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# ROS production and glutathione response in keratinocytes after application of β-carotene and VIS/NIR irradiation

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ABSTRACT

The skin is exposed to many stress factors which, in turn, can promote a shift of the antioxidant (AO) network towards the prooxidative side, supporting the development of various skin disorders. A balanced diet, in combination with a healthy lifestyle could reduce oxidative stress.

Carotenoids are essential nonenzymatic AOs and main components of the exogenous AO system.

To examine the interdependence between endogenous and exogenous AOs, secondary keratinocytes (HaCaT) were treated with various Beta (β-)-carotene concentrations with subsequent stress treatment by moderate irradiation (700–2000 nm). To facilitate the uptake of β-carotene, an innovative nanocrystal solution was used.

Cell viability assay was applied to HaCaT cells to evaluate suitable concentration of β-carotene, whereby the uptake was measured by resonant Raman spectroscopy. The redox status was determined before and after supplementation with two selected β-carotene concentrations (0.02 and 0.1 μg/ml) and irradiation. Reactive oxygen species (ROS) were measured by electron paramagnetic resonance spectroscopy and the AO glutathione (GSH) by a fluorescent-based assay for evaluating the endogenous redox status.

An increase of ROS and a reduction of GSH after irradiation was observed. Interestingly, the applied β-carotene, already induce oxidative stress. Nevertheless, an effective protection against irradiation could be observed for the lower dose. The high dose turned pro-oxidative.

## 1. Introduction

In a physiological system, usually an equilibrium between pro- and antioxidative processes is present. An imbalance in favor of the prooxidative side is referred to as oxidative stress, which could be triggered by exogenous and endogenous factors [1]. The skin is constantly exposed to oxidative stress by reactive oxygen and reactive nitrogen species (ROS, RNS) [2,3] which can promote the oxidation of cell components such as membranes, proteins and nucleic acids leading to inflammatory reactions, cell-/tissue damage and alteration in gene expression [4]. In order to control and minimize damages caused by ROS/RNS, the antioxidant network of the body consists of enzymatic and nonenzymatic antioxidants (AOs) [1,5]. In general, AOs are chemical compounds, which prevent and/or slow down undesirable or damaging oxidations of cellular compartments, stabilizing the balance between pro- and antioxidative processes [6,7]. However, the protection against oxidation processes is not only performed by a single AO; the antioxidant network consist of many components, which partly influence each other (synergistic effect) [8].

AOs are either formed endogenously in the human organism or are ingested with food. The most important endogenous antioxidant representatives are superoxide dismutases (SODs), catalase and glutathione (GSH) [9]. The most important antioxidants that are exclusively absorbed by diet include ascorbic acid, α-tocopherol (vitamin E) and the carotenoids. The most effective carotenoids are β-carotene and lycopene [10], constituting at least 60–70% of the whole carotenoids in skin [11].

The general assumption is that a diet rich in fruits and vegetables is healthy and a lot of studies have shown its benefits for health. Nevertheless, a supplementation with single high doses of carotenoids has shown negative effects: reducing their antioxidant effects and changing into prooxidative substances. Only limited studies have addressed the interaction of the endogenous and exogenous antioxidant status or the redox status. How do these two systems interact? To investigate this interaction directly and dependent on a moderate stress in a skin cell line model, two additional questions had to be solved:

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1. Because of the lipophilic character of carotenoids, the application as a normal dissolved substance in aqueous cell suspensions is not possible. The application of a water soluble AO such as vitamin C is not possible because this would interfere with our ROS measurements. To overcome this limitation, nanocrystals can be applied which have shown to be taken up by cells [12]. So far the uptake of carotenoids into cells has not been shown.
2. Moderate stress should be applied without extra administration of a substance. In this case irradiation is a good choice. UV exposure strongly induces radicals but here a moderate stress should be applied which can be induced using visible and near infrared light.

Zastrow et al. could demonstrate on excised human skin that UVA and UVB radiation are not exclusively responsible for the radical production during sun exposure [13,14]. Several studies indicate that irradiation of lower energy, near infrared (NIR) and infrared (IR), are also responsible for the production of highly reactive radicals; looking at the entire spectrum of the sunlight, a large amount of up to 50% of the radicals is produced by the VIS/NIR radiation [13,15].

Electron paramagnetic resonance (EPR) spectroscopy has shown to be a sensitive noninvasive method for the detection of radical formation in tissue (*in vivo/ex vivo*) [16,17] and also in cells [18]. Depending on the assignment, the application of various EPR probes allows the quantification and characterization of differently treated radicals, e.g. for their redox status after administration with supplements [19] or radical formation and characterization subsequent to sun exposure [20].

In this study, the redox status of secondary keratinocytes (HaCaT) was examined before and after VIS/NIR irradiation and after supplementation with β-carotene in two different concentrations. It was analyzed whether an additional supply with β-carotene influences the endogenous redox status and the ROS formation. The ROS formation and GSH/GSSG concentration was determined before and after VIS/NIR irradiation.

## 2. Material and methods

### 2.1. Synthesis and characterization of β-carotene crystals

β-carotene was obtained from Sigma Aldrich, Germany. β-carotene nanocrystals were produced by applying a modified bead milling technique after [21]. 3 g of the raw suspension, which contained 5% (w/w) β-carotene and 2.5% (w/w) alkyl polyglycoside (Plantecare 2000, BASF, Germany) as stabilizer and purified water to up to 100% were filled into a vial which contained milling beads (3 g of 0.1 mm and 3 g of 0.5 mm zirconia beads, Fritsch, Idar Oberstein, Germany). The suspension was placed on a magnetic stirrer and stirred at 800 rpm for 8 h. During this time cooling was provided to keep the temperature of the suspension below 10 °C [21]. The nanocrystals were analyzed in regards to size and size distribution using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK), laser diffraction (LD, Mastersizer 2000, Malvern Instruments, UK) and light microscopy (DM1000, Leica Microsystems, Germany). The hydrodynamic diameter of the suspension was about 150 nm. LD and light microscopy proved the absence of larger particles. Dynamic light scattering proved the narrow size distribution with a polydispersity index below 0.25.

### 2.2. Cell culture

#### 2.2.1. Secondary keratinocytes

The immortalized human keratinocytes cell line HaCaT (Deutsches Krebsforschungszentrum Heidelberg, Cell line service, item number: 300493 Germany) was chosen as keratinocyte model cell [22,23].

Cell cultivation was performed as described in previous publications [18,24]. In general, HaCaT cells were cultivated in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) with supplements (1% penicillin/streptomycin (Biochrom, Berlin, Germany), 2% glutamine (Biochrom, Berlin, Germany) and 10% FCS (PAA Laboratories, Vienna, Austria). The cells were cultivated in 75 $cm^2$ flasks at 37 °C, 5% $CO_2$ and 100% humidity. Reaching a confluence of about 80%, the cells were harvested by trypsination 0.5% trypsin and 0.2% EDTA (Gibco, Invitrogen, Carlsbad, CA, USA), counted and seeded in new 75 $cm^2$ flasks and/or were used for further investigations.

#### 2.2.2. Cell viability assay (XTT)

The XTT assay (Roche, Berlin, Germany) is a tetrazolium salt-based assay, which is a widely used method to determine the cell viability of metabolic active cells. The amount of detected NAD(P)H through glycolysis, is correlating to metabolically active cells [25]. For the investigations $1.5 \times 10^4$ cells/well were seeded in 96-microwell plates (Falcon®, VWR, Darmstadt, Germany) followed by an incubation for 24 h. After the incubation time, 100 µl RPMI with various β-carotene concentrations was added, and the cells were incubated for 1 h at 37 °C, 5% $CO_2$ and 100% humidity. As positive control the detergent Triton-X (0.1%, Sigma Aldrich, Darmstadt, Germany) was used. After the incubation time, the antioxidant suspensions were removed, cells were washed with PBS and 100 µl RPMI medium without phenol red were added. The XTT was prepared and added as described in the manufacturer's manual. To determine the cell viability the absorption was measured at 492 nm; the reference measurements were performed at 690 nm. The final result of all experiments ($n = 4$) was evaluated by calculating the mean ± SEM.

After every EPR measurement, the analyzed samples were tested for their cell viability by a trypan blue (Trypan Blue solution, Sigma Aldrich, Darmstadt, Germany) dye. Trypan blue is only reacting with damaged membrane because of the negatively charged chromophore.

To show the proportion between living and dead cells, 100 µl of the measured cells were removed and were incubated with 20 µl of trypan blue for 5 min at room temperature. For counting, 10 µl of the suspension were filled into a Neubauer counting chamber (Superior Marienfeld, Lauda Königshofen, Germany). A Leica DMi 1 microscopy (Leica, Wetzlar, Germany) was used for screening and counting.

### 2.3. Radical formation

For the detection of the oxidative stress development in the cell culture, an X-band EPR spectrometer (MiniScope MS 400, Magnettech, Berlin, Germany) was used with the following parameter settings: microwave frequency 9.4 GHz, microwave power: 10 mW, strength of the magnetic field: 336 mT, bandwidth of the magnetic field: 5.3 mT, modulation amplitude: 0.2 mT, microwave attenuation: 10 dB, sweep time: 20s. For the EPR investigations the spin probe 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO, Sigma Aldrich, Steinheim, Germany) was used. The EPR studies were performed as previously described [18].

For the EPR investigations $1 \times 10^6$ cells/ml were incubated with two selected β-carotene concentrations (0.02 and 0.1 µg/ml) for 1 h at 37 °C, 5% $CO_2$ and 100% humidity. Afterwards, the solution was removed and the cells were washed with PBS. The cell pellet was resuspended in a PBS/glucose (Merck, Darmstadt, Germany) (1 mg/ml) solution. During the EPR investigations, the samples were stored in a water bath heated to 37 °C to avoid additional thermal stress. Before the initial measurements were started, the EPR probe TEMPO (dissolved in PBS) was added to the cell suspensions (end concentration: 5 µM). After the initial measurement, every 4 min for a total of 32 min EPR measurements were performed. Therefore, 500 µl of the cell suspension was measured in a cell cuvette (Magnettech, Berlin, Germany). A physiological temperature of 37 °C was maintained during the whole EPR experiment by using a bio temperature controller (BTC01, Magnettech, Berlin, Germany).

All EPR data were analyzed by using the analysis software of the X-band device (Mplot, Maggnettech, Berlin, Germany). For this purpose,