

Antioxidants Compared in a New Protocol to Measure Protective Capacity Against Oxidative Stress – Part II

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ABSTRACT

Previously, we developed a multistep in vitro process combining biochemical and cell-biological methods to compare the protective capacity of commonly used antioxidants. Data were presented for idebenone, DL- α -tocopherol, kinetin, ubiquinone, DL- α -lipoic acid, and L-ascorbic acid, using photochemiluminescence (Photochem[®]; Analytik Jena AG, Germany, and Analytik Jena USA, Inc., Texas), pro-oxidative systems (LDL-CuSO₄, microsomal NADPH/ADP/Fe³⁺) with measurement of primary and secondary oxidation products, and UVB irradiation of human keratinocytes. In an attempt to add to the usefulness of this multistep process, we evaluated the same antioxidants in vivo, using the human sunburn cell (SBC) assay. Correlation and trends between in vitro and in vivo results were established and a standardized test protocol proposed to quantify oxidative-stress-protection capacity of antioxidants. Sunburn cell delta percent reductions evaluated at equivalent concentrations for each of the putative antioxidants were 38%, 30%, 20%, 11%, 9%, and 0% for idebenone, DL- α -tocopherol, kinetin, ubiquinone, DL- α -lipoic acid, and L-ascorbic acid, respectively, resulting in an overall oxidative protection capacity score of 95, 80, 68, 55, 52, and 41 for idebenone, DL- α -tocopherol, kinetin, ubiquinone, L-ascorbic acid, and DL- α -lipoic acid, respectively. The standardization of antioxidant protective capacity should be a valuable tool to assess the anti-inflammatory properties, photoprotective properties, and prevention of UV immunosuppression of topical antioxidants.

MATERIALS AND METHODS

Human Sunburn Cell Assay

Treatments – All applications were made to a 5 cm x 10 cm area site over the midback region once a day for two weeks. Each putative antioxidant was applied to five different healthy adult volunteers between the ages of 18 and 60 (antioxidants were dissolved in ethanol/water at the concentrations indicated in the figure legends). Additionally, one test site was left untreated and served as a control. Approximately ten minutes after the last application, test sites were irradiated to 1½ MED (minimal erythema dose) of UVB light, a shave biopsy taken and prepared histologically, and the number of sunburn cells (SBCs) evaluated microscopically per high power field (HPF).

Light source – The light source used was a 150-watt xenon arc solar simulator equipped with a UV-reflecting dichroic mirror and a 1-mm-thick Schott WG 320 filter (BES Optics Inc., W. Warwick, RI) to produce simulation of the solar spectrum. A 1-mm-thick Schott UG5 filter was added to remove reflected heat and remaining visible radiation.

MED determination – The MED for each subject was determined by exposing a 1-cm-diameter circle of untreated area to a series of exposures in 25%-dose increments from the solar simulator. The MED was defined as the time of exposure required to produce a minimally perceptible erythema 20 ± 4 hours after exposure.

Biopsies – Approximately 10 minutes after the last topical application of the putative antioxidant, a circular area measuring 1 cm in diameter was exposed to a single dose of 1½ MED, using the solar simulator. Approximately 20 hours later, a shave biopsy (4 mm x 4 mm) was obtained from each irradiated and untreated control site, following injection of a local anesthetic (lidocaine). The skin specimens were immediately fixed in 10% buffered formalin.

Histology – The fixed specimens were processed routinely, embedded in paraffin and then sectioned and stained with hematoxylin-eosin. The numbers of sunburn cells (SBCs) were determined in at least 12 sections at 50 μ intervals. A minimum of 70 high power fields (HPFs) was counted from each biopsy and the average number of SBCs per HPF determined. All specimens were counted in a blinded manner.

Photochemiluminescence

The individual antioxidant capacity of the putative antioxidant substances was estimated by the Photochem[®] system (Analytik Jena AG, Jena, Germany, and Analytik Jena USA, Inc., Texas). The system combines the generation of radicals through photochemical excitation with highly sensitive luminometric detection. Samples are diluted with premade buffers (standardized kits) and applied to the device. The relative antioxidative capacity is determined by comparison to blank (without antioxidants) and a standard provided with the kit.

We employed the ACL kit (for lipid-soluble substances) and the ACW kit (for water-soluble substances).

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Pro-oxidative Systems Measuring Primary and Secondary Oxidation Products

Isolation of LDL – Low-density lipoproteins ($d = 1.019\text{--}1.063$ kg/L) were isolated in clean Beckman one-way Quick-Seal® tubes (Beckman Coulter, Inc., Palo Alto, Calif.) by ultracentrifugation from pooled plasma of healthy donors using an established protocol (Havel et al, 1955). After isolation, LDLs were extensively dialyzed against a degassed and nitrogen-saturated Tris-HCl buffer (5 mmol/L, pH7.4) containing 1 mmol/L EDTA. Before oxidation by CuSO_4 , EDTA was removed from LDL by dialysis against a Tris-HCl buffer (5 mmol/L, pH7.4) without added EDTA.

Incubation of LDL with the pro-oxidant system Ham's F-10/ CuSO_4 – LDL oxidation was achieved by incubating (37°C, 95% O_2 , 5% CO_2) 1 g of LDL protein/L with and without the putative antioxidant substances in 2 mL of serum-free Ham's F-10 medium (BioSource International, Camarillo, Calif.) in the presence of 20 $\mu\text{mol/L}$ CuSO_4 for the times indicated in the figure legends.

Preparation of microsomes – Tissue was homogenized in 50 mmol/L HEPES, 250 mmol/L sucrose buffers, pH 7.4, containing 150 $\mu\text{mol/L}$ KCl and 500 $\mu\text{mol/L}$ EDTA, using a KINEMATICA POLYTRON® PT 3000 (Brinkmann Instruments, Westbury, NY) homogenizer. Microsomal vesicles were isolated by removal of the nuclear fraction at 8,000 \times g for 10 minutes at 4°C and removal of the mitochondrial fraction at 18,000 \times g for 10 minutes at 4°C using a Beckman L8-55 ultracentrifuge and a Type 50 Ti-13 rotor. The microsomal fraction was sedimented at 105,000 \times g for 60 minutes at 4°C. The pellet was washed once in 50 mmol/L HEPES and 150 mmol/L KCl, pH 7.4, and collected again at 105,000 \times g for 30 minutes. The resulting microsomal pellet was resuspended in HEPES/KCl, pH 7.4, by careful sonication in ice and stored in portions (10 mg protein/mL) at -80°C until use.

Incubation of microsomes with the pro-oxidant system NADPH/ADP/ Fe^{3+} – The microsomal preparations were incubated in the presence of the pro-oxidant system NADPH/ADP/ Fe^{3+} , consisting of 0.20 mmol/L NADPH, 50 mmol/L ADP, and 0.25 mmol/L FeCl_3 in HEPES/KCl buffer (150 mmol/L KCl, 50 mmol/L HEPES) with and without the putative antioxidant substances. Oxidation of 1-mL aliquots containing 1 mg of protein was started at 37°C by the addition of NADPH and was stopped with EDTA (10 mmol/L) after the times indicated in the figure legends. Control incubations without the pro-oxidant system were performed at 37°C in the presence of EDTA. Antioxidants were dissolved in water or ethanol and added to the incubations at the concentrations indicated in the figure legends.

Lipid peroxidation assays – Lipid peroxidation was followed by determination of lipid hydroperoxides (LDL preparations) or by measurement of malondialdehyde (MDA) equivalents using the TBA method.

Measurement of lipid hydroperoxides – Lipid hydroperoxides were determined with the Cayman LPO Assay Kit (Cayman Chemical, Ann Arbor, Mich.), which measures the hydroperoxides directly utilizing the redox reactions with ferrous ions. Hydroperoxides are reacting with ferrous ions to produce ferric ions, which can be detected using thiocyanate ion as the chromogen. The antioxidative effect of the substances is shown as percentage compared to the blank (incubation without addition of antioxidants).

TBARS method – Microsomal preparations (500 μL) were mixed with 1 mL of thiobarbituric acid (0.67 g/100 mL, 0.05 mol/L NaOH). After the addition of trichloroacetic acid (50% w/v), the samples were heated to 90°C for 30 minutes. After cooling and extraction of the samples with 1 mL of butanol, the absorbance of the butanol phase was determined spectrophotometrically at 532 nm. For quantification, an external standard curve was prepared using 1,1,3,3-tetraethoxypropane, which yields MDA. The antioxidative effect of the substances is shown as percentage compared to the blank (incubation without addition of antioxidants).

UVB Irradiation of Keratinocytes

Keratinocyte collection – Human primary foreskin keratinocytes (second passage) were grown in 6-well plates containing cover slips to 60% confluence in serum-free medium (KGM®, Clonetics®; Cambrex Corporation, E. Rutherford, NJ) containing 0.07 mM CaCl_2 . Six hours before UVB radiation, medium was removed and replaced by fresh growth medium with or without the anti-oxidative substances.

Ultraviolet light (UVB) irradiation of keratinocytes – Keratinocyte cultures were irradiated with a single dose of 200 mJ/cm² UVB, using FS-20/T-12 bulbs (emission range: 280–340 nm; 305 nm max.). Immediately prior to irradiation, the medium was replaced with 1 mL sterile PBS (pH 7.4, 37°C), and after irradiation, PBS was replaced with fresh growth medium. UVB exposure was quantitated using a Goldilux™ ultraviolet radiometer (Oriental Instruments, Stratford, Conn.). Cells were maintained at 37°C (5% CO_2) for 1 hour until fixation with paraformaldehyde (PFA).

Fixation and nuclear thymine-dimer-staining of keratinocytes – Cells were fixed with 4% PFA in PBS for 30 minutes at RT, washed with PBS and permeabilized by incubation with EtOH/PBS (90/10; v/v%) for 30 minutes at 10°C. After fixation and permeabilization, cells were washed twice with PBS containing 1% of bovine serum albumin (BSA). Cells were then incubated for 30 minutes with 10 $\mu\text{g/mL}$ anti-thymine dimer Ab (clone KTM53; Kamiya Biomedical Company, Seattle, Wash.) at RT. After the incubation period cells were washed twice with PBS-BSA and incubated for 30 minutes with 20 $\mu\text{g/mL}$ secondary FITC-conjugated antimouse IgG at RT. After incubation with the secondary antibody, cells were washed twice with PBS-BSA and fixed again with 4% PFA for 15 minutes at RT. Slides were analyzed by confocal microscopy.

Human Sunburn Cell Assay

This investigation determined if the pretreatment of the skin with a putative antioxidant preparation for two weeks was likely to change the response of human skin to UVR. It has been stated that the topical application of certain antioxidants can provide photoprotective properties to the skin via the inhibition of the radical cascade initiated by UV light that ultimately leads to cell death/apoptosis. The extent of UV damage can then be detected by using a more sensitive endpoint than erythema, such as by estimating the degree of direct UVR damage to epidermal keratinocytes through SBC formation. Data depicted below express the photoprotective benefits of the antioxidants tested based on the percent change over baseline (delta percent) for the number of sunburn cells per high power field. Based on the data generated, idebenone outperformed the other antioxidants.

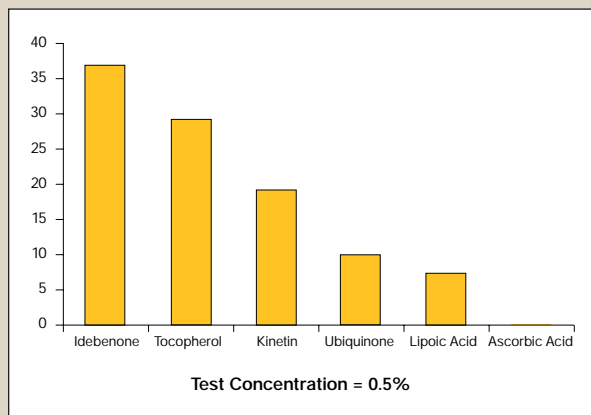
Photochemiluminescence

The Photochem® device (Analytik Jena AG, Jena, Germany, and Analytik Jena USA, Inc., Texas) was used for in vitro measurement of the general antioxidative capacity of substances. After measuring the substances in both lipid-soluble (ACL) and water-soluble (ACW) systems, we used mainly the ACL system since most of the lipid-soluble substances did not show any antioxidative effect in the ACW system. On the contrary, water-soluble substances (ascorbic acid) showed antioxidant properties regardless of the system used. The concentration of the substances needed to achieve an antioxidant effect is shown in Table 1. The Photochem® device serves as an adequate prescreening method for evaluating relative antioxidant capacity for new substances.

Measurement of Primary Oxidation Products

The LDL- CuSO_4 oxidation system was employed to evaluate the protection of lipids over time. The highly reactive lipid hydroperoxides measured in this experiment are the primary products of lipid peroxidation. In our conditions kinetin and idebenone demonstrated a consistent protection against lipid peroxidation over 24 hours. Other substances like ubiquinone, lipoic acid, and ascorbic acid only showed a comparatively short-lasting protective efficiency (Figure 2).

Figure 1
Sunburn Cell Reduction



Measurement of Secondary Oxidation Products

The pro-oxidative NADPH/ADP/Fe³⁺ microsomal system was used as an in vitro model system resembling cell membranes. Oxidation of cell membrane lipids leads to serious consequences in altering cell membrane fluidity and cell function. Antioxidants protecting bulky lipids, such as LDL, are not necessarily good protectors of cell membranes due to their hydrophilic/lipophilic bilayer composition. In our model, lipoic acid and idebenone showed the best protection against oxidation of the cell membrane lipids. Kinetin, which favorably protected bulky lipids (LDL), showed only a weak protective effect (Figure 3).

Figure 3
Microsome Oxidation System: Measurement of Secondary Oxidative Products (TBARS)

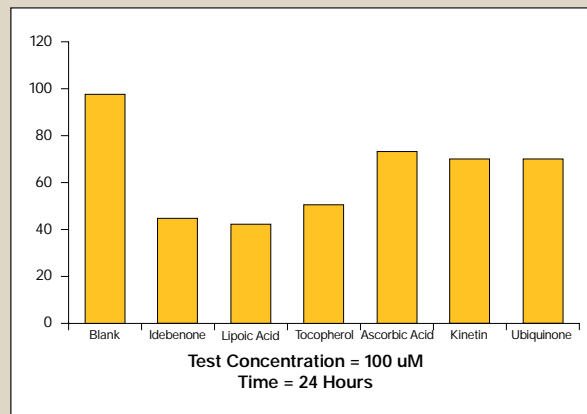


Table 1
Comparison of Antioxidative Concentrations of Compounds

Antioxidant Substance/Combination	Effective Concentration (nmol/L)
Idebenone	10
Ascorbic Acid	10
Tocopherol	10
Ubiquinone	100
Kinetin	1000
Lipoic Acid	> 1000 (not detectable)

UVB Irradiation of Human Keratinocytes

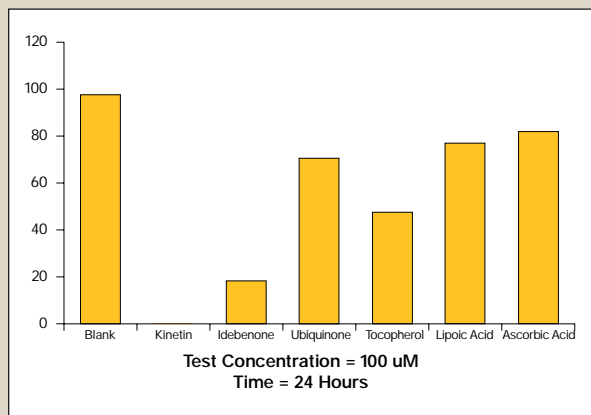
The accumulation of nuclear photoproducts in keratinocytes after UVB radiation with a specific antibody against thymine dimers was measured. This experiment is thought to reflect the in vivo-occurring DNA damage following UVB exposure and the protection of such nuclear damage by antioxidants. The results (Table 2) have to be seen as approximate estimations of the occurrence of nuclear thymine dimer photoproducts. Idebenone provided the highest level of inhibition.

Table 2
Nuclear Anti-thymine Dimer Staining in Keratinocytes After UVB Radiation

Antioxidant Substance	Positive Cells After UVB Radiation*	Inhibition of Photoproduct Generation
No-radiation Control	0%	100%
Idebenone	29%	45%
Ascorbic Acid	34%	36%
Kinetin	34%	36%
Tocopherol	35%	34%
Ubiquinone	51%	4%
Lipoic Acid	53%	0%
200mJ/cm ² UVB radiation	53%	0%

* = percentage of positive cells (above threshold) in three fields (counted cell number 120–150).

Figure 2
LDL-CuSO₄ Oxidation System: Measurement of Primary Oxidative Products (lipid hydroperoxides)



SUMMARY

Establishing a Standardized Way to Summarize Results

Scoring system – Five studies, equally weighted at 20 points each; highest possible score 100 points.

Assigning values – The antioxidant activity that demonstrates the greatest benefit for the test conducted receives 20 points. The remaining ingredients are assigned a percentage of the 20 points based on their relationship to the overall highest-scoring antioxidant.

Example: Idebenone produced the greatest benefit in the sunburn cell assay, a 38% reduction in SBCs; therefore, it receives 20 points. Tocopherol was second, producing a 31% reduction. To determine the relative activity to idebenone you would take $31/38 \times 100 = 82\%$ relative activity. Therefore, tocopherol would receive 82% of the 20 points or approximately 16 points. Likewise, kinetin demonstrated a 20% reduction in SBCs and a relative activity of 53% to receive a score of 11 points. This calculation is repeated for each ingredient tested.

Lastly, because the photochemiluminescence assay results are expressed as the lowest effective concentration and a base-10 serial dilution was used, points were assigned in the following manner:

Effective Concentration	Point Assignment
10 nmol/L	20
100 nmol/L	15
1000 nmol/L	10
> 1000 nmol/L	5

These studies compared the protective capacity of commonly used antioxidant ingredients in both in vitro and in vivo methods. Correlation and trends between the study results obtained allows the establishment of a standardization testing protocol that quantifies the oxidative-stress-protection capacity of the substances studied.

Table 3
Summary of Results

Test	Idebenone	Tocopherol	Kinetin	Ubiquinone	Ascorbic Acid	Lipoic Acid
Sunburn Cell Assay	20	16	11	6	0	5
Photochemiluminescence	20	20	10	15	20	5
Primary Oxidative Products	16	10	20	5	3	4
Secondary Oxidative Products	19	17	10	12	12	20
UVB-irradiated Keratinocytes	20	17	17	17	17	7
Total Points	95	80	68	55	52	41

CONCLUSION

Reviewing the summation of all study results presented (Table 3), one compound — idebenone — appears as a powerful antioxidant most consistently throughout all experiments. Although this potent antioxidant is relatively unknown to dermatology today, it represents a powerful potential use for topical skin care protection.

REFERENCE

Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest.* 1955;34(9):1345-1353.

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