Antioxidants from grape seeds protect hair against reactive oxygen species

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Summary
Oxygen based radicals are predominantly generated in the water phase. Therefore, it is very important to use not only lipophilic antioxidants but also water-soluble radical scavengers in cosmetic formulations. Vitamin C, glutathion and different enzymes are natural water-soluble antioxidants. Unfortunately, most of them are not stable in cosmetic formulations.
We studied a new water-soluble antioxidant based on procyanidins isolated from grape seeds. These procyanidins together with tocopherols form a very powerful antioxidant-complex which is stable in water.
The activity of this antioxidant-complex was investigated by a novel method whereby the toxicity of UV-A irradiated lipids is measured in a cell culture assay. The test results confirm that the combination of water-soluble and oil-soluble antioxidants performs extremely well.

It has been recognized that human hair is exposed to different reactive oxygen species (ROS) generated in wet hair. Thus, we investigated the activity of grape seed antioxidants in different hair care applications. Our experiments show that UV-irradiation of wet hair or drying with a hair dryer can significantly damage it. However, the pretreatment of hair with the antioxidant-complex offered a good protection.
The antioxidant-complex even worked in a hair conditioner used in a rinse-off experiment. Thus, we conclude that the application of antioxidants based on procyanidins from grape seeds in combination with tocopherols can protect hair against reactive oxygen species originating from environmental hazards.

Introduction
Radicals induced by environmental factors, such as air pollution, ozone and sunlight cause skin to age prematurely. Thus, the use of antioxidants and radical scavengers in skin care is very important and widespread. In addition, antioxidants are essential components in cosmetic formulations to increase the shelf life of the products by reducing the oxidative degradation of sensitive ingredients.
Oxygen based radicals are predominantly generated in the water phase. They attack unsaturated lipids, proteins and nucleic acids in the cell. All living organisms protect themselves against them with a combination of lipid-soluble antioxidants, such as vitamin E and carotenoids and water-soluble antioxidants, such as vitamin C, glutathione and different enzymes.

Unfortunately, most of these water-soluble antioxidants are very unstable in cosmetic preparations. Thus, so far, only oil-soluble antioxidants are frequently used in cosmetic formulations.

Water-soluble antioxidants from grape seeds

Grapes, specially the red species such as Pinot Noir, are extraordinary rich in polyphenols. By far the largest portion is found in the seeds in form of procyanidins. Catechins and epicatechins are the basic units of the procyanidins which consist of up to 50 monomers joined by oxidative condensation. Procyanidins are very powerful radical scavengers and often more effective than vitamin C and E.

For our experiments, we isolated procyanidins from grape seeds by a two step process. The final extract contains procyanidins in a mixture of water, glycerin and alcohol.

Antioxidant activity of grape seed extract (GSE)

We investigated the protective (antioxidant) effect of GSE against UV-A-induced lipid peroxidation. For our experiments, we used borage oil containing polyunsaturated fatty acids which are very susceptible to lipid peroxidation. Upon UV-A irradiation, these fatty acids form malonaldehyde which can be detected by the thiobarbituric acid assay (1). Peroxidation occurs very rapidly if the oil is dispersed in water. Therefore, we prepared oil-in-water nanoemulsions encapsulating borage oil. The nanoemulsions are stabilized with phospholipids from soy. They are prepared by high-pressure homogenization using a microfluidizer as described by Mayhew (2). A droplet size of less than 60 nm can be achieved with a pressure of 1200 bar. These nanoemulsions are transparent and can therefore be UV-irradiated without light scattering (3).

The dispersions containing borage oil were UV-irradiated for three hours to generate high concentrations of lipid peroxides measured as thiobarbituric acid reacting substances (TBARS). The formation of lipid peroxides can be inhibited by the addition of antioxidants to the nanoemulsions.

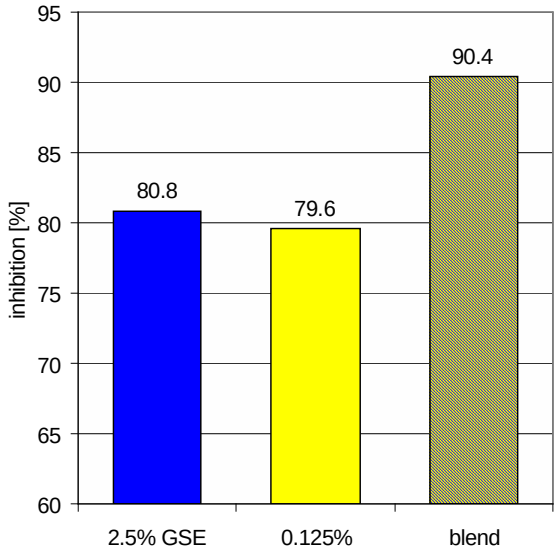

Fig. 1: Inhibition of UV-A induced formation of lipid peroxides in borage oil with grape seed extract and tocopherols.

A nanoemulsion encapsulating borage oil was UV-A irradiated for 3 h (365 nm, 7 mW/cm^2) and subsequently analyzed for lipid peroxides by the thiobarbituric acid assay. The inhibition of this lipid peroxidation by grape seed extract, tocopherols and the combination of both antioxidants (blend) at the indicated concentrations is shown.

Figure 1 shows that our grape seed extract (GSE) significantly inhibits the formation of lipid peroxides at a concentration of 2.5%. This efficacy is very similar to that of tocopherols at 0.125%. Figure 1 shows that their combination further improves the efficacy and results in an almost 100% inhibition of lipid peroxidation.

Biological activity of antioxidants

Antioxidants are usually characterized by chemical reactions. In cosmetic applications however, they are to become part of biological interactions. Thus, we developed a new method to study the biological activity of antioxidants. In this assay, we deliver lipids into cells using our nanoemulsion as a carrier system (4).

In our work, TK6 lymphoblastoid cells were incubated with different concentrations of UV-A irradiated nanoemulsions with encapsulated squalene, one of the most important skin lipids. After two days, the number of surviving cells was compared to those of cultures which had not been treated with the nano-emulsion or which had been treated with a not-irradiated nanoemulsion.

We found that squalene encapsulated in nanoemulsions is very well tolerated by the cells. However, after UV-A irradiation these squalene nanoemulsions were very toxic. In cell cultures which contained 1% of the UV-A irradiated squalene nanoemulsion 50% of the cells died (data not shown).

We studied the properties of our antioxidants to inhibit the formation of UV-A induced toxic compounds in these squalene nanoemulsions.

Figure 2 shows that GSE alone at a concentration of 0.5% and tocopherols alone at a concentration of 0.025% did not inhibit the formation of toxic compounds to a reasonable extent. However, the blend of both antioxidants shows a very good biological activity. The combination of the water-soluble and the oil-soluble antioxidant was able to inhibit the formation of toxic compounds by 100%.

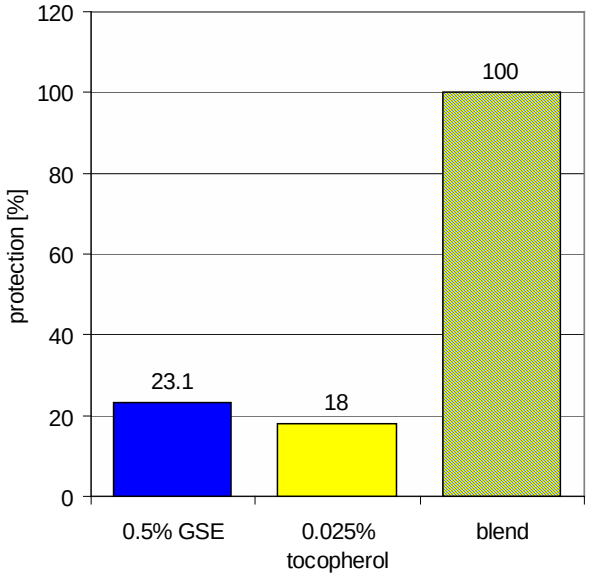

Fig. 2: Biological activity of grape seed extract (GSE), tocopherols and the combination of both antioxidants (blend) in a cell culture experiment.

Squalene nanoemulsions were irradiated with UV-A light, whereby the first sample was protected against the formation of toxic compounds by 0.5% GSE, the second by 0.025% tocopherols and the third by the blend of both antioxidants. A nanoemulsion without antioxidants and a not irradiated emulsion served as controls. TK6 lymphoblastoid cells were treated with these nanoemulsions at a concentration of 1% for two days. Then, the cell counts were determined. The protecting effect of the antioxidants is expressed as increase of surviving cells in % compared to a culture which was treated with a squalene nanoemulsion, which was not protected by antioxidants.

Antioxidants in hair care

Human hair is exposed to a number of chemical and physical hazards, such as combing, brushing, heating, drying and different chemical treatments which render it brittle and dull. Moreover, UV-light, predominantly UV-A irradiation, causes significant hair damage. The culprits are called ROS (reactive oxygen species). Most of these ROS are generated in the water phase; thus wet hair is particularly susceptible. Attention should be paid to the sulfur-containing amino acid cystein which is located in the rigid outer layer of the hair structure, the cuticula. But also the aromatic amino acids tryptophan and tyrosine are easily degraded by light. Damaged hair has a porous surface and is thus more sensitive to free radicals.

We investigated the properties of our grape seed extract in hair care applications. Grape seed extract was mixed with 5% natural tocopherols using a solubilizer (PEG-40 Hydrogenated Castor Oil). The efficacy of this blend of water-soluble and oil-soluble antioxidants in protecting hair against protein damage induced by UV-irradiation, seawater, detergents and the use of a hair dryer was studied:
Samples of European hair were washed under standardized condition with a shampoo. The hair was rinsed with water and incubated in a 2.5% dilution of our antioxidant blend in water. The samples were rinsed again with water and then subjected to UV-irradiation and hair dryer treatments (10 cycles). The hair samples were then extracted with a 2% SDS solution or artificial seawater. To analyze hair damage, the concentration of proteins and peptides in the seawater and the SDS extracts was determined.

All experimental hazards led to a significant damage of the hair samples (data not shown). Already the repeated treatment of wet hair with a hair dryer caused protein degradation due to the heat activation of dissolved oxygen molecules in the water film on wet hair. However, the antioxidant blend based on grape seed extract and tocopherols was able to offer an excellent protection in all experiments (5), even though the antioxidants were used in rinse-off treatments only.
To further elucidate the efficacy of our antioxidant mix, we formulated the blend into a hair protection fluid and a conditioner (see table 1).

Protection of hair against UV-irradiation

The hair protection fluid (see table 1) containing 1.0 or 1.5% of our antioxidant blend made up of grape seed extract and tocopherols was used to study UV-induced hair damage.
Hair samples were washed with shampoo and rinsed with water. The hair was then treated with the two protection fluids or the corresponding placebo formulation under standardized leave-on conditions. The samples were subjected for 20 minutes to UV-light (Ultra-Vitalux lamp Osram) and then extracted with a 2% SDS solution. After filtration, the protein concentration in the different extracts was determined.

Table 1

Hair Protection Fluid (INCI)	**%**	**Conditioner (INCI)**	**%**
Aqua	ad 100	Aqua	ad 100
PEG-60 Hydrogenated Castor Oil	0.80	Cetearyl Alcohol	4.50
Phenoxyethanol	0.80	Behentrimonium Chloride	3.20
Sodium Benzoate	0.35	Glyceryl Stearate	1.00
Citric Acid	0.15	Isopropyl Alcohol	0.68
Polyquaternium-4	0.15	Hydroxyethylcellulose	0.30
Benzophenone-4	0.10	Isopropyl Myristate	0.30
Cetrimonium Chloride	0.05	Hydroxypropyl Guar Hydroxypropyl-trimonium Chloride	0.20
Alcohol Denat.	0.01	Cetrimonium Chloride	0.175
Polyquaternium-11	0.05	Citric Acid	0.10
Disodium EDTA	0.045		
Hydrolized Silk	0.009		
Antioxidant blend	**1.0 or 1.5**	**Antioxidant blend**	**1.0 or 1.5**

Figure 3 shows that the UV-irradiation caused significant damage in the hair sample treated with the placebo formulation. The protection fluid containing the antioxidants was able to reduce this damage in a concentration-dependent manner.

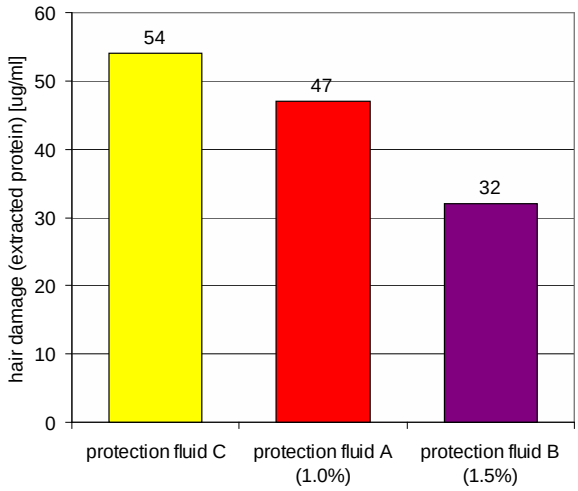

Fig. 3: Protection of hair against UV-A irradiation with a protection fluid containing an antioxidant blend*.*

Hair samples were sprayed with a protection fluid containing 1 % (A) or 1.5 % (B) of the antioxidant blend and then irradiated with UV-light for 20 min (Ultra-Vitalux-lamp Osram). After the irradiation, the protein and peptides of the hair were extracted with a 2% SDS solution and determined by the Bradford assay. The protection fluid without antioxidants (C) served as a control.

Protection of hair against ROS induced by a hair dryer

Obviously, drying wet hair with a conventional dryer damages the hair structure significantly. This damage is not primarily caused by the heat itself but is induced by reactive oxygen species generated in the water film on wet hair. Thus, the application of water-soluble antioxidants should offer adequate protection against this particular hazard hair is subjected to every day.

We investigated the effect of our antioxidant blend containing water-soluble grape seed extract and oil-soluble tocopherols in a hair conditioner (see table 1). Hair samples were washed with shampoo and rinsed with water. The samples were then soaked in a conditioner containing 1.0 or 1.5% of the antioxidant blend or the corresponding placebo formulation. After 5 minutes, the hair was rinsed with water and dried with a hair dryer. The dry hair was watered and dried again and extracted with a 2% solution of SDS after 5 hair dryer cycles. The protein concentrations in the different extracts show that the hair drying experiment significantly damages hair (figure 4) in a manner similar to the damage caused by UV-irradiation (figure 3).

Figure 4 shows that, again, the application of the antioxidant blend in a conditioner can offer a profound protection against this oxidative stress. A single treatment with a conditioner containing 1.5% of the antioxidant blend already results in a 50% protection.

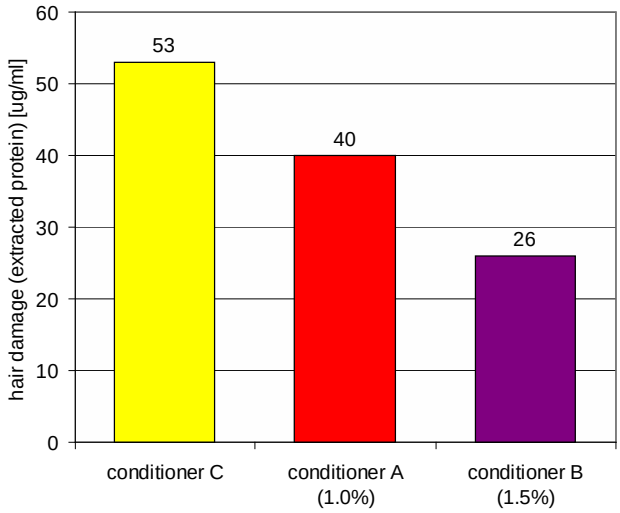

Fig. 4: Protection of hair against oxidative stress induced by a hair dryer with a conditioner containing a blend of antioxidants*.*

Hair samples were pretreated with a conditioner containing 1 % (A) or 1.5 % (B) of the antioxidant blend for 5 minutes and rinsed with water. Then, the hair samples were dried by a hair dryer and sprayed with water again 5 times. Then the proteins and peptides of the damaged hair were extracted with a 2 % SDS solution and determined by the Bradford assay. A conditioner without antioxidants (C) served as a control.

Conclusion

The experiments show that our nanoemulsion technique is very well suited to study the biological effects of antioxidants in cell toxicity assays. With this new method we could prove that it is absolutely necessary to combine water-soluble and oil-soluble antioxidants in order to achieve an adequate biological protection against oxidative stress.

We found that a blend of grape seed extract and natural tocopherols is a highly effective mixture of water-soluble and oil-soluble antioxidants. This antioxidant blend was able to inhibit the formation of UV-induced lipid peroxides in borage oil and to protect nanoemulsions with encapsulated squalene against the formation of cytotoxic compounds.

However, this antioxidant blend is also of special interest given its excellent performance in hair care applications. The application of our blend of water-soluble and oil-soluble antioxidants proved to be very effective in protecting hair against the detrimental effects of different reactive oxygen species caused by sunlight (UV-A irradiation) and seawater. In addition, we showed that even ordinary blow-drying of wet hair can cause significant damage. Our blend of grape seed extract and tocopherols again offered adequate protection.

We conclude that up to date cosmetic formulations - skin or hair care products - must contain not only oil-soluble antioxidants but also water-soluble products, such as grape seed procyanidins, to offer a broader protection against environmental oxidative stress.

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