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Melatonin Suppresses Reactive Oxygen Species in UV-Irradiated Leukocytes More than Vitamin C and Trolox

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Key Words
Reactive oxygen species · UV light · Leukocytes · Antioxidants

Abstract
To prove the relative potency of melatonin as a radical scavenger in UV-irradiated leukocytes, it was compared to other antioxidative substances such as trolox and vitamin C. Human leukocytes were isolated from EDTA whole blood and incubated with melatonin, trolox and vitamin C. The experiments were performed in a wide concentration range from 0.1 n*M* to 1 m*M* and in a small concentration range from 0.5 to 2 m*M* (mel), 5 m*M* (trolox) and 10 m*M* (vit. C). Irradiation was performed with UV-light (280-360 nm) at a dose of 750 mJ/cm^2. Radical formation was measured by the chemiluminescence technique. The maximum effect of radical suppression was seen at a concentration of 10 n*M* ($p = 0.003$) and 1 m*M* melatonin ($p < 0.001$) and vitamin C ($p = 0.002$; $p < 0.001$), respectively. ROS suppression by trolox was only significant at 1 m*M* ($p < 0.001$). In the small concentration range, a linear dose-response relationship was found and melatonin showed the strongest radical suppression ($IC_{50} = 0.21$ m*M*) followed by vitamin C ($IC_{50} = 0.26$ m*M*) and trolox ($IC_{50} = 1.03$ m*M*).

Introduction

Skin aging is an additive process of intrinsic and extrinsic aging, e.g. the UV-induced photoaging. UV irradiation is an important acute and chronic stressor to the skin. Besides UV light-induced inflammatory reactions promoted by proinflammatory mediators, free radicals are supposed to be involved in the skin damaging process by destruction of

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cell membranes, DNA, proteins and lipids [1, 2]. Vitamins E and C are non-enzymatic antioxidants which protect the skin against oxidative damage [3, 4]. Melatonin (N-acetyl-5-methoxy-tryptamine) is an indole produced by the pineal gland. It primarily exhibits seasonal biorhythmic functions, reproduction, daily sleep induction in dependency of light perception, aging and modulation of immunobiological reactions [5]. It has been proven that melatonin is a strong radical scavenger, especially of hydroxyl radicals [6]. These radicals are reported to be the most damaging reactive oxygen species (ROS) induced by UV light [1]. A radical suppressive effect of melatonin has been shown in UV-irradiated leukocytes [7]. To assess the relative potency of melatonin, it was compared with the above-mentioned antioxidants.

Methods

Leukocytes were isolated from human whole blood by density gradient and hemolyzation of erythrocytes. Ten aliquots of PBS-diluted leukocytes were put into UV light-permeable quartz glass Petri dishes (Fisher Scientific, Germany), 2,250 µl each. Then, 250 µl of the test solutions containing different concentrations of melatonin, vitamin C and trolox were added to 9 aliquots. One aliquot was incubated with 250 µl PBS (Sigma, Germany) as a control.

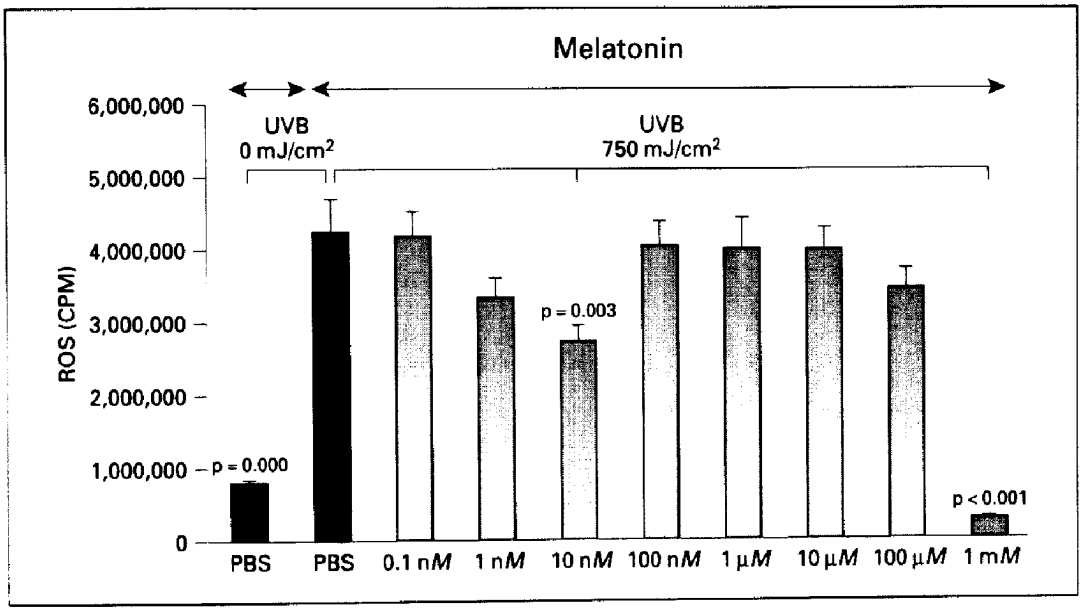
Melatonin (highly purified, MG 232.2, Helsinn Chemicals, Biasca, Switzerland) was prepared in concentrations of 2, 1, and 0.5 m*M*, 100, 10, and 1 µ*M*, and 100, 10, 1, and 0.1 n*M*. Vitamin C (Merck KgaA, Darmstadt, Germany) and Trolox (Calbiochem, Bad Soden, Germany) were prepared with PBS and diluted in the same way as melatonin. The investigations were performed in two different experimental designs: the first series was performed over a wide concentration range of powers of 10 from 0.1 n*M* to 1 m*M*. The second preparation was performed after having detected the optimum effect range. This was a small range of concentrations from 0.1 to 10 m*M* for vitamin C. Trolox was soluble up to 5 m*M* and melatonin up to 2 m*M*.

The UV absorption properties of the applied antioxidants were assessed under UV irradiation by a spectrophotometer (Jasco UV-530 UV/Vis Gross-Umstadt, Germany). The cell solutions incubated with the antioxidants were irradiated using the Waldmann UV800 UV lamp (Waldmann GmbH & Co., Villingen-Schwenningen, Germany) with an UV light spectrum of 280–360 nm. The UV dose was 750 mJ/cm² UVB light. After irradiation, the cell solutions were put in small test tubes (200 µl) and transferred to the luminometer LB 953 (Berthold, Germany) for measurement of ROS. The method is described elsewhere [8]. Prior to the measurement in the luminometer, 20 µl of each preparation were taken for staining with 20 µl trypan blue to assess viability of the cells counted under the microscope. Mean values and standard deviation were calculated by Microsoft Excel software and statistical analyses were performed with SPSS using the Student t test for unpaired samples. To compare the suppression of free radicals under influence of the different antioxidants all CPM-values were calculated as percentage in relation to 100% CPM of the untreated (PBS) leukocytes. To have a comparable m*M*-value of efficacy the probit-transformation was performed. With this statistical technique a dose response curve from 100% to 0% CPM was extrapolated for each substance. Under the assumption of a sigmoid dose-response-curve the IC_{50}-value was calculated by SPSS software.

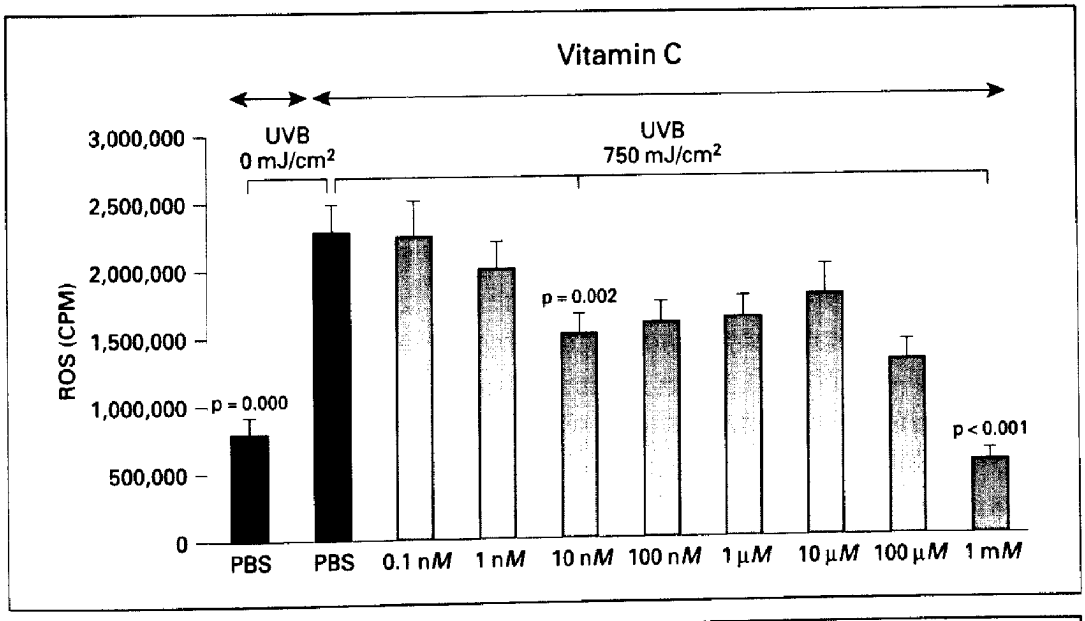
Results

The maximum of ROS suppression in a wide concentration range was at 1 m*M* melatonin ($p < 0.001$) and the second effect maximum at 10 n*M* ($p = 0.003$) (fig. 1). The concentration of 0.5 m*M* melatonin had already led to a significant suppression of ROS in the

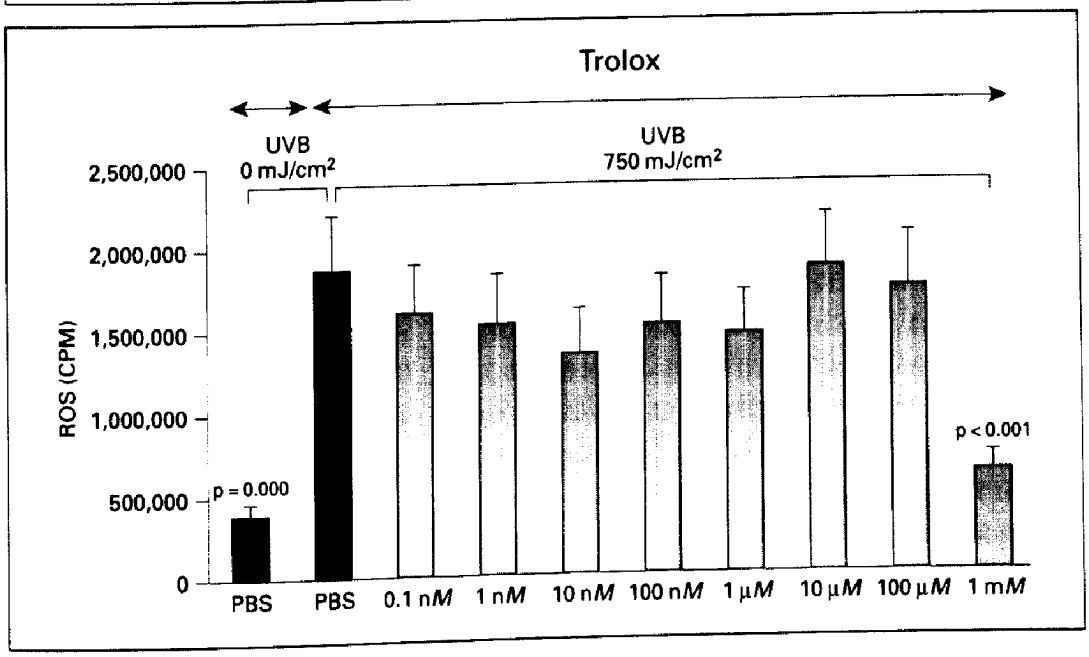
Fig. 1–3. Effects of the radical suppression in leukocytes treated with melatonin (**1**), vitamin C (**2**), and trolox (**3**) in a range from 0.1 n*M* to 1 m*M*. Melatonin and vitamin C show an effect maximum at 10 n*M* and 1 m*M* with statistical significance. Trolox reveals a significant effect optimum at only 1 m*M*, but not at 0.1 n*M* compared with controls (PBS).


Melatonin
6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
ROS (CPM)
UVB
0 mJ/cm²
UVB
750 mJ/cm²
p = 0.000
p = 0.003
p < 0.001
PBS
PBS
0.1 nM
1 nM
10 nM
100 nM
1 µM
10 µM
100 µM
1 mM

1


Vitamin C
3,000,000
2,500,000
2,000,000
1,500,000
1,000,000
500,000
0
ROS (CPM)
UVB
0 mJ/cm²
UVB
750 mJ/cm²
p = 0.000
p = 0.002
p < 0.001
PBS
PBS
0.1 nM
1 nM
10 nM
100 nM
1 µM
10 µM
100 µM
1 mM

2


Trolox
2,500,000
2,000,000
1,500,000
1,000,000
500,000
0
ROS (CPM)
UVB
0 mJ/cm²
UVB
750 mJ/cm²
p = 0.000
p < 0.001
PBS
PBS
0.1 nM
1 nM
10 nM
100 nM
1 µM
10 µM
100 µM
1 mM

3

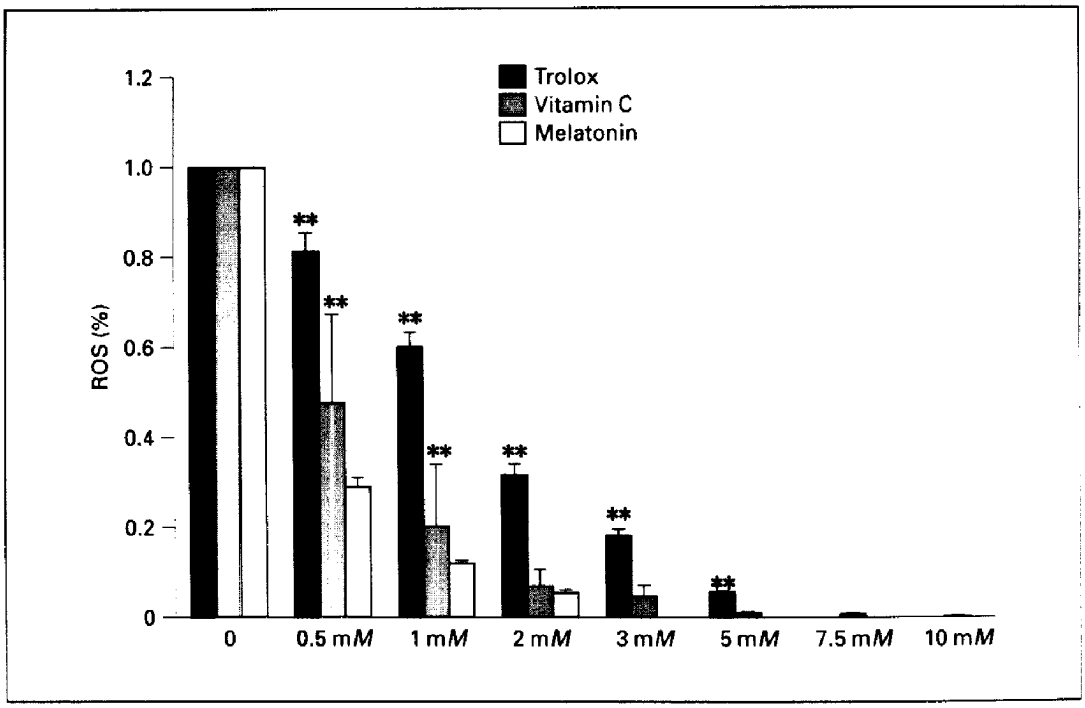


Fig. 4. Suppression of ROS by melatonin compared to vitamin C and trolox. Melatonin suppressed the radical formation significantly better than trolox at all concentrations and better than vitamin C at 0.5 and 1 m*M* (** $p < 0.001$). The ROS values were significantly lower in vitamin C-treated than in trolox-treated cell solutions at concentrations of 3 and 5 m*M*. Cell solutions were irradiated with UV light at a dose of 750 mJ/cm^2.

higher concentration range ($p < 0.001$). Increasing concentrations of melatonin at 1 and 2 m*M* were still stronger showing a linear dose response ($p < 0.001$) (fig. 4).

Analogous to the experiments with melatonin, vitamin C also showed the maximum effect at 1 m*M* in the wide concentration range ($p < 0.001$). The second maximum effect was at 10 n*M* vitamin C ($p = 0.002$) (fig. 2). In the higher concentration range from 0.1 to 10 m*M*, a linear dose-response relationship was seen between concentrations of vitamin C and suppression of ROS. The concentration of 0.5 m*M* led to a significant suppression compared to controls ($p < 0.01$). Increasing doses led to an increasing suppressive effect at 1 m*M* ($p < 0.001$) and at 2 and 3 m*M* vitamin C ($p = 0.000$) (fig. 4).

In a wide range of trolox concentrations from 0.1 n*M* to 1 m*M*, the maximum effect of ROS suppression was measured at 1 m*M* ($p < 0.001$). At 10 n*M* trolox, there was a second effect maximum, but this was not significant compared to untreated leukocyte solutions (fig. 3). In the higher concentration range from 0.1 to 5 m*M*, trolox led to a linear dose-dependent suppression of ROS. At the concentration of 1 m*M* trolox, the suppression was significant with $p < 0.005$. At the concentration of 2, 3 and 5 m*M*, the ROS value was also suppressed significantly ($p < 0.001$) (fig. 4).

The suppression of radicals by the tested antioxidants was compared and analyzed statistically. We found that melatonin had the strongest radical suppressive effect followed

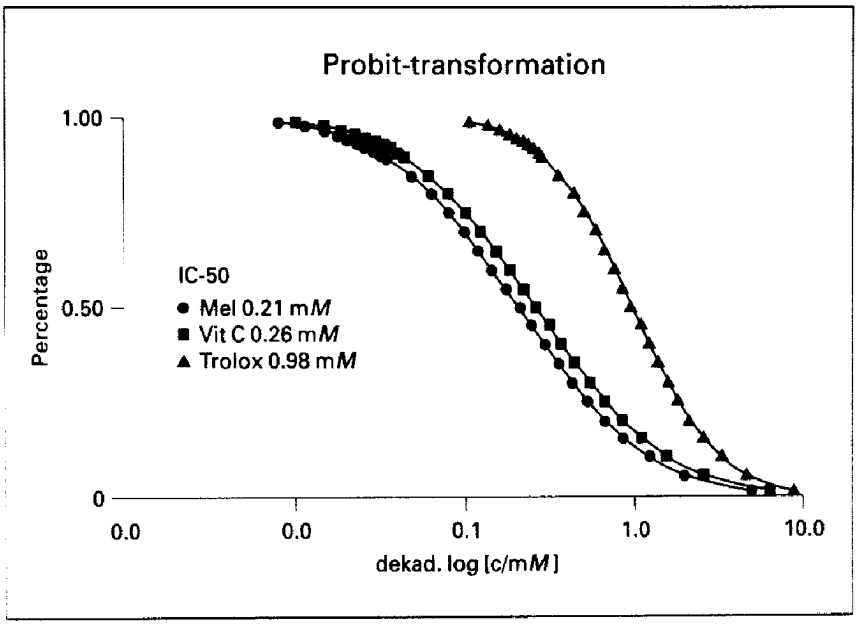


Fig. 5. Under the assumption of a sigmoid dose-response, melatonin had the lowest concentration of the three antioxidants to suppress 50% of the radical formation compared to vitamin C and trolox.

by vitamin C and trolox (fig. 4). Using the probit transformation, a 50% inhibitory concentration (IC_{50}) of 0.21 m*M* was revealed for melatonin, 0.26 m*M* for vitamin C and 0.98 m*M* for trolox (fig. 5). The viability of the cell solutions which was assessed by trypan blue staining was higher than 95% in all preparations with melatonin, vitamin C and trolox.

An UV absorption of all substances was seen in the lower part of the applied UV irradiation spectrum (750 mJ/cm^2 UV light/280–360 nm). The UV absorption maximum was at 260 nm for vitamin C, 280 nm for melatonin and at 290 nm for trolox. The highest light absorption coefficient in the broadband UVB light range from 280 to 360 nm which was applied for the irradiation of the cell solutions was at 0.52 for melatonin, 0.62 for vitamin C and 0.3 for trolox. No light absorption of melatonin, vitamin C and trolox was measured in the wavelength higher than 325 nm.

Discussion

To prove the radical scavenging potency of melatonin in comparison with two other antioxidants, a concentration profile over a wide concentration range from 0.1 n*M* to 1 m*M* was performed showing a nondirect biphasic dose response for melatonin, vitamin C and trolox. In the higher concentrations from 0.5 to 10 m*M*, melatonin revealed a superior radical suppressive effect compared to vitamin C and trolox. The analysis of the UV absorption spectra of the applied substances revealed an UV absorption in the lower part of the UV spectrum applied (260–360 nm). Interestingly, vitamin C had a higher absorption coefficient than melatonin (0.62 vs. 0.52) in the wavelength of 280 nm. But vitamin C had a lower suppressive effect concerning free radical formation than melatonin. In the upper part of the applied UV light up to 360 nm, there is no UV light absorption of melatonin, vitamin C and trolox. The investigation of all substances does not show a consistent relationship between light absorption and free radical suppression. Therefore, we suppose

that the UV light absorption has only a partial influence on the suppression of free radical formation.

The radical suppressive properties of melatonin were already shown by Tan et al. [5] and Reiter et al. [6]. Melatonin was 6-fold and 14-fold stronger than glutathione and mannitol, respectively. In a model with 2-2′-azo-bis(2-amidinopropane)dihydrochloride as a peroxyl radical generator, Pieri et al. [9] also showed a strong suppressive effect of melatonin with double activity compared to trolox.

In a model using lipid bilayers and human skin homogenates, the suppression of peroxidation of liposomes was 250% for vitamin E (alpha-tocopherol) and 80% for melatonin both in combination with alpha-glycolic acid. This seems to be contradictory to our experiments but may be explained by a stronger recycling effect of alpha-glycolic acid to vitamin E than to melatonin [10].

Vitamin C was used in porcine and murine skin homogenates and suppressed UVB-induced erythema, formation of sunburn cells and UVA-induced phototoxic reactions [4, 11].

Concerning the optimum effects, an explanation for the stronger suppressive effect in the lower concentration of 10 n*M* compared with concentrations up to 100 μ*M* may be a change in the membrane fluidity of neutrophils. Melatonin may probably pass easily through the membrane and protects cell compartments from oxidative damage [12]. In the higher concentration of 1 m*M*, melatonin may scavenge radicals outside the cells directly in the solution.

While nothing is known about the presence of melatonin in the skin, there is evidence that melatonin is an important antioxidant in other compartments. These are the bile, the bone marrow and the cerebro-spinal fluid showing much higher levels of melatonin than in the blood [13–15]. Melatonin has not yet been shown to be physiologically present in the skin. It may be speculated that there is a melatonin depot in the skin especially in the stratum corneum because melatonin is a highly lipophilic molecule. The fact that melatonin is produced in the human body by the pineal gland may represent an endogenous protective mechanism against UV light-induced damage in the skin. There is evidence for this thesis because an increase in lipid peroxidation products was observed in the skin of melatonin-deficient rats [16]. This thesis has to be proven in skin physiology studies.

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