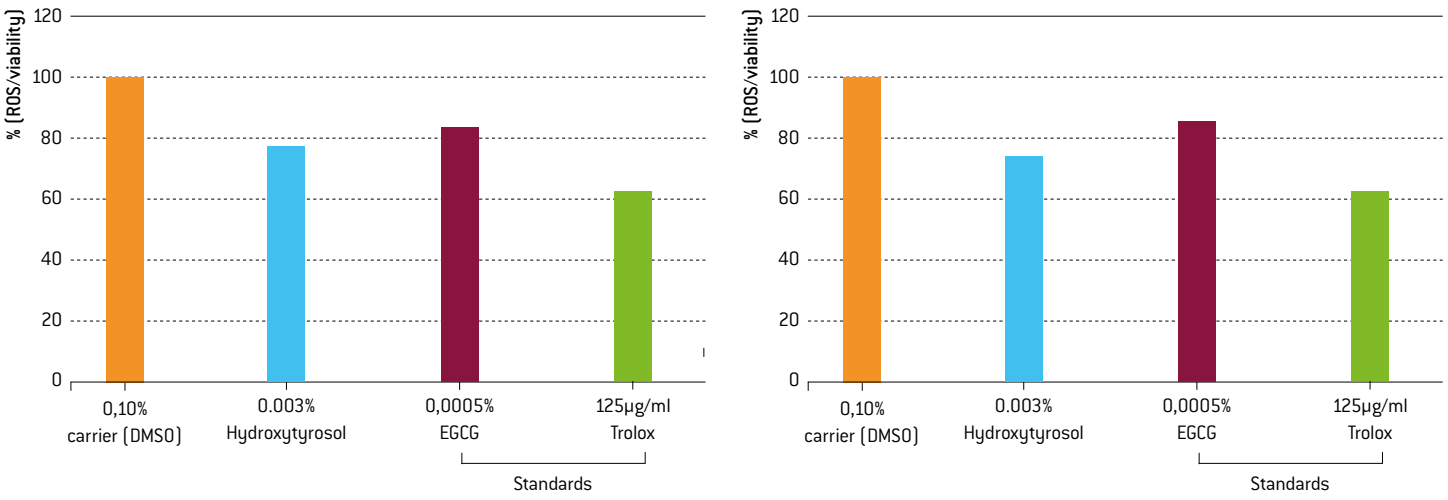

Fig. 3

Hydroxytyrosol when applied to keratinocytes reduces the generation of reactive oxygen species (ROS) under UVA and UVB irradiation. UV light generates ROS which can lead to damage of cellular structures and processes. Hydroxytyrosol performed comparably to EGCG and Trolox to minimize ROS after irradiation with both UVA and UVB.

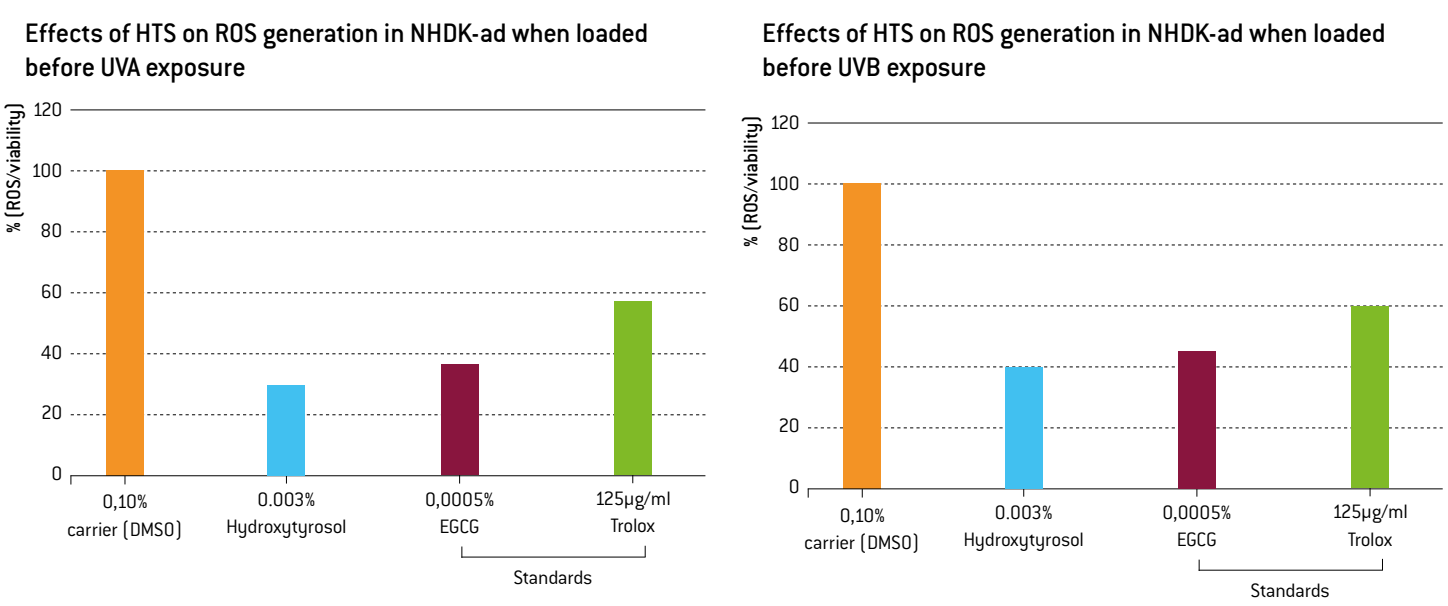

Fig. 4

Hydroxytyrosol reduces generation of ROS in fibroblasts exposed to UVA and UVB. Hydroxytyrosol performed better than EGCG and Trolox to reduce ROS generated in fibroblasts.

Hydroxytyrosol decreases MMP-1 protein expression

Exposure to UV irradiation is known to stimulate MMP-1 protein expression, leading to increased degradation of collagen and the faster formation of skin wrinkles. Since ROS induce MMP-1 and the antioxidants were able to decrease ROS production with varying degrees, the three antioxidants were applied to keratinocytes immediately after exposure to UVA for 24 hours. Hydroxytyrosol was the only antioxidant able to decrease induction of MMP-1. EGCG and Trolox both lead to an even higher increase of MMP-1 compared to the irradiated, untreated control. All three antioxidants were able to decrease ROS production in the cells. An explanation of why only hydroxytyrosol was able to decrease MMP-1 may be that MMP-1 induction is caused by multiple factors, only one being the level of ROS. Hydroxytyrosol may be able to impact multiple MMP-1 upregulation pathways compared to EGCG and Trolox only decreasing ROS.

Induction of MMP-1 Release from NHEK-ad by UVA (related to non-irradiated controls)

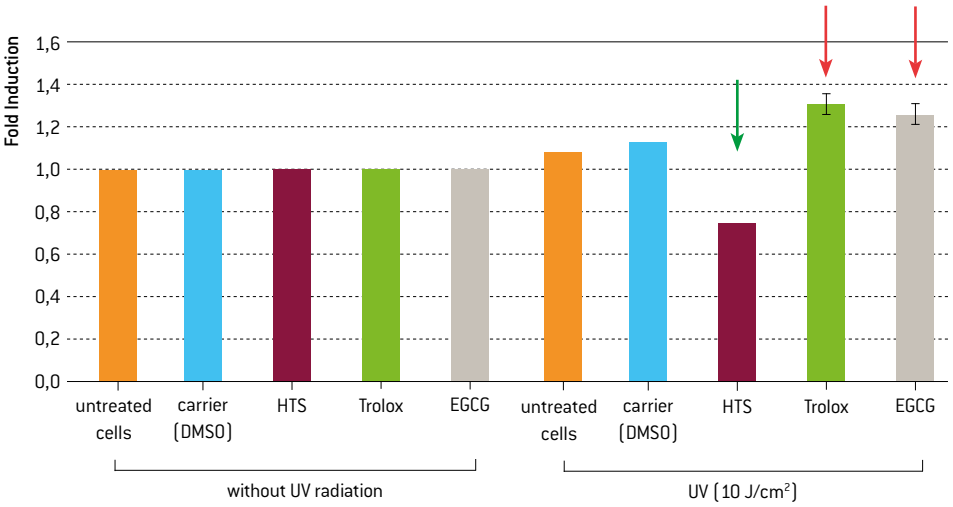

Fig. 5
Hydroxytyrosol reduces expression of MMP-1 in keratinocytes better than Trolox and EGCG.

Conclusion

Exposure to UV irradiation represents a major environmental threat to the skin, increasing its risk of photo oxidative damage by ROS, cellular damage, and even cellular death. UV exposure is known to increase ROS levels in the skin, which has also been correlated to increasing apoptosis. The three antioxidants hydroxytyrosol, EGCG and Trolox were tested to see if they could mitigate some of the negative effects of exposure to UV. In fibroblasts, both hydroxytyrosol and EGCG were able to increase cell viability, with Trolox increasing viability to a lesser degree. In keratinocytes, hydroxytyrosol performed the best at being able to prevent cell mortality. All three antioxidants decreased ROS within the cell with hydroxytyrosol causing the largest decrease in ROS in fibroblasts. Only the antioxidant hydroxytyrosol was able to reduce MMP-1 expression upon exposure to UVA irradiation. These three studies imply that topical application of hydroxytyrosol to the skin may mitigate UV damage and decrease accelerated aging in the cells.

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