Communication

L-Ergothioneine Protects Skin Cells against UV-Induced Damage—A Preliminary Study

Karolina Bazela [1,†,*], **Aleksandra Solyga-Zurek** [2,†], **Renata Debowska** [1], **Katarzyna Rogiewicz** [1], **Ewa Bartnik** [2] **and Irena Eris** [1]

[1] Dr. Irena Eris Cosmetic Laboratories, Centre for Science and Research, Pulawska 107a, Warsaw 02-595, Poland; E-Mails: renata.debowska@eris.pl (R.D.); katarzyna.rogiewicz@eris.pl (K.R.); irena.eris@eris.pl (I.E.)

[2] Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw and Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, Warsaw 02-106, Poland; E-Mails: asolyga@biol.uw.edu.pl (A.S.-Z.); ebartnik@igib.uw.edu.pl (E.B.)

[†] These authors contributed equally to this work.

[*] Author to whom correspondence should be addressed; E-Mail: karolina.bazela@eris.pl; Tel.: +48-22-717-1248; Fax: +48-22-757-0612.

Abstract: Many changes related to aging at the cellular level may be due to the physiological condition of mitochondria. One of the most common types of damage of mtDNA is the so-called "common deletion" referring to a deletion of 4977 base pairs. In the skin cells this phenomenon probably is caused by oxidative damage of mtDNA induced by UV. The present study was aimed at evaluating the effect of the antioxidant L-ergothioneine on UV-induced damage in skin cells. The effect of L-ergothioneine on the reduced glutathione level was studied. The presence of the "common deletion" in human fibroblasts irradiated with UVA and treated with L-ergothioneine was evaluated by a polymerase chain reaction. We have demonstrated that L-ergothioneine enhanced the level of reduced glutathione and protected cells from the induction of a photoaging-associated mtDNA "common deletion". In view of our results, L-ergothioneine could be an effective skin care and anti-photoaging ingredient.

Keywords: L-ergothioneine; common deletion; mitochondria; photoaging

1. Introduction

Ultraviolet irradiation is one of the most important environmental factors in the development of a number of skin conditions, ranging from photoaging to cancer. UVA/B rays penetrate into the skin, reach cells and are absorbed by proteins, lipids, and nuclear and mitochondrial DNA, causing a cascade of oxidative events that can result in progressive deterioration of cellular structure and function [1–3].

There are many theories of aging and a number of them encompass the role of mitochondria in this process. Mitochondria are responsible for producing approximately 90% of the cellular energy in the process of oxidative phosphorylation where electrons from NADH and FADH2 are transferred to oxygen. This generates a significant amount of reactive oxygen species (ROS). Mitochondria are able to counteract the production of ROS with antioxidant defense systems which can detoxify the amount of ROS produced, however, some ROS do evade these processes and are able to damage mitochondrial DNA (mtDNA) as well as proteins and lipids.

Mitochondria contain their own DNA (mtDNA), which is distinct from nuclear DNA and more susceptible to damage. Mitochondrial DNA mutations and deletions have been shown to accumulate in many tissues in mammals during aging. mtDNA mutations are thought to arise due to the close proximity to the inner membrane where reactive oxygen species are continually produced in the electron transport chain. This, along with the absence of protective histones and impaired DNA photoproduct repair makes mtDNA a sensitive marker of UV-induced DNA damage [4,5].

It has been shown that photoaged skin is characterized by increased mutations in the mitochondrial (mt) genome [6]. The most frequently reported deletion is a large-scale (4977 bp) deletion also called the "common deletion" (CD). In human skin CD has been shown to occur more frequently with increasing sun exposure and thus appears to be a useful marker rather for UVA induced alterations than for chronological aging [7–10].

The use of antioxidants is an effective approach to prevent symptoms related to photo-induced aging of the skin. Many antioxidants have long been used in cosmetology. L-Ergothioneine (EGT) is an unusual sulphur-containing derivative of an amino acid, histidine, which is acquired by mammals exclusively in their diet. L-Ergothioneine has two functional groups: L-thiol (SH) group and quaternary ammonium group N–(CH$_3$)$_3$ (Figure 1). EGT's thiol group implies an antioxidant role. The quaternary group EGT resembles carnitine and transfers fatty acids into the mitochondria, therefore increasing the ATP level in cells. EGT is concentrated in cells and tissues frequently exposed to oxidative stress. Numerous *in vitro* assays have demonstrated the antioxidant and cytoprotective capabilities of EGT against a wide range of cellular stressors [11–13].

Figure 1. Chemical structure of L-ergothioneine.
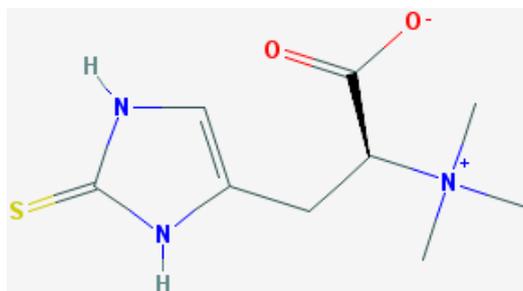
On the basis of these data on the antioxidant activity of L-ergothioneine we found it interesting to study the effect of this substance on UV-induced alterations in skin cell cultures, particularly on mtDNA damage.

2. Experimental Section

2.1. Cell Cultures

KB cells (epidermoid carcinoma cell line KB, American Type Culture Collection (ATCC), Manassas, VA, USA) were grown in standard RPMI medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 0.25 µg/mL streptomycin sulfate) at 37 °C in 5% CO_2. The KB cell line was used as a model representing human keratinocytes.

Skin samples, obtained from donors undergoing cosmetic surgery, were treated with dispase II solution (5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 16 h, followed by separation of dermal and epidermal layers with tweezers. Next, the dermis was cut into ~1 mm^2 fragments and treated with 2 mg/mL collagenase and 0.1 mg/mL DNase (Sigma-Aldrich, St. Louis, MO, USA) overnight. Liberated cells were seeded into cell culture flasks for culture. Primary human fibroblasts were grown in standard DMEM medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA), 10 mM HEPES, 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 0.25 µg/mL streptomycin sulfate) and 50 µg/mL uridine, with or without 20 µM L-ergothioneine (Barnet Products Corp.,Englewood Cliffs, NJ, USA), at 37 °C in 5% CO_2. Confluent passages between 2 and 8 were used.

2.2. UVB Irradiation

Confluent KB cells (control and treated with 20 µM L-ergothioneine (Barnet Products Corp., Englewood Cliffs, NJ, USA)) were exposed to UVB (300 J/m^2, fluorescent bulb TL12 Philips, emission peak at 313 nm) through phosphate buffered saline.

2.3. UVA Irradiation

The presence of the "common deletion" was evaluated in fibroblasts derived from 2 female donors aged 38 and 45 and from 2 male donors aged 30 and 42. Cells were grown in the presence of 50 µg/mL uridine (Sigma-Aldrich, St. Louis, MO, USA), with or without 20 µM L-ergothioneine and irradiated with 3 doses (8 J/cm^2, fluorescent bulb GE F4T5/BLB, emission peak at 368 nm) of UVA per day, through phosphate buffered saline. This procedure was repeated for 4 consecutive days during 3 weeks.

2.4. Colorimetric Determination of Reduced Glutathione (GSH)

GSH level was measured using the GSH/GSSG-412 assay (OxisResearchTM, Portland, OR, USA)—colorimetric determination of reduced and oxidized glutathione. The method employs Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid—DTNB), which reacts with GSH to form a product which is spectrophotometrically detectable at 412 nm. The change in absorbance at 412 nm is

a linear function of the GSH concentration in the reaction mixture [14]. The protein concentration was measured with Bradford Reagent (Sigma-Aldrich, St. Louis, MO, USA) [15]. Three independent experiments were performed. Comparisons between control and post-treatment measurements were performed using an unpaired Student's *t*-test. Probability values of $p < 0.05$ were considered statistically significant.

2.5. "Common Deletion" (CD) PCR

Phenol-chloroform extraction of total DNA and PCR (Fermentas, Lithuania, Vilnius) were performed according to standard protocols. PCR reaction mixture, for both primer pairs, contained: F primer 2 µM; R primer 2 µM; dNTP 20 mM; Taq Buffer with $(NH_4)_2SO_4$ and 20 mM $MgCl_2$ 1×; Taq polymerase 1 U; DNA 100 ng. All reactions were performed in 25 µL volume in the following conditions: the denaturation step was carried out at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 7 min. See Table 1 for primers used.

Table 1. PCR primers for detection of the "common deletion" in mtDNA.

PCR primer	Orientation	Position
MT1A	F	8224-8247
MT1C	F	13176-13198
MT2	R	13501-13477

PCR reaction with primers *MT1A–MT2* was carried out, amplifying a fragment 300 bp in length when the "common deletion" was present. In order to ensure that the absence of CD PCR product is caused by the absence of CD and not by an incorrect PCR reaction, PCR with primers *MT1A–MT2* was performed. A corresponding product 325 bp in length is obtained when the mitochondrial genome without the deletion is present and thus serves as a control for the PCR reaction. As a positive control of the PCR reaction, DNA isolated from a patient with a previously confirmed common deletion in mtDNA was used. PCR products were detected by electrophoresis on a 1.5% agarose gel with ethidium bromide against a DNA molecular-weight marker. The gel was photographed under UV transillumination. Three independent experiments were performed.

3. Results and Discussion

3.1. L-Ergothioneine Increases the Reduced Glutathione (GSH) Level

The tripeptide glutathione (GSH) is the most abundant intracellular non-protein thiol. It is present predominantly in a reduced form (GSH), which is the biologically active form. Over the years, a great deal of information has been gathered on the role of GSH in maintaining the intracellular reduction–oxidation (redox) environment including antioxidant defense via direct interaction with reactive oxygen/nitrogen species (ROS/RNS) or via activities of detoxification enzymes like GSH peroxidases and GSH-*S*-transferases [16]. In our study we tested the effect of L-ergothioneine on the reduced glutathione (GSH) level in the KB cell line using the GSH/GSSG-412 colorimetric assay. Our studies showed that L-ergothioneine at the 20 µM concentration (chosen according to our previous studies [17]) increased the reduced glutathione (GSH) level both in control and UVB irradiated

KB cells (Table 2). Changes between GSH level in control compared with GSH level after treatment with L-ergothioneine, both in cells exposed and unexposed to UVB, were statistically significant. The UVB light was chosen for the irradiation of KB cells since it is the light of the UVB spectrum that is for the most part absorbed by the epidermis *in vivo* [18]. UVB may also damage epidermal cells both directly and indirectly by inducing the formation of reactive oxygen species, *i.e.*, the superoxide anion [19]. Thus, it was deemed necessary to study the antioxidative effect of EGT on UVB irradiated epidermis.

Table 2. Quantitative determination of glutathione (GSH) level in KB cells.

KB cells	μmol GSH/μg protein	
	−UVB	+UVB (300 J/m^2)
control	6.07 ± 0.15	3.03 ± 0.02
20 μM L-ergothioneine	15.37 ± 0.13	4.65 ± 0.06

Our results are in line with numerous studies demonstrating that L-ergothioneine is an integral component of the cellular antioxidant defense system. It scavenges hydroxyl and peroxynitrite radicals as well as the superoxide anion radical and singlet oxygen. *In vitro* tests have also demonstrated that EGT also has strong copper chelating ability—it potentially inhibits tyrosinase activity [20–22].

Moreover, *in vitro* tests have demonstrated that in cultured fibroblasts EGT suppressed the tumor necrosis factor α (TNF α) up-regulation induced by UVB irradiation. In addition in fibroblasts exposed to UVA, EGT suppressed the expression of matrix metalloproteinase 1 (MMP 1) protein [23]. EGT also inhibited nuclear factor kβ (NF kβ) activation and limited the transcriptional activation of the gene for interleukin 8 (IL-8)—a chemotaxin which might account for neutrophil recruitment [24].

It has been demonstrated that EGT is a more powerful antioxidant than either coenzyme Q(10) or idebenone due to its relatively greater efficiency in directly scavenging free radicals and in protecting cells from UV-induced ROS [25].

Recently, a high specific transporter for EGT (ETT) was identified in mammalian tissues, which explains the high tissue levels of EGT and implies a physiological role. Depletion of ETT leads to augmented oxidative stress and cell death. ETT is highly concentrated in the plasma membrane and mitochondria [11].

3.2. L-Rrgothioneine Shows Protective Properties against the Induction of a Photoaging-Associated mtDNA "Common Deletion"

One of the best described mt deletions, the 4977 bp "common deletion", is known to be involved in photoaging of the skin. Intraindividual comparison studies have revealed that the "common deletion" is increased up to 10-fold in photoaged skin, as compared with sun-protected skin of the same individuals [7]. The objective of our study was to evaluate the effect of L-ergothioneine on the occurrence of mtDNA "common deletion" in UV-irradiated human primary fibroblasts obtained from various donors. For the experiments on dermal cells only UVA irradiation was performed, since the great majority of UVB rays is absorbed by the epidermis and hence never reaches the dermal level [18]. UVA is known to cause mainly indirect damage to cellular structures and nucleic acids by inducing the formation of radical oxygen and nitrogen species but may also be responsible for single- and double-strands breaks in DNA [19]. Thus, exposure to UVA may be one of the main causes

of mtDNA mutations including mtDNA deletions [26]. In our experiments, in fibroblasts which were not exposed to UVA we did not observe the "common deletion" after DNA isolation nor during 3 weeks of culture (Figure 2, lanes 9, 11 and 13). When we incubated fibroblasts with L-ergothioneine we also observed no "common deletion" PCR product (Figure 3, lanes 9, 11 and 13). To summarize this part of our study: first of all, cultured fibroblasts isolated from various donors did not initially show the "common deletion". Secondly, L-ergothioneine, itself, had no influence on the presence of the "common deletion".

Figure 2. Representative PCR analysis of the presence of the "common deletion" in a culture of human fibroblasts incubated with and without UVA exposure, without the presence of L-ergothioneine. M, molecular weight marker; C, control PCR reaction, confirming the presence of mtDNA; 1, fibroblasts after 1 week with UVA exposure; 3, fibroblasts after 2 weeks with UVA exposure; 5, fibroblasts after 3 weeks with UVA exposure; 7, positive control—DNA from a patient with a previously confirmed presence of "common deletion"; 9, fibroblasts after 1 week without UVA exposure; 11, fibroblasts after 2 weeks without UVA exposure; 13 fibroblasts after 3 weeks without UVA exposure.
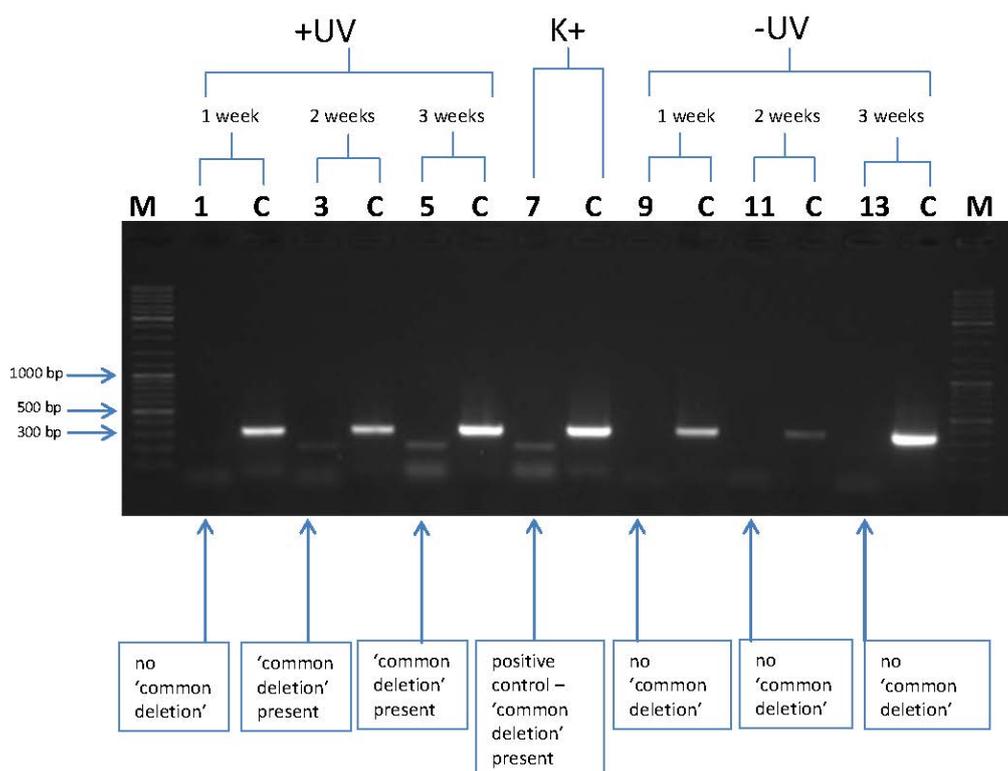
We were able to induce the "common deletion" using repetitive UVA irradiation in cultured human fibroblasts derived from two donors (Figure 2, lanes 1, 3 and 5) suggesting that there is an individual susceptibility to the induction of CD, at least in our *in vitro* model. Due to the fact that our "common deletion" analysis was performed on fibroblasts isolated from four donors, this requires further studies. When fibroblasts were irradiated and co-incubated with 20 μM L-ergothioneine we did not observe the "common deletion" even after 3 weeks with UVA exposure, suggesting that L-ergothioneine may prevent the formation of this particular damage in mtDNA (Figure 3, lanes 1, 3 and 5). Our results are in accordance with the previous studies where repetitive exposure of keratinocytes, fibroblasts or

human skin to UVA at physiological doses was found to induce mutations of mtDNA [7,27–29]. Interestingly, it has been shown that in fibroblasts the level of CD is decreasing with prolonged culture, which can be counteracted with uridine supplementation of culture media [6,7,30]. For this reason, uridine supplementation was employed during all CD analyses in this study.

Figure 3. Representative PCR analysis of the presence of the "common deletion" in a culture of human fibroblasts incubated with and without UVA exposure, in the presence of 20 μM L-ergothioneine. M, molecular weight marker; C, control PCR reaction, confirming the presence of mtDNA; 1, fibroblasts after 1 week with UVA exposure; 3, fibroblasts after 2 weeks with UVA exposure; 5, fibroblasts after 3 weeks with UVA exposure; 7, positive control—DNA from a patient with a previously confirmed presence of "common deletion"; 9, fibroblasts after 1 week without UVA exposure; 11, fibroblasts after 2 weeks without UVA exposure; 13, fibroblasts after 3 weeks without UVA exposure.
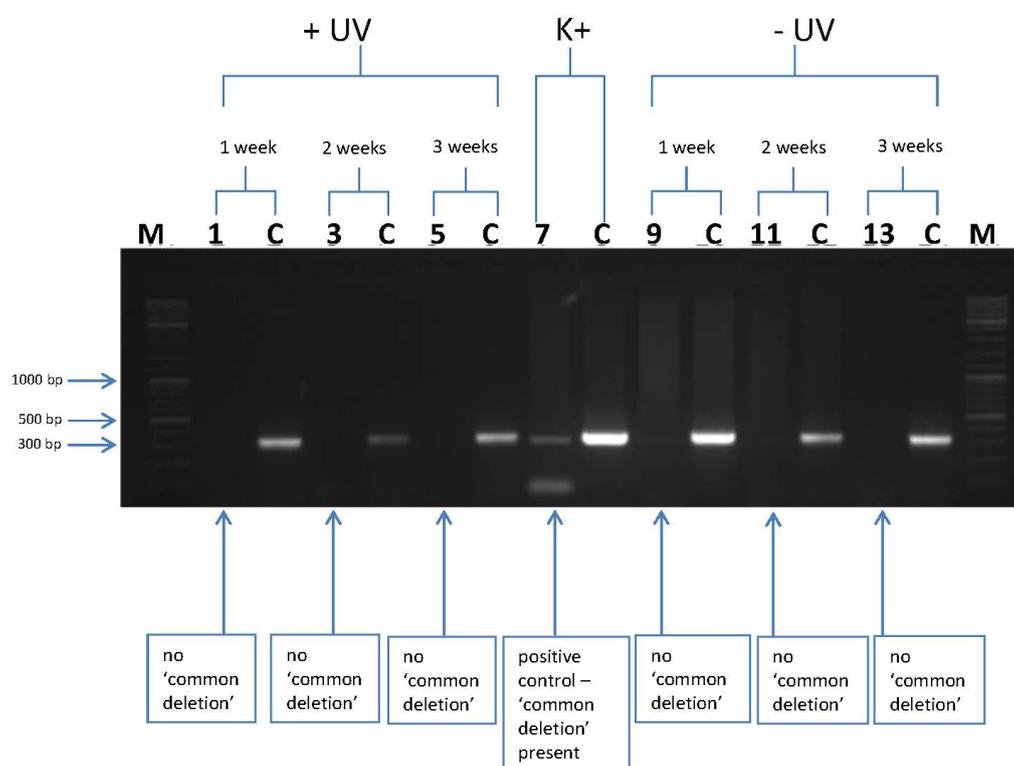
Protection against the induction of a photoaging-associated mtDNA mutation has been also previously shown for two other widely applied cosmetic actives—β-carotene and genistein [31,32].

Our results demonstrated that L-ergothioneine may protect skin cells against some UV-induced damage; in particular against the occurrence of mitDNA "common deletion". Further studies are required to assess the full-spectrum of L-ergothioneine's protective activity. We conclude that L-ergothioneine, as an active compound of cosmetic products, shows considerable promise as an effective agent against the aging process. On the other hand, we have shown that in cosmetology, the detection of mtDNA deletions could be a useful tool to assess the effect of active ingredients on UV-irradiated human skin cells.

4. Conclusions

Aging of human skin has been the subject of a profound interest for cosmetic industry which attempts to counteract this process. In our study we tested L-ergothioneine, one of the antioxidants used in cosmetology, and we found that L-ergothioneine enhanced the level of reduced glutathione and protected against the induction of a photoaging-associated mtDNA "common deletion". L-Ergothioneine might be involved in the protection of mtDNA from ROS generated in the course of electron transport.

In view of these results L-ergothioneine could act as an effective skin care and anti-photo-aging ingredient. On the other hand, we have shown that in cosmetology, the detection of mtDNA deletions could be a useful tool to assess the effect of active ingredients on UV-irradiated human skin cells.

Acknowledgments

Author Contributions

Study conception and design: Karolina Bazela, Aleksandra Solyga-Zurek, Renata Debowska, Katarzyna Rogiewicz, Irena Eris; Acquisition of data: Karolina Bazela, Aleksandra Solyga-Zurek; Analysis and interpretation of data: Karolina Bazela, Aleksandra Solyga-Zurek, Renata Debowska; Drafting of manuscript: Karolina Bazela, Aleksandra Solyga-Zurek; Critical revision: Ewa Bartnik.

Conflicts of Interest

The authors declare no conflict of interest.

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