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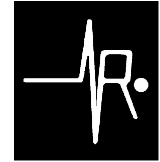
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Original Contribution

# The impact of nitrite and antioxidants on ultraviolet-A-induced cell death of human skin fibroblasts

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## Abstract

Nitrite ($NO_2^-$) occurs ubiquitously in biological fluids such as blood and sweat. Ultraviolet A-induced nitric oxide formation via decomposition of cutaneous nitrite, accompanied by the production of reactive oxygen (ROS) or nitrogen species (RNS), represents an important source for NO in human skin physiology. Examining the impact of nitrite and the antioxidants glutathione (GSH), Trolox (TRL), and ascorbic acid (ASC) on UVA-induced toxicity of human skin fibroblasts (FB) we found that $NO_2^-$ concentration-dependently enhances the susceptibility of FB to the toxic effects of UVA by a mechanism comprising enhanced induction of lipid peroxidation. While ASC completely protects FB cultures from UVA/$NO_2^-$-induced cell damage, GSH or TRL excessively enhances UVA/$NO_2^-$-induced cell death by a mechanism comprising nitrite concentration-dependent TRL radical formation or GSH-derived oxidative stress. Simultaneously, in the presence of GSH or TRL the mode of UVA/$NO_2^-$-induced cell death changes from apoptosis to necrosis. In summary, during photodecomposition of nitrite, ROS or RNS formation may act as strong toxic insults. Although inhibition of oxidative stress by NO and other antioxidants represents a successful strategy for protection from UVA/$NO_2^-$-induced injuries, GSH and TRL may nitrite-dependently aggravate the injurious impact by TRL or GSH radical formation, respectively.
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*Keywords:* Antioxidants; Nitric oxide; Nitrite; Fibroblasts; UVA

## Introduction

Human dermal fibroblasts (FB) are subject to constant photooxidative stress caused mainly by deeply penetrating UVA irradiation [1]. Photosensitized formation of cyclobutane pyrimidine dimers and 8-oxo-7,8-dihydroguanine in the DNA of human cells [2] and/or membrane damage caused by reactive oxygen or nitrogen species (ROS or RNS) represent major promoters of photoaging and photocarcinogenic processes initiated and promoted by long-term UVA exposure of the skin [3,4].

Nitric oxide (NO) is an effective inhibitor of lipid peroxidation serving membrane function and integrity [5]. Furthermore, the coordinated action of NO on gene expression and preservation of membrane function effectively protects against both UVA- and ROS-induced apoptotic and necrotic cell death [6–8]. Thus, it had been repeatedly shown that NO regulates the expression of a large number of genes including protective stress response genes such as vascular endothelial growth factor, heme oxygenase-1, and Bcl-2 [8–10]. Moreover, several

*Abbreviations:* ASC, ascorbate; GSH, glutathione; TRL, Trolox; ROS, reactive oxygen species; RNS, reactive nitrogen species; FB, human skin fibroblasts; NO, nitric oxide; NOS, nitric oxide synthase; LPO, lipid peroxidation; FCS, fetal calf serum; PBS, phosphate-buffered solution; FAMOS, Faraday modulation spectroscopy.

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lines of evidence indicate that in human skin NO is involved in the control of wound-healing processes, allergic skin manifestations, microbicidal activity, antigen presentation, proliferation and differentiation of epidermal cells, and in the regulation of innate immune reactions as well as in inflammatory responses [11,12].

Nitric-oxide-synthase (NOS)-dependent production of NO potentially occurs in all dermal cell types. Some of the NO molecules formed remain at or close to the point of their origin as S- or *N*-nitroso compounds and their oxidation products nitrite ($NO_2^-$) and nitrate ($NO_3^-$) in comparable high concentrations [13]. Nitrite is also present in blood as a stable oxidation product of NO synthesis or as a consequence of intake of nitrite-containing food; as a constituent of sweat, nitrite is found at concentrations up to 15 μM and is assumed to be formed on the skin surface by commensural bacteria [14]. Further, nitrite salts are chemical agents with widespread use in manufacturing processes, and as preservatives for meat, fish, and cosmetics. Moreover, nitrite is used in medicine as broncho- and vasodilating agents and as an antidote against cyanide poisoning [15].

Studies in environmental chemistry revealed that both nitrite anion and nitrous acid ($HNO_2$) in aqueous solutions undergo photodecomposition when irradiated with UV light at 320–400 nm, resulting in formation of NO and ROS or RNS, e.g., hydroxyl radicals or nitrogen dioxide radicals [16–19]. As we have shown very recently for rat cells, nitrite potentially represents an enzyme-independent source for NO during UVA exposure via decomposition, exerting analogue protective capacity against UVA- or ROS-induced injuries as seen with endogenous, NOS-generated NO [8,13,20].

Addition of antioxidants seems the natural strategy to provide protection against oxidative damage caused by external and internal stressors [21,22]. Therefore, the aim of this study was to investigate the potential protective capacity of the antioxidants ascorbic acid (ASC), glutathione (GSH), and the water-soluble vitamin-E-derivative Trolox (TRL) against UVA- and/or nitrite-induced toxicity and lipid peroxidation (LPO) in human dermal fibroblasts. To our surprise, with human dermal fibroblasts photodecomposition of nitrite represents a strong toxic insult due to ROS and RNS formation, as exogenous application of NO or ASC represents a successful strategy to protect cells from UVA/$NO_2^-$-induced cell death.

Interestingly, in contrast to ASC, usage of GSH and TRL strongly augments the injurious impact of nitrite photodecomposition due to radical formation in antioxidant molecules.

## Material and methods

### Material

Chemicals and media were from Sigma (Deisenhofen, Germany) and PAA (Linz, Austria), respectively, if not otherwise indicated. All buffer and culture media were free from nitrate and nitrite. (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-aminoethyl)-amino]diazen-1-ium-1,2-diolate (DETA-NO) was synthesized as described [23,24].

### UVA sources

(1) Sellas 4000-W mercury arc lamp unit (Sellas Medizinische Geräte, Gevelsberg, Germany) emitting a UVA spectrum (340–410 nm) with a maximum of intensity at 366 nm (84 mW/cm$^2$ at a distance of 25 cm). (2) Sellas 2000-W mercury arc lamp unit emitting a UVA spectrum (320–400 nm) with a maximum of intensity between 350 and 370 nm (70 mW/cm$^2$ at a distance of 25 cm).

### Cell culture and cellular characterization

Primary cultures of human fibroblasts (FB) were prepared from reduction mammoplasty specimens as described [25]. Cells were grown in 100-mm cell culture dishes (Greiner, Frickenhausen, Germany) under standard culture conditions in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Cells were passaged every 4–5 days. Cells were subcultured for up to eight passages, and removal from culture dishes for each passage was performed by treatment with 0.05% trypsin/0.02% EDTA in isotonic NaCl for 3 min.

### Experimental design

All measurements were performed with cells from passages 2–8. Fibroblasts ($2 \times 10^5$ cells) were cultured in 12-well tissue culture plates in a humidified incubator at 37 °C in RPMI 1640/10% FCS. During UVA challenge, cells were maintained in phosphate-buffered solution (PBS). Additionally, during UVA irradiation, resident FB were incubated with the respective additives at concentrations indicated. At time points indicated, growth rates and viability, apoptotic and necrotic cell death, and lipid peroxidation were assessed.

### Growth rates and viability

Cell growth was determined by neutral red staining [26]. FB were incubated for 90 min with neutral red (1:100 dilution of a 3% solution), washed twice with PBS, dried completely, and lysed with isopropanol containing 0.5% 1 N HCl. Extinction of the supernatants, which shows a linear correlation to the number of living cells, was then measured at 530 nm. Additionally, viability of FB was routinely controlled at the beginning and the end of every experiment using the trypan blue exclusion assay or propidium iodide staining. Viable cells were defined as cells excluding trypan blue or propidium iodide and positive for active endocytosis as determined by neutral red.

### Detection of apoptotic or necrotic cells

At different time points (1–24 h) after UVA irradiation (25 J/cm$^2$) in the absence or presence of the respective additives, fibroblast cultures grown in 12-well culture plates were washed with PBS and stained with Hoechst dye H33342 (8 μg/ml) and/or propidium iodide (0.5 μg/ml) for 5 min and nuclei or necrotic cells were visualized using a Zeiss fluorescence microscope. In

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