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Cell Toxicity of UV-A Irradiated Squalene

Keywords: UV-A, Squalene, Lipide

Summary

UV-A irradiation is known to induce oxidative stress to the skin. The formation of free radicals causes skin lipid peroxidation and other harmful effects. Squalene is one of the main lipid components of the sebum and is particularly susceptible to photo-oxidation. To study the biological properties of UV-irradiated lipids, we have developed a novel method to deliver lipophilic compounds to cell cultures. The delivery system is based on a lipid nanoemulsion which is stabilized by phospholipids. In our experiments, we have studied the toxicity of a squalene nanoemulsion before and after UV-A irradiation on TK6 lymphoblastoid cells. We observed that UV-A exposure of squalene led to the formation of lipid peroxides and significantly increased the toxicity of the nanoemulsion to the cells. The addition of antioxidants, such as tocopherol and grape seed extract can reduce the formation of peroxides. It also had a positive effect on the toxicity of the UV-A irradiated nanoemulsion. Our results show that the developed test system based on lipid nanoemulsions and cell cultures is a new biological tool to study UV-A induced modifications in lipids.

Introduction

Squalene is one of the major lipids in the stratum corneum. It was shown that even low doses of UV-A irradiation can cause the formation of squalene hydroperoxides in the skin (1) that can be measured by the chemiluminescence method (2, 3). Topical application of some antioxidants can protect the skin against this UV-A induced formation of lipid peroxides (1). An excellent protection against UV-A induced squalene peroxidation in volunteers could be obtained with sodium carboxymethyl betaglucan (CM-Glucan), a water-soluble beta glucan derivative prepared from yeast (4).
Squalene hydroperoxides and other lipid peroxides are thought to be harmful to the skin. The aim of this work is to study the in-vitro formation of peroxides in squalene and other lipids and to characterize their biological properties. We have developed a special carrier system based on a nanoemulsion to deliver lipids to cells. Thus the toxicity of lipids prior or subsequent to UV-A irradiation can be evaluated in a simple cell culture experiment.

Preparation and Properties of Nanoemulsions Suitable for Cell Culture Assays

In vitro testing of cosmetic and pharmaceutical compounds for biocompatibility or toxicity has rapidly gained recognition as it is quick, cost-effective and can replace experimental studies in animals.
In the past, efficient protocols for cell culture assays have been elaborated for the testing of compounds that are soluble in water. However, the characterization of lipophilic agents in such assays is much more difficult.
To overcome this limitation, we have developed special nanoemulsions to deliver lipophilic compounds to cell cultures. Nanoemulsions are oil-in-water dispersions with a very small vesicle size and are already used for example for the parenteral administration of certain drugs (5, 6). Our nanoemulsions were prepared by high pressure homogenization (1 200 bar) using a microfluidizer (M-110T, microfluidics, USA) as described by *Mayhew* (7).
To achieve a small particle size, we used a high ratio of phospholipids to oil to prepare the nanoemulsions (8). Under these conditions, a particle size of less than 60 nm in diameter could be obtained. The size of the nanoparticles was determined by photon correlation spectroscopy (autosizer 2c, Malvern, United Kingdom).
The vesicle size has a great influence on the optical appearance of the nanoemulsions. Preparations with particles bigger than 200 nm in diameter are white, even in diluted dispersions. Preparations containing particles of 100 nm appear opaque. A further reduction of the particle size to below 60 nm results in clear transparent oil-in-water dispersions. These latter preparations are ideal for cell culture assays because they do not disturb the visual observation of the experiment.

Composition of Nanoemulsions

Nanoemulsion 1	
Phospholipids	0.6%
Oleic acid triglyceride	0.5%
Squalene	0.5%
Nanoemulsion 2	
Phospholipids	0.6%
Oleic acid triglyceride	1.0%

All preparations had an average particle size of 60 nm. The nanoemulsions were filtered through 0.1 µm membranes (Millex-VV, Millipore, USA) to obtain sterile preparations suitable for cell culture experiments.

UV-A Induced Lipid Peroxidation

Transparent nanoemulsions are ideal to study the effect of UV-A irradiation on lipids. The dispersed oil phase can be irradiated with only very little light scattering. The small oil droplets of the nanoemulsion are very susceptible to the effects of UV-irradiation. **Fig. 1** shows the very simple experimental set-up which was used to study UV-A induced lipid peroxidation in nanoemulsions.

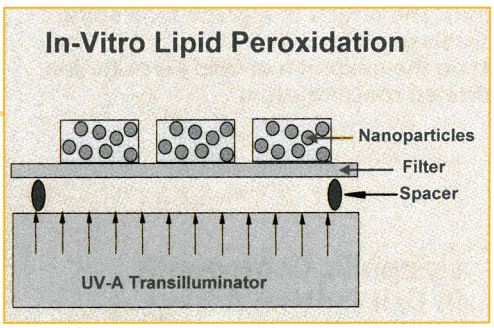

Fig. 1 Transparent nanoemulsions with an average particle size of 60 nm were UV-A irradiated on a transilluminator at 365 nm, 7mW / cm²

Squalene was encapsulated in a nanoemulsion together with oleic acid triglyceride as a carrier oil (Nanoemulsion 1). As a control, we prepared a nanoemulsion without squalene but with the carrier oil (Nanoemulsion 2). The nanoemulsions 1 and 2 were UV-A irradiated and subsequently analyzed by the thiobarbituric acid method and by the Fox assay (9) to measure lipid peroxides.

The UV-A irradiation of the nanoemulsion 1 containing squalene significantly increased the concentration of lipid peroxides measured by the Fox assay, whereas the peroxide level in the control nanoemulsion without squalene (Nanoemulsion 2) remained almost the same. The determination of lipid peroxides by the thiobarbituric acid method which is specific for polyunsaturated fatty acids did not show an increase of peroxides after UV-A irradiation in both nanoemulsions.

The formation of lipid peroxides in the squalene nanoemulsion measured by the Fox assay clearly depends on the dose of UV-A irradiation **(Fig. 2)**. To study the influence of antioxidants, we have used a 3 h UV-A irradiation of the nanoemulsions in the subsequent experiments.

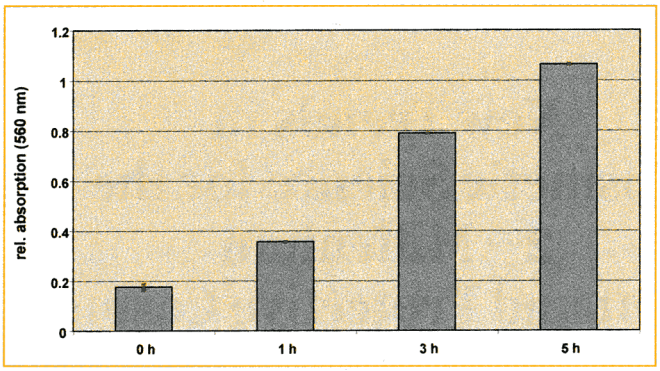

Fig. 2 Nanoemulsion 1 encapsulating squalene was exposed to UV-A irradiation (365 nm 7mW / cm²) and subsequently analyzed for lipid peroxides by the Fox method. The concentration of peroxides is shown as relative absorption at 560 nm for the different exposure doses

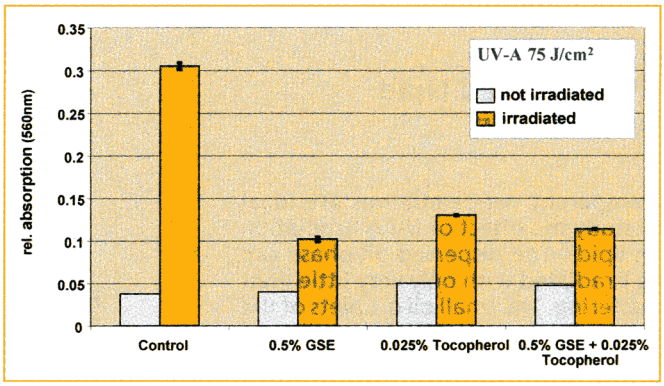

Fig. 3 Nanoemulsion 1 encapsulating squalene was UV-A irradiated for 3 h and subsequently analyzed for lipid peroxides by the Fox method. The concentration of peroxides before and after UV-A irradiation is shown as relative absorption at 560 m (control). The effects of a grape seed extract (GSE), tocopherol (dissolved in alcohol) and the combination of both antioxidants on the inhibition of lipid peroxidation is shown for the indicated concentrations

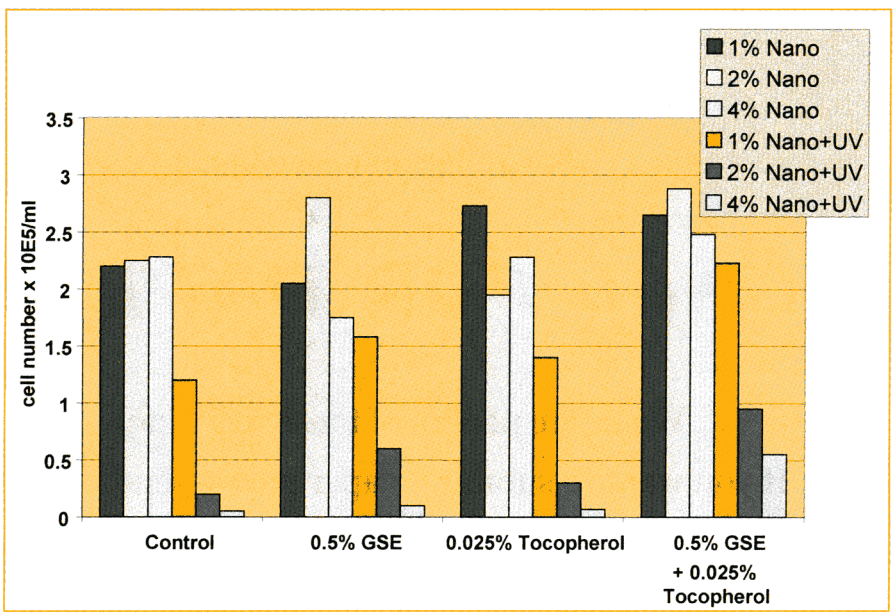

Fig. 4 TK6 lymphoblastoid cells were incubated with different concentrations of the squalene nanoemulsion which was UV-A irradiated for 3 h (75 J / cm²). As a control, the cells were also treated with the not irradiated nanoemulsion 1 at the same concentration. After two days, the viable cells were counted twice for two individual experiments (average number of cells is shown). The effects of a grape seed extract (GSE), tocopherol (dissolved in alcohol) and the combination of both antioxidants on the reduction of the cell toxicity is shown for the indicated concentrations

The encapsulation of lipophilic antioxidants and radical scavengers, such as tocopherol or coenzyme Q10, together with the squalene in the nanoemulsion can reduce the formation of UV-A induced lipid peroxides to some extent (data not shown). However, the addition of antioxidants to the water phase gave much better results. **Fig. 3** shows the effect of a grape seed extract and a tocopherol solution in alcohol which have been added to the water phase of the squalene nanoemulsion. Both antioxidants could significantly reduce the concentration of peroxides in the UV-A irradiated emulsions. But the combination of the two antioxidants did not further enhance the inhibitory effect.

Lipophilic Compounds in Cell Culture Assays

Our nanoemulsions, described above, have been successfully applied to characterize the biocompatibility and toxicity of various lipophilic compounds such as different oils, UV-filters and fragrances in cell culture tests (10). TK6 lymphoblastoid cells have proven to be an ideal cell line for these experiments since the cultures can tolerate relatively high concentrations of nanoemulsions.

TK6 lymphoblastoid cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 5% gentamycin and 10% horse serum (Gibco, Life Technologies, Basel, Switzerland) in sterile flasks at 37 °C in an incubator with 5% carbon dioxide. The nanoemulsions were added to the cell cultures at different concentrations. After two days of incubation, the number of cells were counted. The comparison with untreated cell cultures revealed the inhibitory effect of the different lipophilic compounds.

Toxicity of UV-A Irradiated Squalene Nanoemulsions

We saw that UV-A irradiation of squalene encapsulated in nanoparticles

leads to the formation of lipid peroxides and that the addition of antioxidants can inhibit this lipidperoxidation. To characterize the biological relevance of UV-A induced chemical changes in squalene, we have screened various samples in our cell culture assay using TK6 lymphoblastoid cells.

Nanoemulsion 1 encapsulating squalene was added to the cells at different concentrations. **Fig. 4** shows that the nanoemulsion which was not irradiated was well tolerated by the cells at the applied concentrations. However, the UV-A irradiated nanoemulsion was very toxic. The growth of the TK6 cells was significantly reduced in a concentration dependent manner. This proves that the UV-A irradiation of squalene leads to the formation of toxic compounds.

We have seen that the addition of the antioxidants tocopherol and grape seed extract could significantly reduce the in-vitro formation of lipid peroxides. Thus, we wanted to know if the application of these antioxidants can also reduce the toxicity of UV-A irradiated squalene in our cell culture assay. **Fig. 4** shows that the application of 0.5% grape seed extract or 0.025% tocopherol to the squalene nanoemulsion reduced the formation of toxic compounds induced by UV-A irradiation. However, the biological activity of these antioxidants is much less pronounced compared to the in-vitro effect. On the other hand, the combination of the two antioxidants revealed an additive or synergistic effect which could not be detected in the peroxide studies.

Conclusion

Our results prove that nanoemulsions can be used to characterize lipophilic compounds in simple cell culture experiments. Oil soluble substances encapsulated in nanoemulsions can be dispersed in aqueous media under defined conditions. Therefore, the biological properties of lipids can now easily be screened in cell culture experiments. Transparent nanoemulsions are ideal to study the effects of UV-irradiation on lipids because they form a homogenous dispersion of lipids in water without light scattering.

UV-A irradiation is well known to be a very potent stimulator of the oxidative stress in the epidermis (11). In our earlier studies, we have found that UV-A irradiation of the skin of volunteers leads to the formation of squalene hydroperoxides and that the pretreatment of the skin with sodium carboxymethyl betaglucan (CM-Glucan) can significantly inhibit this lipid peroxidation (4). In this study, we could show that UV-A irradiation of squalene encapsulated in nanoemulsions leads to the formation of toxic compounds determined in a cell culture experiment with TK6 lymphoblastoid cells **(Fig. 4)**. Therefore, we can conclude that the protection of the skin against the formation of lipid peroxides is very important.

In our study, we have evaluated the effects of different antioxidants, such as grape seed extract (hydrophilic) and tocopherol (lipophilic) on the in-vitro inhibition of lipid peroxidation measured by the Fox assay. The biological relevance of these results were then analyzed in cell toxicity experiments with TK6 lymphoblastoid cultures.

Grape seed extract or tocopherol showed a pronounced inhibition on the UV-A induced formation of lipid peroxides in a squalene nanoemulsion. However, the combination of both antioxidants did not reveal an additive effect at the applied concentrations. In other studies, we could find a synergistic effect of the two antioxidants on the inhibition of lipid peroxidation in polyunsaturated fatty acids (borage oil) measured as thiobarbituric acid reacting substances.

In this study, we could show that Grape seed extract and tocopherol not only reduced the formation of lipid peroxides but also reduced the toxicity of the UV-A irradiated squalene nanoemulsions on TK6 lymphoblastoid cells. However, this positive effect in the biological assay is much less pronounced compared to the in-vitro inhibitory effect on lipid peroxidation. On the other hand, we found a synergistic effect of the two antioxidants indicating that they work through different mechanisms in reducing the toxicity of UV-A irradiated squalene. Thus, we can speculate that in general one antioxidant cannot protect lipids against all chemical alterations induced by UV-A irradiation. Therefore, the combination of different antioxidants in a product will give better results.

UV-A induced oxidative stress leads to a number of chemical reactions in lipids. The resulting molecules, such as lipid peroxides are in various degrees toxic to cells. Lipid peroxides can be analyzed by simple chemical methods. However, it is also known that these tests detect only certain species of molecules. Therefore, it is very important to have also other tools available to measure the activity of antioxidants and radical scavengers. In this study, we have shown that the use of nanoemulsions in cell toxicity assays is a quick method to get relevant results on the biological activity of antioxidants.

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