Protective effects of glutamine on human melanocyte oxidative stress model

Liya Jiang¹,², Zhen Guo¹, Yulong Kong¹,⁴, Jianhua Liang², Yi Wang⁵, Keyu Wang¹

¹Department of Dermatology, Qilu Hospital, Shandong University, ²Department of Dermatology, Jinan Central Hospital Affiliated to Shandong University, Jinan, 250012, Departments of ³Dermatology and ⁴General Surgery, Liaocheng People’s Hospital, Liaocheng, 252000, ⁵Department of Dermatology, Affiliated Nanjing Jiangbei Hospital, Medical College of Southeast University, Nanjing, 210048, P. R. China

Abstract

Background: Vitiligo is a disorder caused by the loss of the melanocyte activity on melanin pigment generation. Studies show that oxidative-stress induced apoptosis in melanocytes is closely related to the pathogenesis of vitiligo. Glutamine is a well known antioxidant with anti-apoptotic effects, and is used in a variety of diseases. However, it is unclear whether glutamine has an antioxidant or anti-apoptotic effect on melanocytes.

Aims: The aim of this study was to investigate the protective effects of glutamine on a human melanocyte oxidative stress model.

Methods: The oxidative stress model was established on human melanocytes using hydrogen peroxide. The morphology and viability of melanocytes, levels of oxidants [reactive oxygen species and malondialdehyde], levels of antioxidants [superoxide dismutase and glutathione-S-transferase], and apoptosis-related indicators (caspase-3, bax and bcl-2) were examined after glutamine exposure at various concentrations. Expressions of nuclear factor-E2-related factor 2, heme oxygenase-1, and heat shock protein 70 were detected using western blot technique after glutamine exposure at various concentrations.

Results: Our results demonstrate that pre-treatment and post-treatment with glutamine promoted melanocyte viability, increased levels of superoxide dismutase, glutathione-S-transferase and bcl-2, decreased levels of reactive oxygen species, malondialdehyde, bax and caspase-3, and enhanced nuclear factor-E2-related factor 2, heme oxygenase-1, and heat shock protein 70 expression in a dose dependent manner. The effect of pre-treatment was more significant than post-treatment, at the same concentration.

Limitations: The mechanisms of glutamine activated nuclear factor-E2-related factor 2 antioxidant responsive element signaling pathway need further investigation.

Conclusions: Glutamine enhances the antioxidant and anti-apoptotic capabilities of melanocytes and protects them against oxidative stress.

Key words: Apoptosis, glutamine, melanocytes, oxidative stress, vitiligo

Introduction

Vitiligo is a primary disorder of skin depigmentation with a 1% incidence worldwide.¹ Studies show that the imbalance between oxidation and antioxidation in the local skin microenvironment, melanocyte damage, and apoptosis induced by the accumulation of oxidative stress factors are important causes of vitiligo.¹ Reactive oxygen species are a group of oxidants and major indicators to evaluate oxidative stress within the body. Increased levels of reactive oxygen species cause cellular oxidative stress, cell injury, and even death.² Malondialdehyde is the final oxidation product of membrane lipid peroxidation. It increases reactive oxygen species levels by enhancing the activities of the mitochondrial respiratory chain complex and key mitochondrial enzymes.³ Both reactive oxygen species and malondialdehyde...
levels in the blood and skin lesions of patients with vitiligo are dramatically increased.3  

The components of the antioxidant enzyme system, including superoxide dismutase and glutathione-S-transferase, can effectively remove reactive oxygen species and protect cells against damage.3,5 Various studies have shown that abnormal expression of superoxide dismutase and glutathione-S-transferase is involved in the pathogenesis of vitiligo.5,6 Studies have shown that hydrogen peroxide treatment changed the morphology of melanocytes, decreased cell viability and superoxide dismutase levels, and enhanced levels of reactive oxygen species and malondialdehyde in melanocytes,2,7 which mimics the profile of skin lesions in patients with vitiligo. It has been used as the oxidative stress model of melanocytes to study vitiligo pathogenesis and treatment.2,7 Nuclear factor-E2-related factor 2 is the most important transcription factor that regulates oxidative stress response. Activated nuclear factor-E2-related factor 2-antioxidant responsive element signalling pathway increases the production of nuclear factor-E2-related factor 2 mRNA and protein. Elevated levels of nuclear factor-E2-related factor 2 mRNA and protein, in turn, enhance the production of downstream antioxidant enzymes such as heme oxygenase-1, superoxide dismutase, and glutathione-S-transferase.8 Heme oxygenase-1 is the main antioxidant enzyme in melanocytes through the nuclear factor-E2-related factor 2-oxidant responsive element signalling pathway.9 nuclear factor-E2-related factor 2 and several downstream genes of antioxidant enzymes are increased in vitiligo skin lesions. Impaired activation of nuclear factor-E2-related factor 2 under oxidative stress could result in abnormal expression of antioxidant enzymes and increased death of vitiligo melanocytes, suggesting that nuclear factor-E2-related factor 2 and its downstream genes are involved in skin homeostasis in vitiligo.10,11 Therefore, the nuclear factor-E2-related factor 2-antioxidant responsive element pathway plays an important role in protecting human melanocytes against oxidative stress.12  

Caspase-3, bax, and bcl-2 are considered as important indices to evaluate melanocyte apoptosis.13 Increased activity of caspase-3 was discovered in the melanocytes of skin lesions in vitiligo patients.14 It was confirmed that hydrogen peroxide can upregulate the activity of caspase-3 and the ratios of bax/bcl-2 in vitro studies. However, this upregulating effect is suppressed by antioxidants.15 Heat shock protein 70 is one of the most important members of the HSP family. It exhibits multiple biological functions, such as inhibiting apoptosis, protecting against oxidation, and light-induced ageing. As a molecular chaperone of the nuclear factor-E2-related factor 2-antioxidant responsive element signalling pathway, Heat shock protein 70 can also help antioxidant enzymes to fold properly.15 Heat shock protein 70 expression levels and regions in vitiligo are higher than those in normal skin. This enhanced expression probably plays a role in the pathogenesis of vitiligo and may be related to antioxidative stress and inhibition of apoptosis.16  

Glutamine is the most abundant free amino acid in the body and a well-known antioxidant in a variety of diseases.17 However, the antioxidant role and anti-apoptotic role of glutamine in melanocytes and patients with vitiligo is unclear. In this study, we established an oxidative stress model in melanocytes using hydrogen peroxide and investigated whether glutamine had antioxidant and anti-apoptotic effects on melanocytes. This was done by examining cell morphology, viability, levels of oxidants, antioxidants, and apoptosis-related indicators. Our study aims to provide the theoretical basis for the clinical application of glutamine in treating vitiligo and other relevant diseases.  

Methods 

Materials  

The materials used in this study are as follows: Medium complete with trace elements (M254) and human melanocyte growth supplement (HMGS) (Gibco, USA); 0.25% trypsin (Sigma, USA); cell counting kit-8 cell proliferation and toxicity kit (Dojindo Institute, Japan); malondialdehyde, reactive oxygen species, and superoxide dismutase assay kits (Nanjing Jiancheng Bioengineering Institute, China); glutathione-S-transferase enzyme-linked immunosorbent assay kit (Shanghai Bio-Swamp, China); Penicillin-streptomycin (Beijing Biotop, China); heme oxygenase-1 and nuclear factor-E2-related factor 2 antibodies (Beijing Bios, China); Heat shock protein 70 antibody, actin antibody, and goat anti-rabbit and goat anti-mouse secondary antibodies (Zhongshan Bio, Beijing, China); Caspase-3, bax and bcl-2 enzyme-linked immunosorbent assay kit (XinqdiBio, Wuhan, China). All other reagents were analytical-grade products purchased from current laboratory suppliers.  

Melanocyte primary culture  

Normal human foreskin tissue (skin phototypes III and IV) was obtained from 13 patients, aged 18–30 years. They were screened for human immunodeficiency virus (HIV), hepatitis B virus, human papilloma virus, mycoplasma and chlamydia infection. They were medication free for at least three months prior to obtaining the tissue. They had no history of smoking or alcohol intake. Patient consent and hospital ethics committee approval was obtained. The melanocytes were cultured in vitro, and cells in passages 3–9 were used for cellular experiments. Cells were set in triplicate for each group, and the experiments were repeated four times.  

Hydrogen peroxide and glutamine dose screening  

Various concentrations of hydrogen peroxide (0–2 mmol/L) were used to treat melanocytes, and cell viability was detected after treatment. Melanocytes (2 × 10^4 cells/mL) were seeded in 96-well plates (100 µL/well) and randomly assigned into different groups:  

1. Glutamine group: M254 culture medium containing glutamine at 0.5–60 mmol/L was added at 24 hours after cell seeding  

2. Glutamine-hydrogen peroxide group: cells received the same exposure as glutamine group at 24 hours after seeding, and then 1.5 µl hydrogen peroxide at 50 mmol/L was added to make the final concentration of hydrogen peroxide 0.75 mmol/L at 48 hours after seeding  

3. Hydrogen peroxide-glutamine group: 1.5 µl hydrogen peroxide at 50 mmol/L was added at 24 hours after cell seeding to make the final concentration of hydrogen peroxide 0.75 mmol/L, and then M254 culture medium containing glutamine at 0.5–60 mmol/L was added after removing the supernatant 48 hours after cell seeding  

4. Blank (culture medium only, without cells) and control (no glutamine added) subgroups were included in each group. At 72 hours after cell seeding, the cell viability was measured using a cell counting kit-8 kit.  

Cell morphology, viability assay, and measurement of reactive oxygen species  

Melanocytes (2 × 10^4 cells/mL) were seeded in 96-well plates (100 µL/well). After culturing for 24 hours, the medium was
removed and the cells were randomly divided into different groups: group 1 (normal control group: no glutamine, no hydrogen peroxide) and group 2 (hydrogen peroxide control group: no glutamine, with hydrogen peroxide), which were added to 100 µL of normal culture medium; groups 3–7 were added to 100 µL of normal culture medium, containing various concentrations of glutamine (2, 5, 10, 15, 20 mmol/L); and group 8 was added to 94 µL of culture medium containing hydrogen peroxide 0.75 mmol/L. After culturing for 48 hours, 1.5 µL of phosphate buffered solution was added to group 1, 1.5 µL of hydrogen peroxide (50 mmol/L) was added to groups 2–7 (final concentration: 0.75 mmol/L), and 7.5 µL of glutamine (200 mmol/L) was added to group 8 (final concentration: 15 mmol/L). Seventy-two hours after cell seeding, cell morphology was analyzed and photographed using an inverted microscope without any staining. Cell viability and levels of reactive oxygen species were detected by cell counting kit-8 kit and reactive oxygen species kit.

Detection of malondialdehyde, superoxide dismutase, and glutathione-S-transferase
Melanocytes (2 × 10⁶ cells/mL) were seeded in 24-well plates (1 mL/well). The cell density, grouping, and drug treatments were the same as mentioned above. Seventy-two hours after seeding, the cells were digested, collected, and lysed using ultrasonic degradation. The supernatant was stored at −80°C until further use. Cellular malondialdehyde levels, superoxide dismutase levels, and glutathione-S-transferase levels were measured by following the manufacturer’s instructions for each kit. Protein concentrations were examined using the bicinchoninic acid (BCA) assay.

Western blot analysis
Melanocytes (2 × 10⁶ cells/mL) were seeded in 25 cm² flasks (5 mL/flask). Cell grouping and subsequent processing was done as discussed above. Twenty-five micrograms of protein was used for western blot analysis in each group.

Detection of caspase-3, bax and bcl-2
Melanocytes (2 × 10⁶ cells/mL) were seeded in 24-well plates (1 mL/well). Cell grouping and subsequent processing was done as per the manufacturer’s instructions for each kit. Protein concentrations were examined using the bicinchoninic acid (BCA) assay.

Results
Hydrogen peroxide and glutamine dose screening
Various concentrations of hydrogen peroxide (0–2 mmol/L) were used to treat the melanocytes. Results showed that cell viability was reduced after hydrogen peroxide treatment in a dose-dependent manner and reached 50% of normal levels after 0.75 mmol/L hydrogen peroxide treatment [Figure 1]. Therefore, 0.75 mmol/L of hydrogen peroxide was selected as it effectively induced oxidative stress in melanocytes and also maintained cells half functional. In addition, 0.5–60 mmol/L of glutamine pre-hydrogen peroxide-treatment and post-hydrogen peroxide-treatment was performed. The melanocyte oxidative stress model viability was significantly enhanced after glutamine 2–40 mmol/L pre-treatment, or 5–25 mmol/L post-treatment, even though glutamine at concentrations lower than 30 mmol/L had no obvious effect on normal melanocytes [Table 1]. Therefore, we chose glutamine at concentrations of 2, 5, 10, 15 and 20 mmol/L to treat cells.

Effect of glutamine on cell morphology
Compared to the control group, melanocyte cell bodies treated with hydrogen peroxide (0.75 mmol/L) for 24 hours were crimped and round. The dendrites were shortened, which eventually disappeared. These changes were rescued by pre-treatment with glutamine 2–20 mmol/L, as well as post-treatment with glutamine 15 mmol/L. It increased the number of dendrites and its length, compared to the hydrogen peroxide treated group. This effect was particularly dramatic with glutamine 15 mmol/L pre-treatment [Figure 2].

Table 1: Effect of glutamine treatment on melanocyte viability (dose screening)

| Groups (%) | Glutamine-H₂O₂ (%) | H₂O₂-glutamine (%) |
|------------|---------------------|---------------------|
| 0          | 101.0±4.2           | 51.0±4.8            | 49.8±5.7          |
| 0.5        | 99.2±3.1            | 50.4±5.7            | 50.0±4.9          |
| 1          | 100.3±3.3           | 51.2±4.9            | 51.3±5.7          |
| 2          | 100.3±3.8           | 55.5±4.6*           | 53.6±3.8          |
| 5          | 100.8±2.6           | 59.0±4.2**          | 55.7±3.7**        |
| 10         | 99.2±2.9            | 63.0±3.8*           | 59.8±3.6*         |
| 15         | 100.5±3.3           | 69.5±3.8*           | 63.7±3.5**        |
| 20         | 98.9±4.3            | 63.4±4.1*           | 59.7±3.9*         |
| 25         | 100.3±3.7           | 61.2±4.7**          | 57.2±3.5**        |
| 30         | 98.4±4.1            | 58.5±4.4**          | 52.2±5.1          |
| 40         | 95.1±3.4**          | 54.6±3.4*           | 52.0±4.2          |
| 60         | 91.7±5.4**          | 51.0±3.9            | 49.2±7.3          |

Glutamine (%) indicates cells were treated with various concentrations of glutamine only. Glutamine-H₂O₂ (%) means cells were pre-treated with glutamine prior to be exposed to H₂O₂ (0.75 mM). H₂O₂-glutamine (%) were cells treated with H₂O₂ (0.75 mM) before various concentrations of glutamine added. *P<0.05 versus control group; **P<0.01 versus control group. H₂O₂: Hydrogen peroxide
Effect of glutamine on cell viability, superoxide dismutase activity, and levels of reactive oxygen species, malondialdehyde, and glutathione-S-transferase

After treatment with hydrogen peroxide (0.75 mmol/L) for 24 hours, the melanocyte viability which was indicated by levels of cell counting kit-8 and superoxide dismutase levels, dropped dramatically compared to the control group ($P < 0.01$); reactive oxygen species, malondialdehyde ($P < 0.01$), and glutathione-S-transferase ($P < 0.05$) levels increased dramatically compared to the control group ($n = 12$ for each group). Glutamine 2–20 mmol/L pre-treatment (groups 3–7) and glutamine 15 mmol/L post-treatment (group 8) successfully increased cell counting kit-8 levels, inhibited reactive oxygen species production, and reduced malondialdehyde levels in comparison to the hydrogen peroxide group ($P < 0.01$). Glutamine 5 mmol/L pre-treatment (group 3) increased superoxide dismutase and glutathione-S-transferase levels in comparison to the hydrogen peroxide group ($P < 0.05$). Glutamine 10–20 mmol/L pre-treatment (groups 4–7) and glutamine 15 mmol/L post-treatment (group 8) enhanced superoxide dismutase and glutathione-S-transferase levels in melanocytes ($P < 0.01$), compared to the hydrogen peroxide group. The highest levels of cell counting kit-8, superoxide dismutase, and glutathione-S-transferase; and the lowest levels of reactive oxygen species and malondialdehyde were seen in the glutamine 15 mmol/L pre-treatment group [Figure 3].

Discussion

Oxidative stress plays a role in the pathogenesis of vitiligo. Hydrogen peroxide treatment of melanocytes can trigger membrane lipid peroxidation, thus inducing cell apoptosis, which mimics the oxidative stress status of melanocytes during the evolution of vitiligo. Our results are similar to earlier studies, indicating that hydrogen peroxide-induced oxidative stress in melanocytes is a successful model that mimics vitiligo pathogenesis. We found that hydrogen peroxide promoted the expression of heme oxygenase-1, nuclear factor-E2-related factor 2, and heat shock protein 70 expressions. Western blot showed that heme oxygenase-1, nuclear factor-E2-related factor 2, and Heat shock protein 70 expression was significantly increased after hydrogen peroxide treatment compared to the control group. Glutamine 2–20 mmol/L pre-treatment and glutamine 15 mmol/L post-treatment enhanced heme oxygenase-1 and Heat shock protein 70 expression levels in melanocytes ($P < 0.01$) compared to the hydrogen peroxide group. Glutamine 5–20 mmol/L pre-treatment and glutamine 15 mmol/L post-treatment significantly enhanced nuclear factor-E2-related factor 2 expression levels in melanocytes ($P < 0.01$) compared to the hydrogen peroxide group. The highest heme oxygenase-1, nuclear factor-E2-related factor 2, and heat shock protein 70 expression level was observed in the glutamine 15 mmol/L pre-treatment group ($P < 0.01$) [Figure 4a and b].

Effect of glutamine on heme oxygenase-1, nuclear factor-E2-related factor 2, and heat shock protein 70 expressions

Western blot showed that nuclear factor-E2-related factor 2, and Heat shock protein 70 ($P < 0.01, n = 12$ for each group) expression was significantly increased after hydrogen peroxide treatment compared to the control group. Glutamine 2–20 mmol/L pre-treatment and glutamine 15 mmol/L post-treatment significantly enhanced nuclear factor-E2-related factor 2 expression levels in melanocytes ($P < 0.01$) compared to the hydrogen peroxide group. Glutamine 5–20 mmol/L pre-treatment and glutamine 15 mmol/L post-treatment significantly enhanced nuclear factor-E2-related factor 2 expression levels in melanocytes ($P < 0.01$) compared to the hydrogen peroxide group. The highest nuclear factor-E2-related factor 2, and Heat shock protein 70 expression levels were observed in the glutamine 15 mmol/L pre-treatment group ($P < 0.01$) [Figure 5].
nuclear factor-E2-related factor 2, Heat shock protein 70 and glutathione-S-transferase; increased caspase-3 and bax levels; and decreased bcl-2 levels. The results suggest that these were slightly increased in oxidative stress conditions as a compensation to protect cells against oxidative damage. At the same time, oxidative stress initiated the apoptosis process of melanocytes.

It is now recognized that glutamine has cytoprotective effects by inhibiting oxidative-stress induced apoptosis and damage, increasing antioxidant enzyme levels in vivo, and decreasing oxidative metabolite production. Studies report that antioxidant intervention activates the nuclear factor-E2-related factor 2-antioxidant responsive element signalling pathway and induces its downstream antioxidants. According to our findings, we propose that the antioxidant effects of glutamine may be regulated by the enhanced expression of nuclear factor-E2-related factor 2, which binds to nuclear antioxidant responsive element and triggers the transcription of a series of downstream genes. These encode phase II detoxifying enzymes, antioxidant proteins, and molecular chaperones, thus enhancing the expression of superoxide dismutase, glutathione-S-transferase, heme oxygenase-1 and Heat shock protein 70. A powerful antioxidant effect against oxidative stress is activated by inhibiting reactive oxygen species and malondialdehyde production, improving cell morphology and promoting cell viability.

We found that glutamine significantly decreased the expression of bax and caspase-3, increased the expression of bcl-2, and demonstrated significant anti-apoptotic effects in melanocytes. This increases the stability of the mitochondrial outer membrane, maintains the normal permeability of mitochondrial permeability transition pore (MPTP) and prevents the release of factors that promote apoptosis. Caspase-3 acts as the main effective factor, activation of which is followed by an irreversible stage of apoptosis. Studies show that nuclear factor-E2-related factor 2 knockout aggravates oxidant-induced apoptosis, and nuclear factor-E2-related factor 2 signalling pathways regulate bcl-2 anti-apoptotic protein expression, proving that this pathway plays a protective role in cell apoptosis. Glutamine significantly induces Heat shock protein 70 expression, which is an important apoptosis regulator. It inhibits apoptosis by interfering in all major apoptotic pathways. In summary, the nuclear factor-E2-related factor 2-antioxidant responsive element signalling pathway is involved in apoptosis resistance as well as antioxidant effects. These effects echo our results, where glutamine increased nuclear factor-E2-related factor 2 expression and its downstream proteins such as superoxide dismutase, glutathione-S-transferase, heme oxygenase-1 and Heat shock protein 70. Apoptosis suppression depends on glutamine concentration.

It was found that vitiligo melanocytes exhibited hypersensitivity to hydrogen peroxide-induced oxidative injury because of reduced nuclear factor-E2-related factor 2 nuclear translocation and transcriptional activity, leading to an aberrant redox balance. We hypothesize that if melanocytes from vitiliginous skin tissue was used in our experiment, the melanocyte reaction to hydrogen peroxide-induced oxidative stress would have been more significant.
However, the role of glutamine in vitiliginous melanocytes needs further investigation.

**Study limitations**
The molecular mechanisms of glutamine activated nuclear factor-E2-related factor 2 antioxidant responsive element signalling pathway and the role of glutamine in vitiliginous melanocytes must be thoroughly studied.

**Conclusions**
Our results have proved that glutamine has excellent antioxidative and anti apoptotic effects in a dose dependent manner. Moreover, glutamine has both prophylactic and therapeutic effects against oxidative stress induced melanocyte damage. The prophylactic effect is more significant than the therapeutic effect at the same dose. The results of this study shed light on the pathogenesis and treatment of vitiligo, and can guide clinical therapy for vitiligo patients.

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**Conflicts of interest**
There are no conflicts of interest.

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