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Pterostilbene's protective effects against photodamage caused by UVA/UVB irradiation

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Aim: The aim of this study was to further elucidate the mechanism of pterostilbene against UVA/UVB irradiation and the Nuclear factor E2-related factor 2 (Nrf2) signal pathway. *Methods:* A photo-damage model with UVA/UVB irradiation in HaCat cells was established and used in this study. The dose of pterostilbene was selected through MTS assay. Cell proliferation and apoptosis in Nrf2 and knockdown Nrf2 cells was detected by MTS assay. Expression of CAT, HO-1, and SOD in Nrf2 and knockdown Nrf2 cells was explored by qPCR. Western blot was used to analysis of Nrf2 nuclear translocation changes in Nrf2 and knockdown Nrf2 cells. Protein carbonyl content and MDA content was tested. *Results*: Our photo-damage model was successfully established and 20J/cm^2 UVA and 57mJ/cm^2 UVB irradiation was the suitable dose for HaCaT cell damage study. UVA/UVB irradiation would affect Nrf2 protein location, especial for 9.75 μM pterostilbene dose. In addition, cell proliferation could be significantly inhibited by UVA/UVB treatments ($P<0.05$), whereas, 9.75 μM pterostilbene treatment can alleviate the photo-damage. UVA/UVB irradiation would lead to decreased expressions of CAT, HO-1, and SOD. Carbonyl content and MDA was significantly changed by UVA/UVB treatments ($P<0.05$). The adverse events could be reversed by adding 9.75 μM pterostilbene. Western blot analysis showed that Nrf2 cytoplasm content in UVA/UVB treated cells was reduced and Nrf2 nuclear content was increased, which are different with the normal HaCaT cells without knockdown Nrf2 treatment ($P<0.05$). The results of cell proliferation, apoptosis, and cell antioxidant capacity in knockdown Nrf2 treated HaCaT cells were also significantly different with the normal HaCaT cells without knockdown Nrf2 treatment ($P<0.05$). *Conclusion:* We hypothesize that pterostilbene could play an anti-oxidation role *via* the Nrf2 signal pathway.

1. Introduction

As the first defense line against various physical and chemical stimuli, Skin is the largest human organ, which is inevitably damaged by ultraviolet rays (UV) and environmental pollutants (Bickers and Athar 2006). According to the wavelengths differences, UV is mainly divided into UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). Of them, UVA and UVB are considered the main factors causing skin damage (Krutmann 2000; Nishigoriet al. 2003). 95% of UV reaching the human epidermis is UVA, which can penetrate the epidermis to the deep layer of dermis, and damage collagen and elastin. UVA could activate tyrosinase causing new melanin formation and melanin deposition. Meanwhile, low dose UVB is the main factor leading to sunburn erythema. UVB wavelength is similar with the absorbance values of DNA and protein. Therefore, UVB would result in the 800-1,000× skin damage intensity than the same dose UVA (Krutmann 2000). The mechanisms of UV induced skin damage are complex. The elaborated mechanisms include oxidative stress injury (Kruk and Duchnik 2014), DNA damage (Dinkova-Kostova et al. 2002; Kawachi et al. 2008; Marrot et al. 2008), protein damage (Kammeyer and Luiten 2015; Sprecher 2007), and skin immune suppression (Kulms and Schwarz 2000; Yanaka 2011). Of the mechanisms mentioned above, oxidative stress injury is crucial (McMahon et al. 2001). Therefore, removing excessive ROS and increasing the antioxidant level in cells would be helpful to prevent UV-induced damage.

The nuclear factor E2-related factor 2 (Nrf2) signal pathway is an important anti-oxidative stress mechanism. Nrf2 belonging to CNC (cap 'n'collar) is one member of leucine zipper transcriptional activator family (Sykiotis and Bohmann 2010). Many studies suggested that Nrf2 signal pathway plays an important role in UV damage protection (Schafer and Werner 2015). Nrf2-related signals are different when the skin is irradiated by different wavelengths and doses. For example, Nrf2 in keratinocyte was not sensitive to low dose (20J/cm^2) UVA irradiation. However, high dose (40 J/cm^2) irradiation would promote Nrf2 related gene expressions (Gruber et al. 2010). In HaCaT cells and melanocytes, 10 mJ/cm^2 UVB irradiation could reduce the Nrf2 and its target proteins' expression in a dose-dependent manner (Kannan and Jaiswal 2006). Moreover, 25 J/cm^2 UVA irradiation could activate Nrf2 and induce down-stream HO-1 gene expression (Zhong et al. 2010). In addition, an UV protective function of Nrf2 has also been identified in a series of gene silence experiments and in the human body (Kawachi et al. 2008; Zhong et al. 2010).

Pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) is an effective ingredient derived from plants such as red sandalwood, blueberries, grapes and palm trees. It is a non-flavonoid polyphenol compound, which is developed from resveratrol (3,4',5-trihydroxystilbene). Previous studies have indicated that resveratrol is beneficial to heart disease, nervous system diseases, and metabolic diseases. However, pterostilbene owns higher biological activity and bioavailability than resveratrol (McCormack and McFadden 2012). Meanwhile, there are no obvious toxic and side effects in animals with 3000 mg/kg/day treatment, which demonstrates the cpompounds safety (Ruiz et al. 2009). Pterostilbene could reduce oxidative stress injury and promote the expressions of hydroge-

nase, total glutathione, glutathione peroxidase, glutathione reductase, and SOD in cells (McCormack and McFadden 2013). Chiou et al. (2011) have revealed that pterostilbene could active Nrf2 and increase the expressions of HO-1 and glutathione reductase in rectal cancer (Chiou et al. 2011). Moreover, pterostilbene could active Nrf2 to reduce ROS and IL-1β expressions in chondrocyte damage (Xue et al. 2017). Pterostilbene analogues have been identified to relieve skin erythema, edema, and sunburn cells formation induced by UV irradiation (Pastore et al. 2012). In addition, pterostilbene has protective functions in acute skin injury in UVB (360 mJ/cm^2) treated SKH-1mice (Sirerol et al. 2015). However, the detailed mechanism has not been revealed.

In summary, UV irradiation can cause various skin injuries. Oxidative stress respond is an important factor in the injury induced by UV irradiation. Therefore, activation of antioxidant signaling pathways would protect skin cells against UV radiation damage. Nrf2 signal pathway is the core mechanism that protect cell against oxidative stress reaction. Of this study, we chose HaCat cells as study objective to explore the protective functions of pterostilbene to UV radiation damage in HaCat cells. This work would provide theoretical basis for UV protectors

2. Investigations and results

2.1. UV damage model and UV dose

In order to study the UVA/UVB damage model of HaCat cells, we investigated different doses of UVA/UVB irradiation. For the UVA damage study, we have assigned the HaCat cells to control, 2.5 J/cm^2, 5 J/cm^2, and 10 J/cm^2 UVA irradiation groups. Figure 1A shows that there were no significant cell morphology changes with 10 J/cm^2 dose UVA irradiation. UVA dose irradiation between 20 and 40 J/cm^2 could lead to cell deformation, including reduced size and nuclear condensation. With radiation dose increasing, cell death and cell debris were also promoted. OD values in 20 J/cm^2 and 40 J/cm^2 UVA treated groups were significantly lower than that in the control group (P<0.05). Meanwhile, there are no differences of OD values between 2.5 J/cm^2, 5 J/cm^2, and 10 J/cm^2 treated groups and the control group. However, OD values could be affected by UVA in a dose-dependent manner. Higher UVA dose would result in lower OD values. 20 J/cm^2 UVA irradiation is the cutoff value that leads to a significant difference of OD values compared with that in in the control group (Fig. 1B). Meanwhile, the rate of early apoptosis, late apoptosis and necrotic cells in the control, 2.5 J/cm^2, 5 J/cm^2, and 10 J/cm^2 UVA irradiation groups were less than 10 %. However, the rates under 20 J/cm^2 and 40 J/cm^2 UVA irradiation were more than 10 %. Therefore, 20J/cm^2 UVA irradiation was the threshold value to trigger cell apoptosis and/or necrosis (Fig. 1C). In addition, Figure 1D indicates that the rate of early apoptosis is promoted with radiation dose increasing. Based on the results mentioned above, 20 J/cm^2 UVA irradiation was the suitable dose for the HaCaT cell damage study.

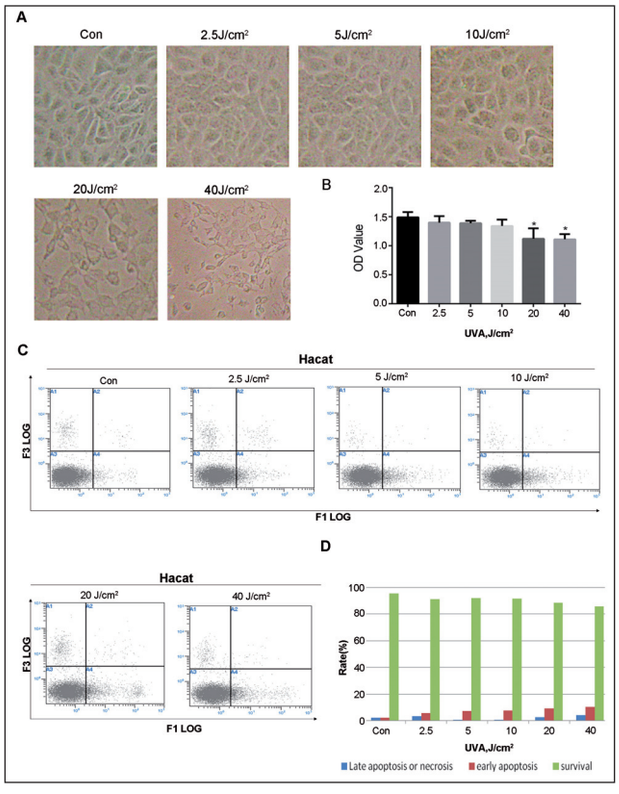

Fig. 1: Acute UVA damage model in HaCaT cells and UVA dose selection. (A) HaCaT cells damage with different UVA doses was observed under light microscope (40×). (B) Effect of UVA irradiation on HaCaT cell proliferation. (C) Effect of UVA irradiation on HaCaT cells apoptosis and necrosis. (D) Cell apoptosis study in different treated groups.

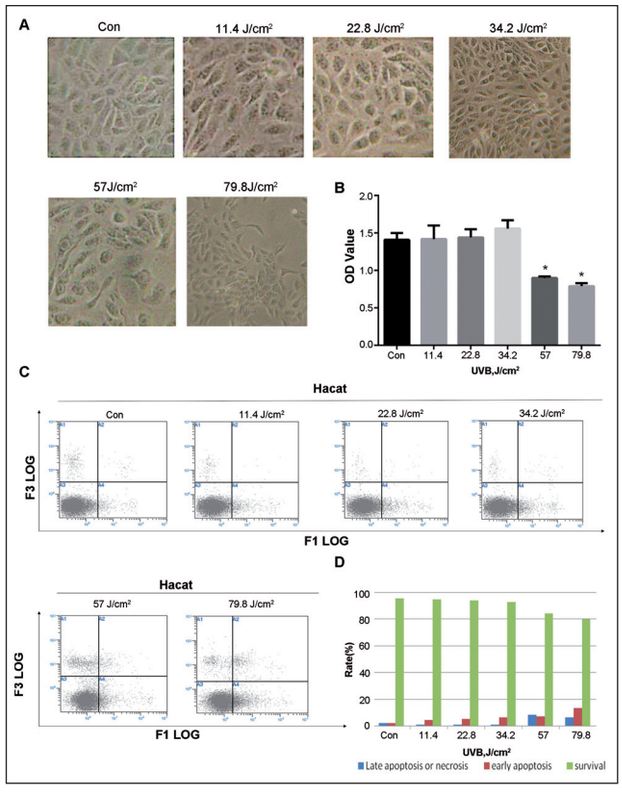

Fig. 2: Acute UVB damage model in HaCaT cells and UVB dose selection. (A) HaCaT cells damage with different UVB doses was observed under light microscope (40×). (B) Effect of UVB irradiation on HaCaT cell proliferation. (C) Effect of UVB irradiation on HaCaT cells apoptosis and necrosis. (D) Cell apoptosis study in different treated groups.

For the UVB damage study, we have divided HaCat cells into control, 11.4 mJ/cm^2, 22.8 mJ/cm^2, 34.2 mJ/cm^2, 57 mJ/cm^2 and 79.8 mJ/cm^2 groups. There were no significant cell morphology changes with UVB dose irradiation between 11.4 and 34.2 mJ/cm^2. UVB dose irradiation between 57 ans 79.8 mJ/cm^2 could lead to HaCaT cell deformation, including reduced size and nuclear condensation (Fig. 2A). OD values in 57 mJ/cm^2 and 79.8 mJ/cm^2 UVB treated groups were significantly lower than that in the control group (P<0.05). There were no differences of OD values between the 11.4 mJ/cm^2, 22.8 mJ/cm^2, 34.2 mJ/cm^2 treated groups and the control group. 57 mJ/cm^2 UVB irradiation was the cutoff value that leads to a significantly difference of OD values compared with that in the control group (Fig. 2B). Moreover, the rate of early apoptosis, late apoptosis and necrotic cells in control, 11.4 mJ/cm^2, 22.8 mJ/cm^2, and 34.2 mJ/cm^2 UVB irradiation were less than 10 %. However, the rates under 57 mJ/cm^2 and 79.8 mJ/cm^2 UVB irradiation were higher. Therefore, 57 mJ/cm^2 UVB irradiation was

the threshold value to trigger cell apoptosis and/or necrosis (Fig. 2C). In addition, the rate of early apoptosis was promoted with radiation dose increasing (Fig. 2D). Therefore, 57 mJ/cm^2 UVB irradiation was a suitable dose for the HaCaT cell damage study.

2.2. Pterostilbene dose

In order to study the influences of different pterostilbene doses on HaCaT cell apoptosis, we assigned HaCaT cells to eight groups according the different pterostilbene doses (control, 2.44 μM, 4.88 μM, 9.75 μM, 19.5 μM, 39 μM, 78 μM, and 156 μM). Figure 3A shows that the rate of HaCaT cell apoptosis and necrosic was promoted with increasing pterostilbene concentration. However, there was no difference in apoptosis and necrotic rates between control and pterostilbene treatments (<9.75μM). Figure 3B reveals that the rate of early apoptosis was promoted with increasing pterostilbene doses. Therefore, 2.44 μM, 4.88 μM, and 9.75 μM was considered as the suitable dose for further study.

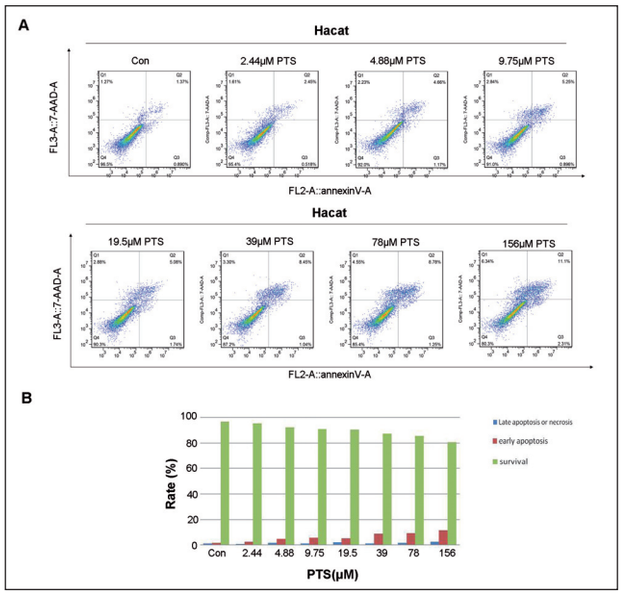

Fig. 3: Pterostilbene concentration selection. (A) Effect of different pterostilbene concentration on HaCaT cell apoptosis and necrotic. (B) Cell apoptosis study in different treated groups.

2.3. Western blot analysis of Nrf2 nuclear translocation changes

Nrf2 nuclear translocation indirectly reflects the protein function changes in different cellular locations. In this study, we probed the Nrf2 protein location in HaCaT cells, which were treated with or without pterostilbene before and after UVA/UVB irradiation. For the non- pterostilbene-treated groups, there were no differences of Nrf2 cytoplasm content between groups with and without UVA/UVB irradiation (Fig. 4). However, Nrf2 nuclear contents in UVA/UVB treated groups were higher than that in non- UVA/UVB-treated groups (P>0.05). For pterostilbene treatment groups, UVA/UVB irradiation treatments could induce Nrf2 concentration in cytoplasm following increasing pterostilbene concentrations. Meanwhile, UVA/UVB irradiation treatments could promote Nrf2 concentration in cell nuclei, which was also pterostilbene concentration dependent.

2.4. Cell proliferation and apoptosis with pterostilbene and UV treatments

In order to study the effect of pterostilbene and UV treatments on HaCaT cell proliferation and apoptosis, we divided HaCaT cells into three groups based on pterostilbene dose (2.44, 4.88, and 9.75 μM). There were no significant differences of HaCaT cell prolif-

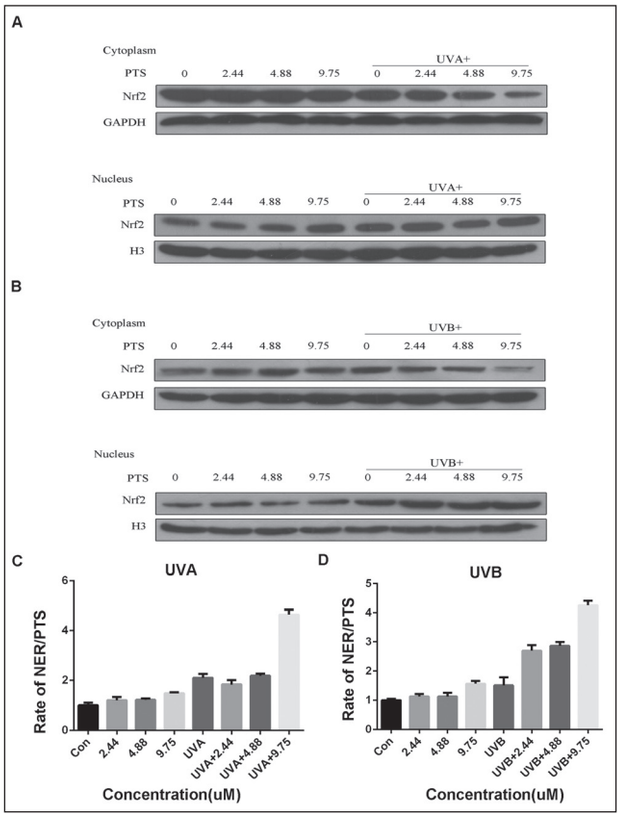

Fig. 4: Nrf2 nuclear translocation changes in pterostilbene treated HaCaT cells before and after UVA/UVB irradiation. (A) Nrf2 nuclear translocation changes in pterostilbene treated HaCaT cells before and after UVA irradiation. (B) Nrf2 nuclear translocation changes in pterostilbene treated HaCaT cells before and after UVB irradiation. (C) Nrf2 nucleus/cytoplasm ratio in UVA irradiation treated HaCaT cells before and after pterostilbene treated. (D) Nrf2 nucleus/cytoplasm ratio in UVB irradiation treated HaCaT cells before and after pterostilbene treated.

eration compared with that in the control group (P>0.05), which indicated that low dose pterostilbene has no influence on HaCaT cell proliferation. However, cell proliferation could be significantly inhibited by UVA/UVB treatment (P<0.05). Whereas 9.75 μM pterostilbene could effectively rescue an inhibiting effect caused by UV damage (P<0.05) (Fig. 5A). Moreover, Fig. 5B shows that different pterostilbene doses had no influence on HaCaT cell apoptosis. UVA/UVB irradiation could promote cell apoptosis, which can be relieved by 9.75 μM pterostilbene treatment (Fig. 5B).

2.5. Cell antioxidant capacity

Pterostilbene and UVA/UVB treatments could be potentially related with cell antioxidant capacity. Therefore, we employed a Q-PCR method to examine the mRNA abundance of CAT, HO-1, and SOD in different treated groups. UVA/UVB irradiation would lead to decreased expressions of CAT, HO-1, and SOD genes, which could be alleviated by adding pterostilbene (Fig. 6A). Especially, SOD gene expression level in pterostilbene and UVA/UVB treatments were significantly higher than that in control and individual UVA or UVB treatment. Meanwhile, protein carbonyl content in the group with UVA (20 J/cm^2) or UVB (57 mJ/cm^2) irradiation was significantly higher than that in the control and the pterostilbene treated groups (Fig. 6B). When HaCat cells were pretreated with various pterostilbene doses for 24 h before irradiation (UVA 20 J/cm^2 or UVB 57 mJ/cm^2), and then cells were cultured with the same pterostilbene dose for 18 h. Carbonyl content was significantly reduced compared with that in UVA or UVB individual treatments. In addition, MDA analysis suggested that malondialdehyde content with UVA (20 J/cm^2) or UVB (57 mJ/cm^2) irradiation were significantly higher compared with that

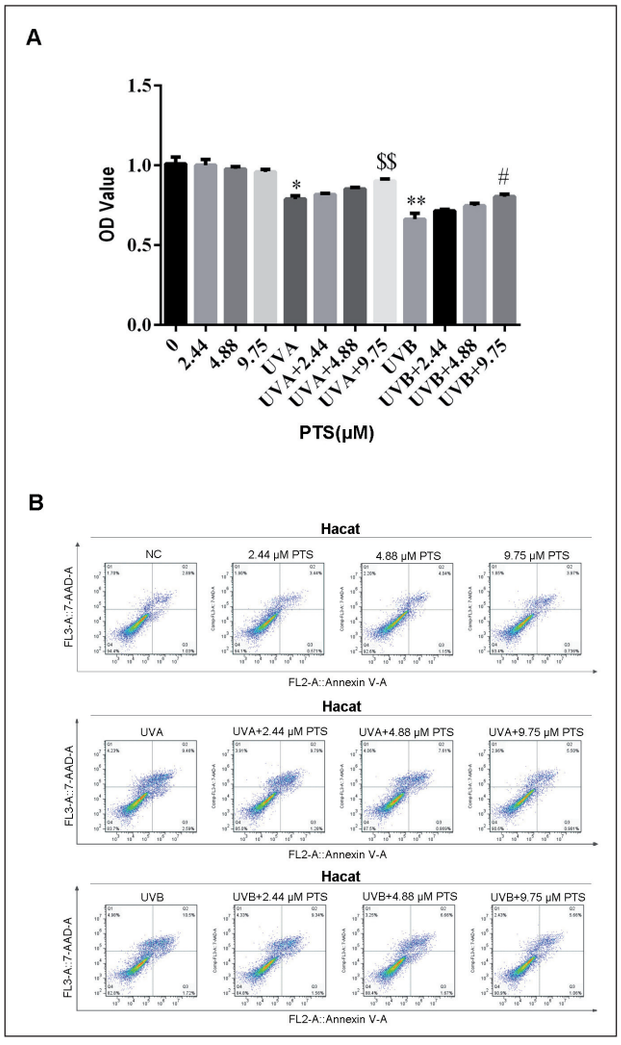

Fig. 5: Effect of different pterostilbene concentrations on HaCaT cells proliferation and apoptosis. (A) Cells proliferation of different pterostilbene concentrations (2.44, 4.88, and 9.75uM) and UVA/UVB treatments on HaCaT cells proliferation (* means the significant difference between various treated groups and group without treated (P<0.05, T test); ** means the extremely significant difference between various treated groups and group without treated (P<0.001, T test); $ means the significant difference between various treated groups and group with UVA treated (P<0.05, T test); $$ means the extremely significant difference between various treated groups and group with UVA treated (P<0.001, T test); # means the significant difference between various treated groups and group with UVB treated (P<0.05, T test)). (B) Cells apoptosis of different pterostilbene concentrations (2.44, 4.88, and 9.75uM) and UVA/UVB treatments on HaCaT cells apoptosis.

in the control and the pterostilbene treated groups (Fig. 6C). When HaCat cells were pretreated with various pterostilbene doses for 24 h before irradiation (UVA 20 J/cm^2 or UVB 57mJ/cm^2), and then cells were cultured with the same pterostilbene dose for 18 h. MDA content was significant reduced compared with that in UVA or UVB individual treatments.

2.6. Nrf2 knockdown in HaCaT cells

Based on the results describe above, pterostilbene could active Nrf2 nuclear translocation after UVA/UVB irradiation. Therefore, it was necessary to study whether pterostilbene exerts its protective role in UV irradiation through the Nrf2 signal pathway. We thus performed knockdown treatment to HaCaT cells with siRNA transfection. Figure 7A shows the immunofluorescence results of HaCaT cells with or without Nrf2 knockdown treatments. Moreover, we carried out Q-PCR analysis to test the mRNA expression level (Fig. 7B). The results suggest that the knockdown treatment was effective.

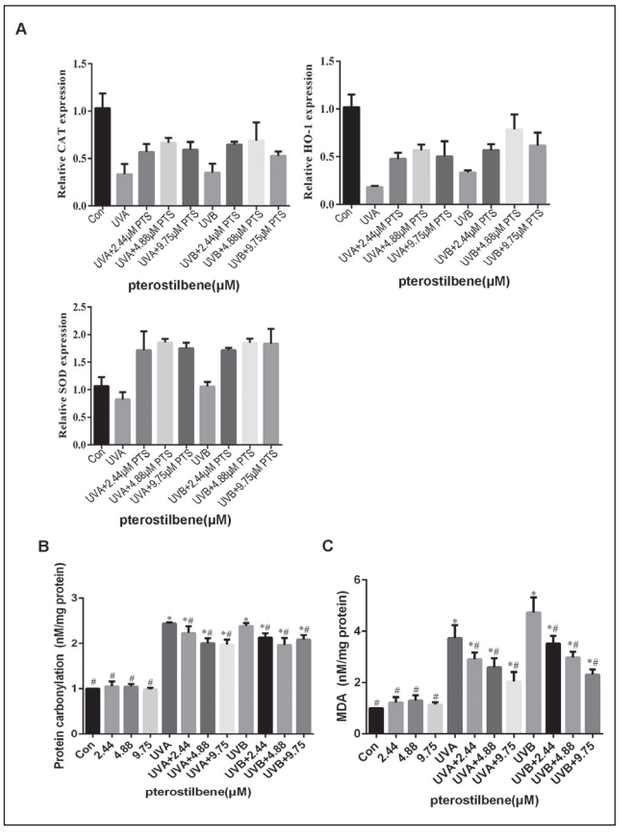

Fig. 6: Antioxidant capacity influences of different pterostilbene concentrations on UVA/UVB treated HaCaT cells. (A) Gene (CAT, HO-1, and SOD) expressions of detoxifying enzymes were examined by Q-PCR. (B) Protein carbonyl detection. Protein carbonyl content with UVA (20J/cm^2) or UVB (57mJ/cm^2) irradiation was significantly higher compared with Control and pterostilbene treated groups. (C) Malondialdehyde (MDA) examination * means the significant difference between various treated groups and control group (P<0.05, T test); # means the significant difference between various treated groups and UVA/UVB group (P<0.05, T test).

2.7. Western blot analysis of Nrf2 nuclear translocation changes in knockdown Nrf2 cells

In this study, we investigated the Nrf2 protein location in knockdown Nrf2 treated HaCaT cells, which were treated with or without pterostilbene and UVA/UVB irradiation. Pterostilbene (9.75 μM), UVA (20 J/cm^2) and UVB (57 mJ/cm^2) were applied in this study. For no knockdown Nrf2 treatment groups, Nrf2 nuclear translocation changes were similar to that in the previous steps of this study. For knockdown Nrf2 treatment groups, Nrf2 cytoplasm content in UVA/UVB treated cells was reduced. However, Nrf2 nuclear content in UVA/UVB treated cells was increased. It is notable that pterostilbene and UVA/UVB treatments in knockdown Nrf2 cells can almost eliminate Nrf2 in cytoplasm (Fig. 8A). Additionally, we calculated the ratio of nucleus to cytoplasm in different groups. The results indicated that this rate was higher in knockdown Nrf2 cells than in the control and other groups (P>0.05).

2.8. Proliferation and apoptosis of knockdown Nrf2 cell with pterostilbene and UV treatments

In order to study the effect of pterostilbene and UV treatments on knockdown Nrf2 cells proliferation and apoptosis, we divided HaCaT cells into six groups: NC, NC+UV, NC+UV+PTS, shNrf2, shNrf2+UV, and NC+UV+PTS. Figure 9A shows that cell proliferation in the NC and shNrf2 groups was significantly decreased after 20 J/cm^2 UVA irradiation. Cell proliferation in the shNrf2+UVA group was significantly lower than that in the NC+UVA group, which could be rescued by pterostilbene treatment. Meanwhile, cell proliferation in NC and shNrf2 groups was

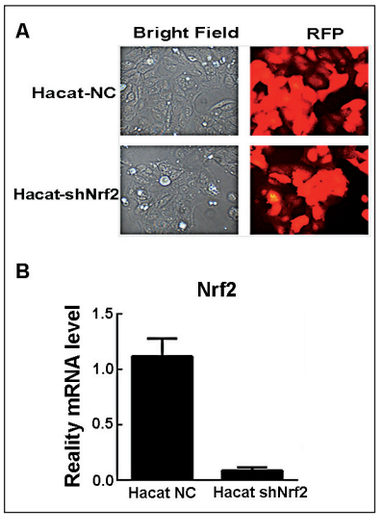

Fig. 7: Nrf2 knockdown HaCaT cells. (A) Immunofluorescence analysis of HaCaT cells with or without Nrf2 knockdown treatments. (B) Q-PCR analysis of Nrf2 mRNA expression in HaCaT cells with or without Nrf2 knockdown treatments.

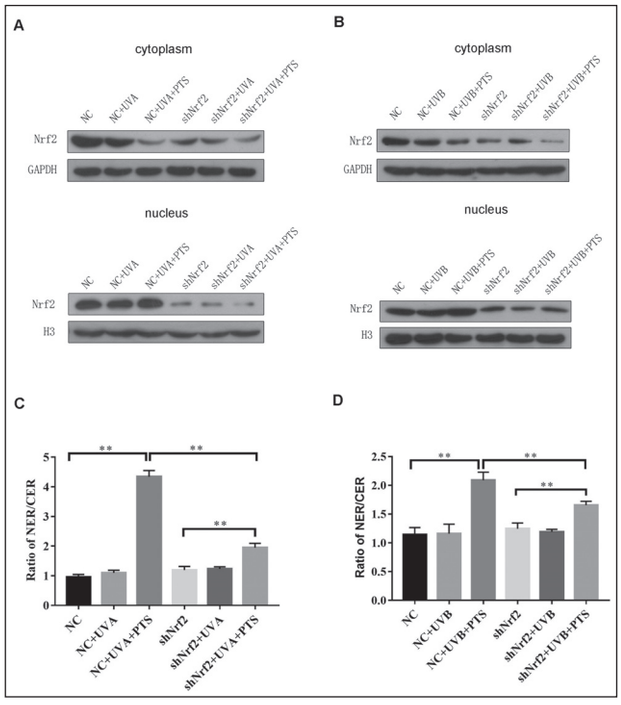

Fig. 8: Western blot analyses of Nrf2 expressions in nucleus/cytoplasm. (A) Nrf2 expressions in nucleus and cytoplasm of UVA treated HaCaT cells with or without knockdown Nrf2 treatments. (B) Nrf2 expressions in nucleus and cytoplasm of UVB treated HaCaT cells with or without knockdown Nrf2 treatments. (C) After Nrf2 knockdown treatment, Nrf2 nucleus/cytoplasm ratio in UVA treated HaCaT cells with or without pterostilbene treatments. (D) After Nrf2 knockdown treatment, Nrf2 nucleus/cytoplasm ratio in UVB treated HaCaT cells with or without pterostilbene treatments.

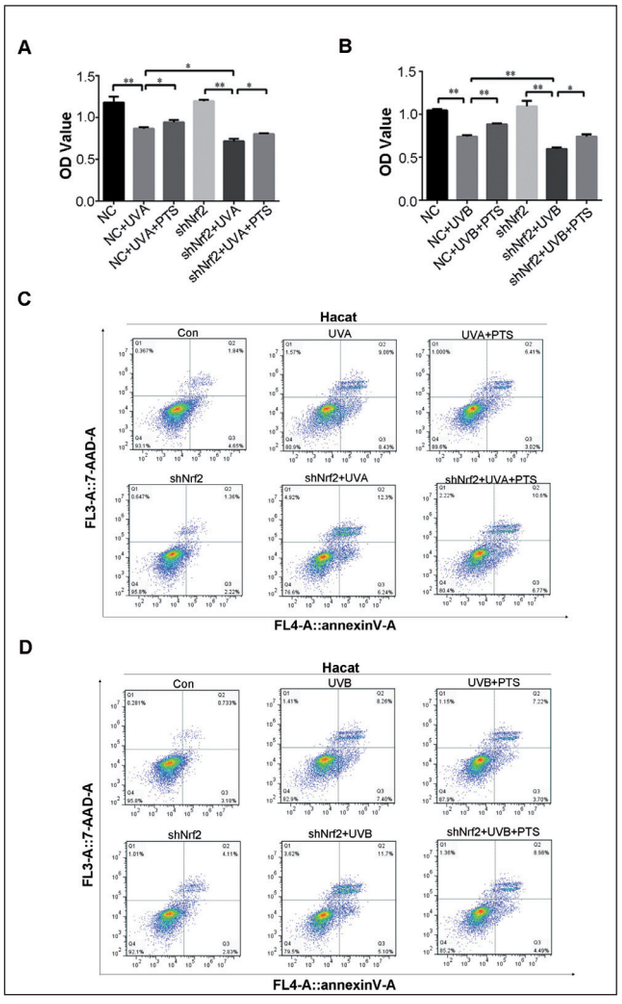

Fig. 9: Effect of UVA/UVB irradiation on cell proliferation and apoptosis of pterostilbene treated HaCaT cells after Nrf2 knockdown treatment. (A) Effect of pterostilbene on cell proliferation of Nrf2 knockdown treated HaCaT cells after UVA treatment. (B) Effect of pterostilbene on cell proliferation of Nrf2 knockdown treated HaCaT cells after UVB treatment. (C) Effect of pterostilbene on the apoptosis Nrf2 knockdown treated HaCaT cells after UVA treatment. (D) Effect of pterostilbene on the apoptosis Nrf2 knockdown treated HaCaT cells after UVB treatment. * means the significant difference between various treated groups and group without treated ($P<0.05$, T test); ** means the extremely significant difference between various treated groups and group without treated ($P<0.001$, T test.

significantly decreased after 57 mJ/cm^2 UVB irradiation. Cell proliferation in shNrf2+UVB group was significantly lower than that in NC+UVB group, which could be rescued by pterostilbene treatment (Fig. 9B). Moreover, the number of apoptosis cell in NC and shNrf2 were increased after UVA (20 J/cm^2) and UVB (57 mJ/cm^2) irradiation, which could be rescued by adding 9.75 μM pterostilbene (Figs. 9C and D). The number of apoptotic cells in shNrf2+UVA/UVB+PTS group was higher than that in the NC+UVA/UVB+PTS group.

2.9. Cell antioxidant capacity in knockdown Nrf2 cell

In order to study the potential relation between pterostilbene and UVA/UVB treatments and cell antioxidant capacity, we examined the mRNA expressions of CAT, HO-1, and SOD in UVA treated HaCaT cells. After Nrf2 knockdown treatment, gene expressions of CAT, HO-1, and SOD in UVA/UVB treated HaCaT cells were decreased. Meanwhile, genes expressions after UVA (20 J/cm^2) or UVB (57 mJ/cm^2) treatment were lower than that before UVA/UVB treatments. These phenomena could be relieved by adding pterostilbene (Figs. 10A and B). After Nrf2 knockdown treatment, protein carbonyl in different treated groups was higher than that in NC group. Meanwhile, protein carbonyl in NC and shNrf2 groups with UVA (20 J/cm^2) or UVB (57 mJ/cm^2) treatment was significant higher than in the control group. However, there were no differences of protein carbonyl between shNrf2+UVA/UVB and NC+UVA/UVB groups. Pterostilbene treatments in different groups could significantly reduce protein carbonyl (Fig. 10C). In addition, MDA in NC+UVA/UVB and shNrf2+UVA/ UVB were higher than that in the control group ($P<0.05$) (Fig. 10D). Meanwhile, MDA in NC and shNrf2 groups with UVA (20 J/cm^2) or UVB (57 mJ/cm^2) treatment were significantly higher than that in

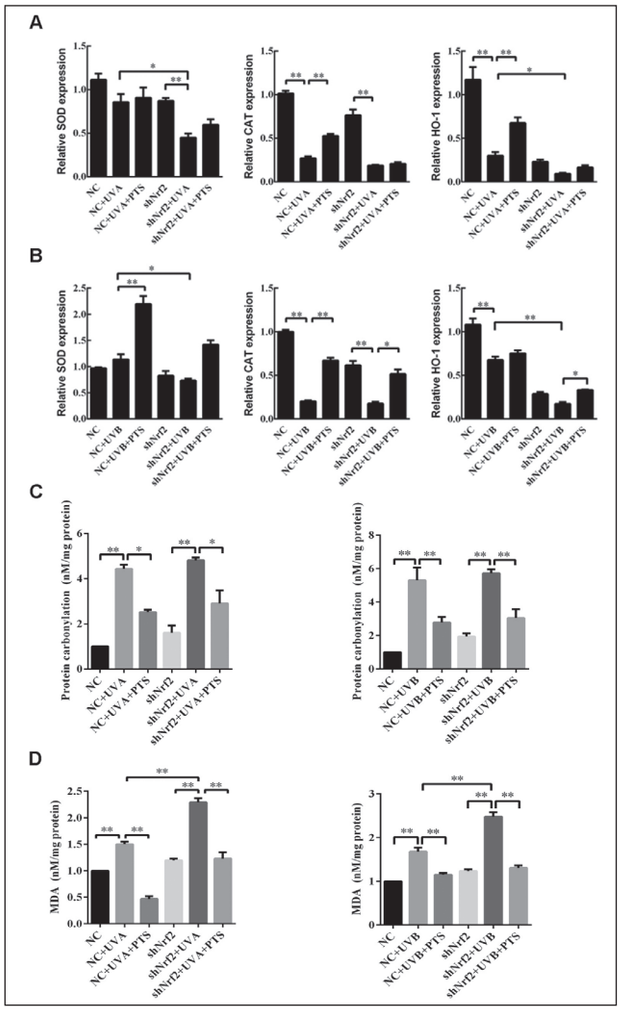

Fig. 10: Antioxidant capacity examination after Nrf2 knockdown treatment. (A) After Nrf2 knockdown treatment, effect of pterostilbene to gene expressions of CAT, HO-1, and SOD in UVA treated HaCaT cells is exmained. (B) After Nrf2 knockdown treatment, effect of pterostilbene to gene expressions of CAT, HO-1, and SOD in UVB treated HaCaT cells were examined. (C) After Nrf2 knockdown treatment, effect of pterostilbene to cellular protein carbonyl in UVA/UVB treated HaCaT cells. (D) After Nrf2 knockdown treatment, effect of pterostilbene to MDA in UVA/UVB treated HaCaT cells were examined. * means the significant difference between various treated groups and group without treated (P<0.05, T test); ** means the extremely significant difference between various treated groups and group without treated (P<0.001, T test.

the control group (P<0.05). However, there were no differences of MDA between shNrf2+UVA/UVB and NC+UVA/UVB groups. Pterostilbene treatments in different groups could significantly reduce the MDA.

3. Discussion

Pterostilbene is a natural antioxidant, functions of which were presented as following: 1) Reducing oxidative stress and reactive oxygen species (ROS) in the cell, for example, H_2O_2 and superoxide anion (O_2); 2) Increasing catalase expression in the cell, for instance, total glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase, etc. (McCormack and McFadden 2013). The antioxidant functions of pterostilbene work through multiple mechanisms. Zhang et al. (2012) have revealed that pterostilbene could activate the expression of Bcl-2 related X protein, which promote manganese superoxide dismutase expression in mitochondrial matrices. Moon et al. (2013) suggested that pterostilbene can interact with LOX-1, which can reduce ROS generation and metalloproteinases expression in cytoplasmic matrix. Moreover, pterostilbene has been proven to activate Nrf2 to exert anti-oxidative functions (Chiou et al. 2011). Bhakkiyalakshmi et al. (2014) also suggested that pterostilbene can activate Nrf2, which promotes the expression of heme oxygenase-1, Glutathione reductase, and Bcl-2 in pancreatic beta-cell apoptosis. On the other hand, UV irradiation keeps close relationships with skin diseases. For example, excess UV radiation stimulates abnormal skin cells proliferation, which can eventually evolve into cancer cells (Armstrong Kricker, 1993). Paul et al. (2010) thought that pterostilbene could induce cell apoptosis, and inhibit cancer cell proliferation and metastasis. Ferrer et al. (2005) have proved that pterostilbene can inhibit metastasis and proliferation of metastatic melanoma cells. Tsai et al. (2012) indicated that pterostilbene is a novel component to prevent inflammation-related skin cancer , whereas, the detailed roles of pterostilbene in skin diseases remained unclear.

In this study, we employed different doses of UVA/UVB to establish an UV damage model. For UVA irradiation, we chose 2.5 J/cm^2, 5 J/cm^2, 10 J/cm^2, 20 J/cm^2, and 40 J/cm^2 to treat HaCaT cells. Cell morphology, proliferation, and apoptosis analysis all indicated that 20 J/cm^2 UVA irradiation was the threshold value to affect cell viability. In parallel, 57 mJ/cm^2 UVB irradiation was the suitable dose for the HaCaT cell damage study. The results mentioned above were consistent with previous studies (Ishikawa et al. 2008; Li et al. 2016). We also tested the effects of different pterostilbene doses (2.44 μM, 4.88 μM, 9.75 μM, 19.5 μM, 39 μM, 78 μM, and 156 μM) to HaCaT cells. A MTT method was carried out to measure the OD value of cell numbers. However, there are limitations for this method to accurately determine cell numbers. For example, the intermediate product of MTT is insoluble in water, and the supernatant must be discarded before entering the organic solvent, which may cause errors. The final results suggested that low pterostilbene doses (2.44 μM, 4.88 μM, and 9.75 μM) were suitable for further study.

Pterostilbene would promote Nrf2 concentration in cell nuclei and decrease Nrf2 concentration in cytoplasm after UVA/UVB irradiation. However, Nrf2 concentration in nucleus and in cytoplasm was not changed in pterostilbene treatments without UVA/UVB irradiation. The results indicated that UVA/UVB irradiation would affect Nrf2 protein location, in particular for the 9.75 μM pterostilbene dose. In addition, we studied cell proliferation and apoptosis with pterostilbene and UV treatments. Cell proliferation could be significantly inhibited by UVA/UVB treatments, whereas 9.75 μM pterostilbene could effectively rescue UV damage. UVA/UVB irradiation would lead to decreased expressions of CAT, HO-1, and SOD genes. Meanwhile, carbonyl content and MDA was significantly changed by UVA/UVB treatments. The adverse events could be rescued by adding 9.75 μM pterostilbene. Is has been suggested that pterostilbene can induce CAT, HO-1, and SOD expressions which is consistent with the current study (McCormack and McFadden 2013). Therefore, pterostilbene could promote SOD activity and eliminate ROS caused by UVA/UVB irradiation.

Based on the results described above, we further investigated whether pterostilbene plays a protective role via the Nrf2 signal pathway in HaCaT cells. Knockdown Nrf2 treatment was performed in HaCaT cells. Western blot analysis showed that Nrf2 cytoplasm content in UVA/UVB treated cells was reduced. Nrf2 nuclear content in UVA/UVB treated cells was increased, which is different to the normal HaCaT cells without knockdown Nrf2 treatment. In addition, the other results, including cell proliferation, apoptosis, and cell antioxidant capacity, in knockdown Nrf2 treated HaCaT cells were also significantly different from normal HaCaT cells without knockdown Nrf2 treatment. Therefore, we hypothesize that pterostilbene could play its anti-oxidative role through the Nrf2 signal pathway.

In conclusion, and based on the results mentioned above, we can conclude that pterostilbene has a protective effect against HaCaT cell's acute photodamage caused by UVA/UVB radiation. The protective mechanism could be activation of antioxidant signaling pathways and increasing cellular activity. Moreover, pterostilbene could exert its anti-oxidative role through the Nrf2 signal pathway

4. Experimental

4.1. UV damage model and UV dose

HaCaT cells were purchased from ATCC (Virginia, USA), and maintained in RPMI 1640 with 10% (v/v) FBS (Invitrogen, Carlsbad, CA). Trypsin was used to digest logarithmic growth phase HaCaT cells. 1×10^4 cells were distributed into each well of a 24-well plate. For the UVA study, we divided HaCaT cells into six subgroups, a control group (normal HaCaT cells), a 2.5 J/cm^2 group (2.5 J/cm^2 UVA treated HaCaT cells), a 5 J/cm^2 group, a 10 J/cm^2 group, a 20 J/cm^2 group, and a 40 J/cm^2 group. For the UVB study, we also formed six subgroups, including a control group (normal HaCaT cells), a 11.4 mJ/cm^2 group (11.4mJ/cm^2 UVB treated HaCaT cells), a 22.8 mJ/cm^2 group, a 34.2 mJ/cm^2 group, a 57mJ/cm^2 group, and a 79.8 mJ/cm^2 group. Hand-held UVA/UVB irradiation meters were used to treat the cells. Before irradiation, the culture medium was discarded. After a small amount of PBs solution was covered, the irradiation was started. After the irradiation, fresh medium was added. The control group was covered with tin box paper.

4.2. Cell proliferation assay

A cell proliferation assay was conducted with a commercial MTT kit (Beyotime Biotechnology, China) following the manufacturer's instruction. 500 cells were placed into each well of 24-well plate and maintained in media containing 10 % FBS for 4 h. The supernatant was discarded and 150 µl DMSO was added to each well. Triplicate wells were measured in each treatment group.

4.3. Cell apoptosis assay

HaCaT cells were seeded into 6-well plates at 5×10^5 cells per well. The culture medium was constituted with DMEM, 10 % FBS and 1 % penicillin/streptomycin. Cells were cultured at 37 °C with 5 % CO_2 for 24 h. Different treatments, including various UVA/UVB doses, pterostilbene, and their co-treatments were applied. After 48 h, treated HaCaT cells were collected. Annexin V-FITC apoptosis detection kit I (eBioscience, USA) was employed to examine the apoptotic cells with flow cytometry. Flowjo 7.1.6 software was used to analysis the results harvested by different treatments.

4.4. Western blot analysis

Differently treated HaCaT cells were seeded at 3.0×10^5 cells per 10 cm dish. Nuclear and cytoplasmic extraction was performed with ProteoExtract® Subcellular Proteome Extraction Kit (Merck KGaA, USA) according to the manufacture's instruction. Nuclear and cytoplasmic proteins derived from different treatments were transferred to a polyvinylidene difluoride membrane (Merck KGaA, USA). Subsequently, Membranes were incubated with corresponding primary antibodies: Nrf2 rabbit polyclonal (1:1000, Abcam), GAPDH (1:1000, Abcam), Histone H3 (1:1000, Abcam). Next, the membranes were incubated with the secondary antibodies: HRP-conjugated anti-mouse IgG secondary antibody (1:2000, Abcam). Immunostar-LD was used to view the bands (Wako, Japan). GAPDH and Histone H3 were used as the loading control.

4.5. qPCR

Total RNA from different treated HaCaT cells was extracted by a TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan) and first-strand DNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Japan). PCR was carried out using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) in an I-Cycler Thermocycler (Bio-Rad, USA). Thermal conditions and PCR reaction system were followed as described (Kathirvel 2009). GAPDH gene was employed as reference genes. The primers are used as follows: Catalase (CAT: NM_009804), Forward primers (5′-ACATGGTCTGGGACTTCTGG-3′), Reverse primers (5′-CAAGTTTTTGATGCCCTGGT-3′); Heme oxygenase 1 (HO-1: NM_010443), Forward primers (5′-CACGCATATACCCGCTACCT-3′), Reverse primers (5′-CCAGAGTGTTCATTCGAGCA-3′); Superoxide dismutase (SOD), Forward primers (5′-GAGACCTGGGCAATGTGACT-3′), Reverse primers (5′-GTTTACTG-CGCAATCCCAAT-3′); Nuclear-related factor 2 (Nrf-2, U70475), Forward primers (5′-CTCGCTGGAAAAAGAAGTGG-3′), Reverse primers (5′-CCGTCCAG-GAGTTCAGAGAG-3′), GAPDH, Forward primers (5′-TGCACCACCAACTGCT-TAGC-3′), Reverse primers (5′-GGCATGGACTGTGGTCATGAG-3′). PCR results were compared using the relative Delta-Delta Ct method.

4.6. Protein carbonyl and lipid peroxidation

Lipid peroxidation and protein break-down products (carbonyls) have been considered as the main indicators for ROS-mediated damage. The lipid peroxidation assay is based on measuring malondialdehyde (MDA) generated from lipid damage. Lipid peroxidation products were measured with a Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, USA) following manufacturer's instructions. Protein carbonyl concentrations were examined with a Protein Carbonyl Content Assay Kit (Sigma-Aldrich, USA) according to manufacturer's instructions.

4.7. siRNA transfection

Dual strand modified siRNA of Nrf-2 were obtained from Santa Cruz Biotechnology (Nrf2 siRNA (h), shRNA and Lentiviral Particle Gene Silencers, USA), and used as indicated. Primary HaCaT cells were seeded in 24-well plates at 1.5×10^5 cells ml^{-1}. The cells were transfected with 10 nM of targeting siRNA. Cells were stimulated 24 h after treatment with siRNA.

4.8. Immunofluorescence

siRNA treated HaCaT cells were planted on coverslips. Cells were fixed with methanol at −20 °C. Subsequently, cells were treated with 0.5% Triton-X in PBS for 12 min and incubated with blocking solution for 1 h (5% FBS, 0.2% Tween-20 in PBS). Cells were stained with primary antibodies of Nrf-2 (1:600 in blocking solution) at 25 °C over night. Next, Coverslip was incubated for 3 h with fluorescently labelled secondary antibodies (Anti-mouse IgG labelled with AlexaFluor647, Life Technologies). LSM710 laser scanning microscope (Zeiss) was used to take images with a ×100 magnification.

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