Title: Oral supplementation of L-glutathione prevents UVB-induced melanogenesis and oxidative stress in BALB/c mice

Tava Shelan NAGAPAN, Wenna Nallance LIM, Dayang Fredalina BASRI and *Ahmad Rohi GHAZALI

Programme of Biomedical Science, Centre of Health & Applied Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

*Corresponding author:

Ahmad Rohi Ghazali, Ph.D

Affiliation: Programme of Biomedical Science, Centre of Health & Applied Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

Email: rohi@ukm.edu.my
Abstract

Dietary antioxidant supplements such as L-glutathione have gained considerable attention in dermatology and cosmeceutical fields. L-Glutathione possesses antiaging, antimelanogenic, antioxidant, and anticancer properties. This study aimed to investigate the inhibitory effects of L-glutathione on melanogenesis activity and oxidative stress in UVB-irradiated BALB/c mice. Eighteen female BALB/c mice were randomly divided into 3 groups: a control group (n=6), a group without UVB irradiation and L-glutathione administration; a UVB irradiated group (n=6), a group irradiated with a UVB dose of 250 mJ/cm² for 3 minutes; and a treatment group (n=6), a group irradiated with UVB and treated with 100 mg/kg of L-glutathione by oral gavage. Treatment was given for 14 days, and UVB irradiation was given on days 9, 11, and 13. Oral L-glutathione significantly (p<0.05) reduced lipid peroxidation and elevated superoxide dismutase activity and glutathione level. L-Glutathione also inhibited melanin content and tyrosinase activity significantly (p<0.05) as compared with the UVB-irradiated group. Histopathological examination also showed that L-glutathione reduced the deposition of melanin pigment in the basal layer of the epidermis as compared with that in UVB-irradiated mice. All in all, the present study demonstrated that L-glutathione has the potential to be developed as a photoprotection agent against UVB-induced oxidative stress and melanogenesis.

Key words: L-Glutathione, melanogenesis, oxidative stress, photoprotection, UVB
Introduction

The skin is the largest organ of the body and comprises two primary layers, which include epidermis and dermis, which together are made up of epithelial, mesenchymal, glandular, and neurovascular components [8]. Skin functions as the primary first-line defense against external antigens and environmental stressors such as ultraviolet (UV) radiation [13]. UV radiation includes UVA (315-400 nm), UVB (280-315 nm), and UVC (200-280 nm). Among all of the UV spectrum, UVB is the most genotoxic and damaging to the skin [22]. Excessive exposure to UVB can lead to hyperpigmentation, erythema, cutaneous edema, and even skin cancer [16].

Melanin is the skin-protective barrier that acts by absorbing UV radiation [25]. It is formed from melanogenesis conducted in melanocytes located in the basal layer of the epidermis [7]. Prolonged exposure to UV radiation may cause the accumulation of abnormal melanin induces pigmentation disorders, such as melasma, freckles and ephelide. One of the noninvasive strategies to treat these disorders is to control melanogenesis by inhibiting tyrosinase gene expression. Tyrosinase is the key enzyme that initiates the melanogenesis biosynthesis pathway [14].

Additionally, acceleration of melanogenesis activity due UV radiation produces hydrogen peroxide (H$_2$O$_2$) and other prooxidants such as reactive oxygen species (ROS) that would expose the melanocyte to high levels of oxidative stress. ROS scavengers have the ability to downregulate the UV-induced melanogenesis [17, 32].

Therefore, the use of antioxidant supplementation has become a new trend in dermatology and skin care products in recent years[18, 1]. L-Glutathione, also known as reduced glutathione, low-molecular-weight, water-soluble tripeptide antioxidant. L-Glutathione plays a vital role in detoxification of metabolic products such as lipid peroxides and xenobiotic compounds like
pollutants, heavy metals, and drugs [1,9]. Several studies have also proven that L-glutathione is able to slow down carcinogenesis, attenuate immune response, and remove toxic xenobiotics [24,11,15]. Besides, its role as anti-melanogenic properties have led to its promotion as a skin lightening agent [26]. In the present study, we aimed to determine the inhibitory effect of L-glutathione on melanogenesis activity and oxidative stress in UVB irradiated BALB/c mice.

Materials and Methods

Preparation of L-glutathione

L-glutathione was a gift from Dr Nabisarr Mustan, Cambridge Herbal Sdn Bhd (Sungai Buloh, Malaysia). Mice were treated with L-glutathione at a dose of 100 mg/kg [31]. The dose was freshly prepared daily by dissolving L-glutathione in corn oil and then vortexing it until the powder dissolved completely. All mice were fasted for 4 hours before being treated with 0.2 ml of dissolved glutathione by oral gavage [19].

Animals and experimental design

Eighteen female BALB/c mice were supplied by the Faculty of Veterinary Medicine, University Putra Malaysia. The animals were kept in the animal house of the Department of Biomedical Science, Centre of Health and Applied Sciences, Universiti Kebangsaan Malaysia, under 12-hours light/dark cycles at controlled room temperatures. All mice were given free access to a standard pellet diet and drinking water.

The animals were randomly divided into three groups: a vehicle control group (n=6), which was not exposed to UVB irradiation and not treated with L-glutathione; an exposure group (n=6), which was exposed to UVB irradiation only; and a treatment group (n=6), which was exposed to UVB irradiation and also treated with 100 mg/kg L-glutathione. The L-glutathione was
administered throughout the treatment period. The treatment was administered for fourteen successive days. Briefly, the dorsal parts of the mice were shaved using an electric shaver (Phillips, Malaysia). Mice from both groups (exposure and treatment groups) were then exposed to UVB irradiation for 3 minutes at a dose of 250 mJ/cm² on the 9th, 11th, and 13th days of treatment [21]. The source of irradiation was a 15 watt lamp emitting UV light with a wavelength of 312 nm (UVP, USA).

The usage of the mice was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC; UKMAEC No: FSK/2017/AHMAD ROHI/22-NOV./887-NOV.-2017-JULY-2018), and guidelines were strictly followed.

Preparation of skin tissue homogenate

The skin tissue was washed with normal saline and then minced using a pestle and mortar. After being weighed, the skin was then homogenized in phosphate buffer solution at pH 7.8. After centrifugation, the supernatant was removed and stored at −40˚C. The whole procedure was conducted under cold conditions. This homogenate was used for evaluation of oxidative stress and melanogenesis activity evaluation.

Determination of glutathione (GSH) level

GSH level was measured in the skin homogenate based on the Ellman method [10], with some modifications. Skin homogenate was treated with metaphosphoric acid solution and centrifuged at 3000x g for 10 minutes at 4˚C. The obtained supernatant was mixed with reaction buffer at pH 8.0 and DTNB for 15 minutes. Then it was measured at 412 nm using a microplate reader. The GSH level is expressed as µmol/mg protein.
**Determination of superoxide dismutase (SOD) activity**

SOD activity was measured in the skin homogenate based on the Beyer and Fridovich method [4]. Briefly, an aliquot of (20 μl) of the homogenate was mixed with a substrate, which contained PBS (EDTA), L-methionine, NBT.2HCl, Triton-X, and riboflavin. The mixture was incubated in an aluminium box under 20 watt lamp for 7 minutes. The SOD activity was measured spectrophotometrically at 560 nm. SOD activity was calculated and expressed in U/min/mg protein.

**Determination lipid peroxidation activity**

Lipid peroxidation activity was measured in the form of malondialdehyde using thiobarbituric acid based on Stocks and Dormandy method with some modifications [27]. Briefly, a 0.5 ml aliquot of skin homogenate was added to a TCA/HCl solution, vortexed, and incubated at room temperature for 15 minutes. The mixture was added to TBA/NaOH, vortexed and heated in a boiling water bath (100°C) for 30 minutes. The MDA in the sample reacted with the thiobarbituric acid to form a pink chromogen containing thiobarbituric acid reactive substances (TBARS). The level of TBARS in the supernatant was measured using a spectrophotometer at 532 nm. The MDA concentration was expressed as nmol/g protein of tissue protein.

**Determination of dopa oxidase activity**

The dopa oxidase assay used was the new 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH) assay based on the Winder and Harris method with a minor modification [30]. The rate production of the pink pigment was used as a quantitative measure of enzyme activity. The reaction mixture (1 ml) contained 490 μl assay buffer A (100 mM sodium phosphate pH 7.1, 4 % by volume N, N’-dimethylformamide), 200 μl 5 mM L-DOPA, and 290 μL 20.7 mM MBTH. The reaction
mixture, without enzyme, was prepared up in a 1.6 ml plastic cuvette and incubated at 37°C for 10 minutes. The temperature of the reaction mixture was measured using a thermometer, 20 µl of homogenate was added into the cuvette, and the cuvette contents were mixed by inversion. The initial rate of absorbance increase at 505 nm was monitored spectrophotometrically, routinely over a period of 5 minutes. The temperature of the assay mixture was rechecked at the end of the assay.

**Determination of melanin content level**

Melanin content was measured using the Talwar method [28]. Melanin in the resulting homogenate (0.35 ml) was solubilized by treatment with 2 N NaOH at 60°C for four days. An equal volume of distilled water was then added, followed by the addition of 0.7 ml of chloroform:phenol (1:1). The mixture was vortexed and centrifuged at 5000 x g for 10 minutes to separate the phases. Melanin content in the water layer was pipetted into a 96-well microplate and measured by a microplate reader at 400 nm. Melanin content is expressed as micrograms of melanin per milligram wet weight of skin.

**Histological evaluation**

At the end of the experiment, the animals were anesthetized with by ketamine and xylazine and euthanized by cervical dislocation. Tissue samples were taken from the dorsal part of skin. The tissue specimens were fixed using 10% neutral buffered formalin for 24 hours and embedded in paraffin blocks. Sections were cut at thickness of 5 µm for histopathological observation. Fontana Masson staining was performed to assess the melanin pigmentation on the mouse skin. In the histological examination, melanin was quantitated based on using Billings method [5].
Statistical analysis

The data analysis was performed using the IBM SPSS Statistics version 23.0 and values were expressed as the mean ± standard error mean (SEM). One-way ANOVA was used to compare the GSH, SOD, MDA, dopa oxidase, and melanin content level between groups. Differences among means were examined using Tukey’s post hoc test and were considered to be statistically significant at p < 0.05.

Results

Effects of L-glutathione on GSH level in UVB-irradiated mice

Figure 1 showed the results for glutathione level in the various groups. The glutathione level of the UVB-irradiated group (0.376 ± 0.025 µmol/mg) decreased significantly as compared with that of the vehicle control group (0.554 ± 0.045 µmol/mg; p<0.05). Oral administration of L-glutathione 100 mg/kg increased the glutathione level (0.468±0.029 µmol/mg) as compared with the level of UVB-irradiated group (0.376±0.025 µmol/mg).

Effects of L-glutathione on SOD activity in UVB-irradiated mice

Figure 2 showed the superoxide dismutase (SOD) activity level in various group. The SOD activity in UVB irradiated group (0.561 ± 0.034 U/min/mg) decreased significantly as compared to the vehicle control group (1.142± 0.156 U/min/mg). However, L-glutathione treatment group (1.153 ± 0.09 U/min/mg) increased significantly (p<0.05) as compared to UVB irradiated group (0.561 ± 0.034 U/min/mg).
Effects of L-glutathione on lipid peroxidation activity in UVB irradiated mice

Figure 3 shows the levels of malondialdehyde (MDA), which indicated the lipid peroxidation activity, in various groups. The MDA level in the UVB-irradiated group increased (13.343 ± 1.35 nmol/g) significantly (p<0.05) as compared with that in the vehicle control group (2.67± 0.613 nmol/g). The MDA level in the L-glutathione treatment group (5.586±0.746 nmol/g) was significantly reduced (p<0.05) as compared with that in the UVB-irradiated group (13.343 ± 1.35 nmol/g).

Effects of L-glutathione on melanin content in UVB-irradiated mice

Figure 4 shows the levels of melanin content in the various groups. The melanin content in the UVB-irradiated group (1.546 ±0.097 µg/mg of skin) was significantly increased (p<0.05) as compared with that in the vehicle control group (1.188 ± 0.091 µg/mg of skin). However, oral administration of L-glutathione (1.122 ± 0.056 µg/mg of skin) reduced the melanin content significantly (p<0.05) as compared with the level in the UVB irradiated group (1.546 ±0.097 µg/mg of skin). Meanwhile, the melanin content in the vehicle control group (1.188 ± 0.091 µg/mg of skin) showed no significant difference as compared with that in the L-glutathione treatment group (1.122 ± 0.056 µg/mg of skin).

Effects of L-glutathione on tyrosinase activity in UVB-irradiated mice

Figure 5 shows the levels of dopa oxidase, which indicated the tyrosinase activity, in the various groups. The dopa oxidase level in the UVB-irradiated group (10.907 ± 2.059 units/min) was significantly increased as compared with that in the vehicle control group (1.533 ± 0.349 units/min). Meanwhile, the L-glutathione treatment group (3.933±0.858 units/min) showed
significantly reduced dopa oxidase activity as compared with the UVB-irradiated group (10.907 ± 2.059 units/min).

*Effects of L-glutathione on histopathological changes in UVB-irradiated mice*

Figure 6 shows the observed histopathological changes in the various groups. UVB irradiated group showed an obvious deposition of melanin in the basal layer of the epidermis as compared with the vehicle control group. Meanwhile, the L-glutathione treatment group showed very minimal deposition of melanin in the basal layer as compared with the UVB irradiated group. Figure 7 shows the melanin pigmentation indexes of the various groups. The UVB irradiated group (index: 15.082 ±1.001) showed a significant increase in pigmentation index as compared with the vehicle control group (index: 3.621 ± 0.442). Meanwhile, the L-glutathione group (4.766 ± 0.337) showed a significantly decreased the pigmentation index compared with UVB-irradiated group (index: 15.082 ±1.001).

**Discussion**

Solar radiation is one of the important external factors in skin pathogenesis that can lead to many complications. Exposure to UVB irradiation can generate ROS which can increase oxidative stress and results in an imbalance between ROS and endogenous antioxidant defense system [20]. It also activates melanocytes to produce melanin, which accelerates the melanogenesis biosynthesis pathway [2]. The use of antioxidant supplements that absorb UV rays and protect skin against UVB irradiation is of great interest in dermatology and cosmeceutical fields. In the present study, we investigated the inhibitory effects of L-glutathione on oxidative stress and melanogenesis activity in mice.
Exposure of skin to UVB irradiation leads to depletion of the antioxidant defense system, including SOD and GSH, which are used up to counter the surge in ROS production [23]. In this study, we discovered that both SOD and GSH decreased significantly after exposure of mouse skin to UVB irradiation, suggesting that the levels of antioxidants depleted. L-Glutathione treatment increased the SOD level significantly. However, no significant difference in reduced glutathione level was observed, which suggested that L-glutathione quenched the ROS by increasing the enzymatic antioxidant (SOD) level.

Oxidative stress is one of the detrimental outcomes of UVB-induced skin photodamage [12]. MDA is the end product of lipid peroxidation and can also serve as an important indicator of the presence of ROS [34]. Our results showed that the lipid peroxidation activity was higher in the UVB-irradiated group, suggesting that oxidative degradation of lipids occurred in the mice. Interestingly, L-glutathione protected the UVB-irradiated mice by decreasing the lipid peroxidation activity. This finding is similar a finding in another study [33] that showed that quercetin significantly reduced the lipid peroxidation induced by UVB. In addition, caffeic acid, a dietary antioxidant supplement- also result reduction of lipid peroxidation in UVB-induced mice skin [3].

Melanin plays a vital role in protecting the skin from photodamage. However, overexposure to UV radiation could lead to hyperpigmentation, melasma, and melanoma [6]. In our study, the melanin content increased in UVB-irradiated mouse skin suggesting that UVB activated the melanogenesis process and resulted in accumulation of melanin in skin. Treatment with L-glutathione had decreased the melanin content in the UVB-irradiated mice skin, suggesting that L-glutathione was able to inhibit or downregulate the melanin biosynthesis pathway. To understand the mechanism behind the significant decrease in melanin content caused by L-
glutathione, we investigated tyrosinase which is the key enzyme involved in melanogenesis activity [2]. L-Glutathione decreased the tyrosinase enzyme level in UVB-irradiated mice and this showed that the mechanism of the photoprotecting action of L-glutathione could be the direct inactivation of tyrosinase by binding with the copper-containing active site [29]. Histopathological observation also showed that UVB-irradiated mouse skin has more visible melanin pigmentation, meanwhile mice treated with L-glutathione showed less melanin pigmentation. The findings are also supported by another similar study conducted on the effects of Diospyros lotus leaf extract against UVB-induced skin damage in BALB/c mice.

In short, we demonstrated that oral administration of L-glutathione prior to UVB irradiation could influence the cutaneous response. L-Glutathione was able to improve the antioxidant status level, decreased lipid peroxidation activity, and could inhibit melanogenesis activity.

**Conclusion**

Oral administration of L-glutathione was able to inhibit melanogenesis activity by decreasing the melanin content and tyrosinase activity. L-glutathione also reduced the oxidative stress by increasing antioxidant levels and preventing lipid peroxidation activity. Hence, it could protect the skin from photodamage and has potential as a natural alternative agent for photoprotection.

**Acknowledgment**

This research work was funded by Universiti Kebangsaan Malaysia (grant number: GUP-2016-081).
References


Figure 1. Determination of glutathione levels in UVB-irradiated mice. The bar chart shows results for the concentration of glutathione in the different groups presented as the mean ± standard error mean (SEM); n = 6.

* Statistically significant difference in comparison with the vehicle control group (P <0.05).
Figure 2. Determination of superoxide dismutase (SOD) activity in UVB-irradiated mice. The bar chart shows the results of concentration of glutathione in the different groups presented as by the mean ± standard error mean (SEM); n = 6.

* Statistically significant difference in comparison with the vehicle control group (P <0.05).

# Statistically significant difference in comparison with the UVB-exposure group (P <0.05).
Figure 3. Determination of lipid peroxidation activity in UVB-irradiated mice. The bar chart shows the results for the concentration of malondialdehyde (MDA) in the different groups presented as the mean ± standard error mean (SEM); n = 6.

* Statistically significant difference in comparison with the vehicle control group (P <0.05).

# Statistically significant difference in comparison with the UVB-exposure group (P <0.05).
Figure 4. Determination of melanin content in UVB-irradiated mice. The bar chart shows the results for the melanin level in the different groups presented as the mean ± standard error mean (SEM); n = 6.

* Statistically significant difference in comparison with vehicle control group (P < 0.05).

# Statistically significant difference in comparison with UVB-exposure group (P < 0.05).
Figure 5. Determination of tyrosinase activity in UVB-irradiated mice. The bar chart shows the results of dopa oxidase activity level in different groups presented as the mean ± standard error mean (SEM); n = 6.

* Statistically significant difference in comparison with vehicle control group (P < 0.05).

# Statistically significant difference in comparison with UVB-exposure group (P < 0.05).
Figure 6. Histological observation of melanin pigmentation (arrow) in the basal layer of the epidermis of mice skin. (A) Vehicle control group; (B) UVB-irradiated group. (C) L-glutathione treatment group. Fontana Masson staining. Images are shown at x 40 magnification. Scale bar: 100 µm.
Figure 7. Determination of melanin pigmentation index in histology of various groups. The bar chart shows the results for the pigmentation index in the different groups presented as the mean ± standard error mean (SEM); n=6.

* Statistically significant difference in comparison with vehicle control group (P <0.05).

# Statistically significant difference in comparison with UVB-exposure group (P <0.05).