An *In Vitro* Model for Assessing The Effectiveness of Antioxidants in Cosmetics.

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ABSTRACT

Oxidative stress in skin has been linked to the development of wrinkles, the aging process and other more serious effects. *In vitro* cell-based systems that can rapidly and efficiently evaluate whether a new chemical or formulation has antioxidant properties are important in the development of new products for the cosmetic industry. The aim of this study was to induce oxidative stress in normal human epidermal keratinocyte (NHEK) cells and then demonstrate protection in the presence of Trolox (Vitamin E) and test products. Oxidative stress was induced with two model compounds: menadione, which requires metabolism to reactive quinone intermediates, and cumene hydroperoxide, which does not require metabolism in order to produce oxidative stress. Formation of reactive oxygen species was monitored with 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) and glutathione (GSH) status. Cell viability was measured with propidium iodide. NHEK cells were seeded into 96-well culture plates (10,000 cells/100 µl) and cultured with human keratinocyte growth medium and 10% BSA. Exposures to menadione (20 µM) and cumene hydroperoxide (100 µM) were for 3, 6, or 24 hr. Menadione increased DCFDA signal by approximately 2- to 3-fold after 6 hr and reduced GSH to levels less than 50% of controls after 6 and 24 hr. These effects were greatly reduced in the presence of Trolox (50 µM). Cumene hydroperoxide (20 and 100 µM) also produced significant oxidative stress, increasing DCFDA signal by 8- to 10-fold relative to controls and reducing GSH levels after a 6 hr exposure. These oxidative stress effects were also significantly reduced in the presence of Trolox. When the NHEK model was used to evaluate the antioxidant properties of new products, it was possible to demonstrate clear antioxidant properties.

INTRODUCTION

The production of reactive oxygen species in the skin by exposure to UV radiation or through the metabolism of environmental chemicals to reactive intermediates can have a significant impact on skin health and the aging process. Therefore the development of new and efficacious antioxidants for use in cosmetic products is important. A key component to the success of new products is the ability to demonstrate efficacy. One needs to show not only that a compound can protect against oxidative stress, but also to understand the mechanism by which it protects. A model that can reproduce oxidative stress in a human skin cell system could be used to evaluate the efficacy of antioxidants. In addition, when adequate dose-response relationships can be shown it should be possible to evaluate relative potency between existing antioxidants that are well characterized, and new molecules. The objective of this study was to develop a human skin cell model (NHEK) in which oxidative stress could be produced by exposure to classical oxidant and pro-oxidant compounds. Oxidative stress was determined by monitoring the formation of peroxides and reactive oxygen species with DCFDA and by measuring the depletion of intracellular reduced glutathione. Once established, the ability of known antioxidants to prevent or reduce the effect could be tested.

METHODS

Cell Culture Conditions
Cryopreserved human foreskin-derived primary NHEK keratinocyte cells were used as the test system. The culture medium used for these cells was Human Keratinocyte Growth Medium with 10% bovine serum. Keratinocyte media was obtained from PromoCell GMBH and calf serum was procured from Invitrogen.

Description of Experimental Setup and Biochemical Assays
Flat bottom 96-well plates were seeded with 10,000 cells/well 48 hr prior to dosing. The day after seeding, the antioxidant compounds in medium were added to the plates for overnight pre-treatment. Media was removed the following morning and replaced with media containing pro-oxidants plus individual test compounds.

Experimental Protocol
Dosing solutions were prepared in complete culture medium. The human keratinocyte (NHEK) cell line was used as the test system. Cells were seeded into 96-well plates and allowed to equilibrate for approximately 48 hr. Following the equilibration period the cells were pretreated for 18 hours with known antioxidant (50 µM Trolox) or NAC in keratinocyte media. Following the pre-exposure period, the pre-treatment media was removed and replaced with media containing antioxidants 50 µM Trolox, or NAC, plus pro-oxidant (20 µM menadione or 100 µM cumene hydroperoxide). Based on the assay, cells or their supernatant (culture medium) were collected and analyzed for changes in cell viability or oxidative stress.

Reactive Oxygen Species – 2,7-Dichlorofluorescein Diacetate (2,7-DCFDA)
2,7-Dichlorofluorescein diacetate (DCFDA) a cell-permeant indicator for reactive oxygen species that is non-fluorescent until removal of the acetate groups by intracellular esterases and oxidation occurs within the cell, was used to measure intracellular production of reactive oxygen species (ROS). For these experiments, the 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) form of DCFDA was used. NHEK cells were preloaded with CM-H2DCFDA for one hour prior to administration of test articles. At the end of each incubation period, fluorescence was measured using a Packard Fusion plate reader or equivalent reader at 492 nm excitation and 517 nm emission.

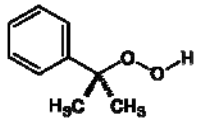
Intracellular Glutathione (GSH) Levels
Intracellular glutathione levels were determined essentially as described by Griffith (1980) with modifications. At the end of the exposure period, the medium was removed from the cells and metaphosphoric acid (MPA) was added to each well. Plates were then shaken for 5 min at room temperature and stored at –20ºC until needed.

Cell Viability
Cell viability was determined in a separate plate using a specific nucleic acid binding dye (propidium iodide (PI)). PI is impermeable to healthy cells. In dead and dying cells the dye enters the cell binds to DNA/RNA and fluoresces. There is a direct correlation between fluorescence and cell death. The percent change relative to controls was calculated by dividing the treatment cell number by the control cell number and multiplying by 100.

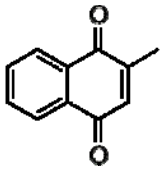
RESULTS

FIGURE 1

Chemical Structures of The Oxidants Tested



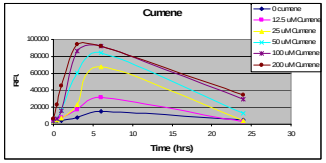
Cumene Hydroperoxide



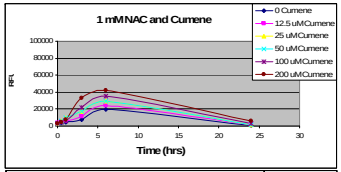
Menadione

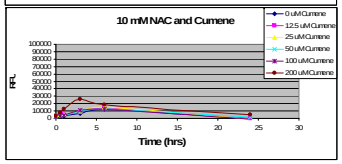
FIGURE 2

Cumene is a Direct Acting Oxidant and Produces Oxidative Stress Related to Dose and Time



NAC Protects Against Oxidative Stress

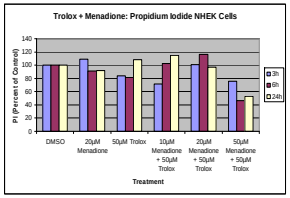


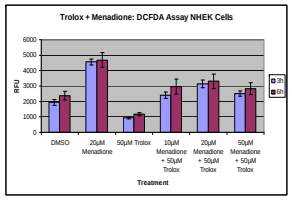


Cumene hydroperoxide produced a dose and time related increase in oxidative stress as measured by the increase in DCF relative fluorescence units (RFUs). Maximum response was observed after 3 to 6 hours of exposure for all concentrations tested. The loss of signal at extended time points was due in part to increased toxicity at concentrations above 50 µM, and in part was due to the inherent nature of this assay. Pretreatment with N-acetylcysteine (NAC) reduced the oxidative response in a dose-dependent manner.

FIGURE 3

Menadione Induced Oxidative Stress was Reduced by Vitamin E





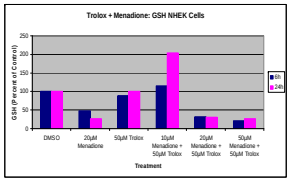
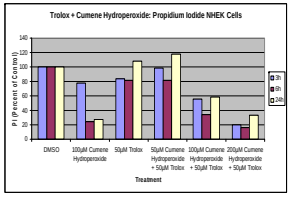
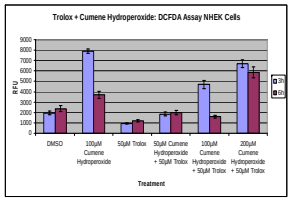
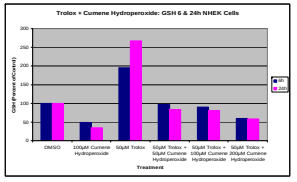


FIGURE 4

Cumene Hydroperoxide Induced Oxidative Stress was Reduced by Vitamin E



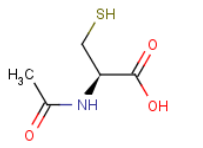




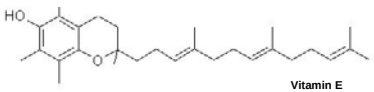
Menadione requires metabolic activation by CYP1A in order to produce oxidative stress. Menadione increased DCF-RFUs 2-fold over controls and reduced GSH levels (Figure 3). In the presence of soluble vitamin E both of these effects were reduced. These oxidative effects occurred at exposure concentrations that had little effect on cell viability. In comparison, the direct acting oxidant cumene hydroperoxide, increased DCF-RFUs by more than 4-fold after 3 hr and reduced GSH by 2-fold. In the presence of the antioxidant vitamin E these effects were reduced to near control values (Figure 4). Compared to vitamin E, N-acetylcysteine (NAC) was considerably more effective at protecting cells from oxidative stress (Figure 2).

FIGURE 5

Chemical Structures of The Antioxidants Tested



N-Acetylcysteine



Vitamin E

SUMMARY

Normal human epidermal keratinocytes (NHEK) cells responded well to known inducers of oxidative stress.

The DCFDA assay was an effective and sensitive endpoint to monitor oxidative stress.

The optimal exposure time for detecting DCFDA signal was between 3 and 6 hours.

Multiple endpoints monitoring cell health, as well as oxidative stress improved the model's performance.

The oxidative effects produced by the indirect oxidant (menadione) and the direct oxidant cumene hydroperoxide could be significantly reduced in the presence of two well characterized antioxidants, vitamin E, and NAC.

CONCLUSIONS

The NHEK cell system responded well to known oxidants, and these effects were reduced in the presence of known antioxidants. The system can effectively demonstrate not only efficacy, but also the relative potency between antioxidant agents. A potential disadvantage of the model is that test compounds with low solubility may not be taken up by the cells. In situations where finished products or ingredients with low solubility are to be evaluated, the use of three dimensional models where cells are grown at an air-liquid interface may improve and extend this approach.

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