
EXPERIMENTAL PAPERS

Rutin Ameliorates H₂O₂-Induced Oxidative Stress Injury in HaCaT Cells via the Nrf2-Regulated Pathway

G.-P. Lang^a and Y.-Y. Han^{b, *}

^aKey Laboratory of Basic Pharmacology of Ministry of Education and Joint International Research Laboratory of Ethnomedicine of Ministry of Education, Zunyi Medical University, Zunyi, China

^bThe Special Key Laboratory of Oral Diseases Research Institution of Higher Education in Guizhou Province, Zunyi Medical University, Zunyi, China

*e-mail: hanyingying5219@163.com

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Abstract—Oxidative stress induced by harmful substances activates inflammatory signaling pathways and causes excessive proliferation of keratinocytes, which is related to the occurrence of psoriasis. Rutin, a natural citrus flavonoid glycoside, exhibits protective effects against oxidative stress. However, whether rutin is able to influence oxidative stress in keratinocytes remains unclear. In the present study, an in vitro cell model of human keratinocytes (HaCaT) treated with H₂O₂ was used to explore whether rutin can prevent oxidative stress. The present findings suggest that rutin protected HaCaT cells against oxidative damage by inhibiting ROS, NO and MDA secretion, increasing SOD activity and restoring GSH-Px activity. In addition, rutin supplement limited IL-6, IL-1β and IL-23A production in HaCaT cells. Moreover, rutin upregulated Nrf2 expression, and promoted the downstream NQO1 and HO-1 expression. These results suggest that rutin may inhibit HaCaT cell oxidative stress by modulating the Nrf2-regulated pathway.

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Psoriasis is clinically characterized by red patches of skin with a pronounced boundary, which are often covered with multiple silvery white scaly layers and accompanied by keratinocyte proliferation, obvious itching and scaling. Psoriasis is a common chronic immune-related inflammatory disease that mainly affects the skin and joints and is mediated by overactive Th17 response [1, 2]. Several studies have reported that oxidative stress plays an important role in the

development and progression of various skin diseases, such as psoriasis [3–5], and the role of reactive oxygen species (ROS) in psoriasis has been demonstrated [6]. Oxidative stress is often related to exogenous factors such as environmental pollution, ultraviolet rays and chemical substances, leading to inflammatory reactions and tissue damage, which subsequently promote the generation of endogenous oxidants [7].

Keratinocytes are a major component of the

epidermis [8]. Oxidative stress-induced ROS and reactive nitrogen species (RNS) can activate inflammatory signaling pathways and cause excessive proliferation of keratinocytes [9]. Therefore, the oxidative stress is closely related to the occurrence and development of the disease, and drug intervention against such pathophysiological phenomena may be a new target for the treatment of psoriasis.

Rutin is a common dietary flavonoid glycoside widely found in vegetables and citrus fruits that has a variety of regulatory effects on cell functions, including antioxidative, anti-inflammatory, antiallergic, antiviral, anticancer, and antiangiogenic effects [10]. Previous study has shown that rutin is capable of reducing the secretion of important mediators of inflammatory and immune responses such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), promote the migration of white blood cells, and increase immunoglobulin levels in the body [11]. Furthermore, rutin effectively protects skin fibroblasts and keratinocytes from oxidative stress following UV exposure [12, 13]. The positive contributions of rutin in aging and cardiovascular diseases have been shown in previous studies [14, 15]. However, the effectiveness of rutin in the treatment of psoriasis remains to be further clarified.

In this study, human immortalized keratinocytes (HaCaT) treated with H₂O₂ were used to determine the effects of rutin *in vitro*. Moreover, we also investigated the important factors related to oxidative stress and inflammation to further explore the possible regulatory mechanisms of rutin in psoriasis.

MATERIALS AND METHODS

Cell culture

The HaCaT cell line (GAINING BIOLOGICAL, Shanghai, China) was cultured in high-glucose Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (BioInd, Inc., Shanghai, China) and 1% penicillin/streptomycin (Solarbio Science & Technology Co., Ltd., Beijing, China) at 37°C and 5% CO₂. Rutin (Solarbio Science & Tech-

nology Co., Ltd., Beijing, China) was prepared as a 20 mg/mL stock solution in phosphate buffered saline (containing 0.1% dimethyl sulfoxide, DMSO) and diluted in DMEM to the specified concentrations used in each experiment. H₂O₂ was obtained from Thermo Fisher Scientific, Inc. (Thermo Fisher Scientific, Inc.) and prepared as a 10 mM stock solution in DMEM. HaCaT cells in the control group were pretreated with DMSO, and cells in the experimental groups were pretreated with rutin for 1 hour before incubation with or without H₂O₂ (200 μ M, diluted in DMEM) for 24 hours as described before [16].

Cell viability assay

A cell viability CCK-8 assay was performed to measure the effect of rutin and H₂O₂ on HaCaT cell viability. In this assay the water-soluble tetrazolium salt in the reagent can be reduced by dehydrogenase in cell mitochondria and thereby converted to formazan dye, which has an absorbance at 450 nm, and the optical density is positively related to the number of viable cells. HaCaT cells (4×10^3 cells/well) were cultured in 96-well plates and treated with rutin at various concentrations (0, 6.25, 12.5, 25, 50, 100 and 200 μ g/mL) for 1 h. In addition, cells (4×10^3 cells/well) were cultured in 96-well plates and treated with H₂O₂ (0, 50, 100, 200, 300, 400, 500, 600, 700, and 800 μ M) for 24 h. Subsequently, the effect of rutin on the viability of HaCaT cells treated with H₂O₂ was studied, and the cells (4×10^3 cells/well) were first treated with rutin (0, 6.25, 12.5, 25, 50, 100 and 200 μ g/mL) for 1 h followed by incubation with 200 μ M H₂O₂ for 24 h. Cells then were treated using CCK-8 kits (Cat# CK04-11, Dojindo, Shanghai, China), and the optical density at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Measurement of oxidative stress factors

The content of intracellular ROS in HaCaT cells was detected by ROS assay kit (Cat# S0033M, Beyotime, Shanghai, China). For this, cells (4×10^4 cells/well) were seeded into 96-well plates and pretreated with rutin (100 μ g/mL) for 1 hour, followed by incubation with or without H₂O₂ (200 μ M) for 24 h. Cells then were washed

with DMEM and incubated with 10 μ L of dichlorodihydro-fluorescein diacetate (DCFH-DA) (1 μ M) at 37°C for 20 min in the dark, then ROS positive populations of HaCaT cells were measured by a flow cytometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 488 nm laser wavelength and 525 nm detection wavelength. The content of nitric oxide (NO) in HaCaT cells was determined in the following experiments using a commercial kit (Cat# A013-2-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). For this, cells (3×10^5 cells per mL) were seeded into 6-well plates and treated with rutin and H₂O₂ as described above. The absorbance of the reaction mixtures was measured at 540 nm with a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and NO content was calculated using the NaNO₂ standard calibration curve. The malondialdehyde (MDA), total superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) content and total antioxidant capacity (T-AOC) in HaCaT cells were evaluated by the corresponding test kit (Cat# A003-1, A001-3, A005-1, A015-2-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions. For this, cells (2×10^6 cells/well) were seeded into 6-well plates and treated as mentioned above. MDA content was analyzed by the thiobarbituric acid (TBA) method. Briefly, MDA reacts with TBA to generate an MDA-TBA adduct whose absorbance can be measured at 532 nm. The activity of SOD was measured by the water-soluble tetrazolium salt (WST) method, 20 μ L supernatant, 20 μ L enzyme working solution and 200 μ L substrate solutions were mixed and incubated at 37°C for 20 min. The absorbance was measured on a microplate reader at 450 nm. GSH-Px activity was measured using the GSH assay kit based on the manufacturer's instruction. A series of enzymatic reactions was activated by GSH-Px in cells which subsequently led to the conversion of GSH to oxidized glutathione (GSSG). The change in absorbance during the conversion of GSH to GSSG was measured by microplate reader at 412 nm.

Protein content analysis

In all experiments after administration with rutin or H₂O₂ or both, cells were homogenized in

PBS (Cat# G4202, Servicebio Technology Co., Ltd., Wuhan, China) at 4°C and centrifuged at 12,000 g for 5 min, the supernatant was collected and protein content was quantified using a bicinchoninic acid (BCA) protein assay kit (Cat# PC0020, Solarbio Science & Technology Co., Ltd., Beijing, China).

Enzyme-linked immunosorbent assay (ELISA)

HaCaT cells (1×10^6 cells/well) were cultured in 6-well plates and treated with rutin (100 μ g/mL) for 1 hour, followed by incubation with or without H₂O₂ (200 μ M) for 24 h, culture supernatants in each well were collected respectively and centrifuged at 3000 rpm for 20 min at 4°C, then supernatants were harvested and analyzed for IL-6, IL-1 β , and IL-23A in ELISA kits (MEIMIAN Co., Ltd., Jiangsu, China). The concentrations of the inflammatory factors in cell supernatants were determined according to the manufacturer's protocols. The optical density (OD) of the samples after performing the tests was read at 450 nm by a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Western blotting

HaCaT cells (1×10^6 cells/well) were cultured in 6-well plates and treated as mentioned above, then cells were washed three times with ice-cold PBS and lysed using 100 μ L of RIPA buffer (Solarbio Science & Technology Co., Ltd., Beijing, China) containing a cocktail of protease inhibitors. Total proteins from HaCaT cells were extracted, and concentrations were determined by the BCA assay. 50 μ g of protein was uploaded in each lane. Protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane. The membranes were blocked in blocking buffer (Beyotime, Shanghai, China) for 2 h at room temperature and immunoblotted overnight at 4°C with the following primary antibodies: rabbit monoclonal anti-Nrf2 antibody (1 : 1000, Cat# ab62352), mouse monoclonal anti-Keap1 antibody (1 : 1000, Cat# ab119403), rabbit monoclonal anti-Heme Oxygenase 1 (HO-1) antibody (1 : 2000, Cat# ab52947), mouse monoclonal anti-NQO1 antibody (1 : 500, Cat# ab28947), rabbit mono-

clonal anti-GCLC antibody (1 : 1000, Cat# ab190685), and mouse monoclonal anti- β -actin antibody (1 : 5000, Cat# ab6276). The membrane was washed with washing buffer (Beyotime, Shanghai, China) and incubated with the corresponding secondary HRP-conjugated goat anti-rabbit antibody (1 : 2000, Cat# A0208, Beyotime) or goat anti-mouse antibody (1 : 2000, Cat# A0216, Beyotime) for 1 h at room temperature. Protein bands were visualized with enhanced chemiluminescence substrate (Beyotime) and quantified using FujiFilm Multi Gauge Ver. 3.0 software (Fuji Photo Film Co., Tokyo, Japan).

Statistical analysis

All data were analysed with GraphPad Prism software v5.01 (GraphPad Software Inc., San Diego, CA, USA) and are shown as the mean \pm SEM. Comparisons between groups were analysed by one-way analysis of variance followed by Tukey's multiple comparison test. $p < 0.05$ was considered as statistically significant.

RESULTS

Effects of rutin on H_2O_2 -induced HaCaT cell viability

In this study, a commercial CCK-8 kit was used to test the viability of the cells using as a measured parameter the absorbance of formazan dye at 450 nm and assess the optimal concentration of H_2O_2 treatment. As shown in Fig. 1a, HaCaT cells exhibited approx. 80% viability after 24 h of incubation with 200 μ M H_2O_2 . Therefore, 200 μ M H_2O_2 was employed to treat HaCaT cells in the following experiments. To assess the potential cytotoxicity induced by rutin treatment in HaCaT cells, cells were treated with various concentrations of rutin for 1 h, and cell viability was evaluated by CCK-8 assay. The results indicated that 200 μ g/mL rutin administered for 1 h revealed significant toxicity. Thus, 100 μ g/mL rutin or less was selected for the subsequent experiments (Fig. 1b). Next, the effect of rutin on the viability of HaCaT cells treated with H_2O_2 was studied. HaCaT cells were pretreated with rutin at 0, 6.25, 12.5, 25, 50, 100 and 200 μ g/mL for 1 h

followed by treatment with H_2O_2 (200 μ M) for 24 h. As shown in Fig. 1c, 100 μ g/mL rutin treatment successfully rescued H_2O_2 -induced cell viability reduction, indicating a promising protective effect of rutin in HaCaT cell oxidative stress injury.

Rutin attenuated H_2O_2 -induced oxidative stress in HaCaT cells

The increase of ROS and NO content are important causes of oxidative stress injury. Therefore, we investigated the capability of rutin to inhibit H_2O_2 -induced ROS and NO production in HaCaT cells by using ROS assay kit and NO test kit respectively. The results in Figs. 2a–2b indicated that H_2O_2 treatment increased ROS and NO generation compared to the control group, which was significantly inhibited by 100 μ g/mL rutin pretreatment. In addition, MDA levels were significantly enhanced following H_2O_2 treatment, and rutin was capable of antagonizing MDA elevation (Fig. 2c). Apart from ROS, NO and MDA, we also assessed the T-AOC, SOD and GSH-Px activities to evaluate the antioxidative properties of rutin. As shown in Figs. 2d–2f, 100 μ g/mL rutin boosted the antioxidant capacity of HaCaT cells by increasing SOD activity and restoring GSH-Px activity reduced by H_2O_2 .

Rutin inhibited cytokine production in H_2O_2 -treated HaCaT cells

To validate the effect of rutin on the inflammatory response in H_2O_2 -treated HaCaT cells. ELISA analysis was performed to measure the concentrations of IL-6, IL-1 β and IL-23A in the cell supernatants. As shown in Fig. 3, H_2O_2 administration markedly enhanced IL-6, IL-1 β and IL-23A content compared with the control group (Figs. 3a–3c). As expected, the alterations that occurred above were reversed by rutin pretreatment (Figs. 3a–3c).

Effect of rutin on the levels of Nrf2 and antioxidant system proteins in H_2O_2 -induced HaCaT cells

Nrf2 is an important factor in the cellular antioxidant defense system. In the following experiment, the expression levels of Keap1 (kelch-like

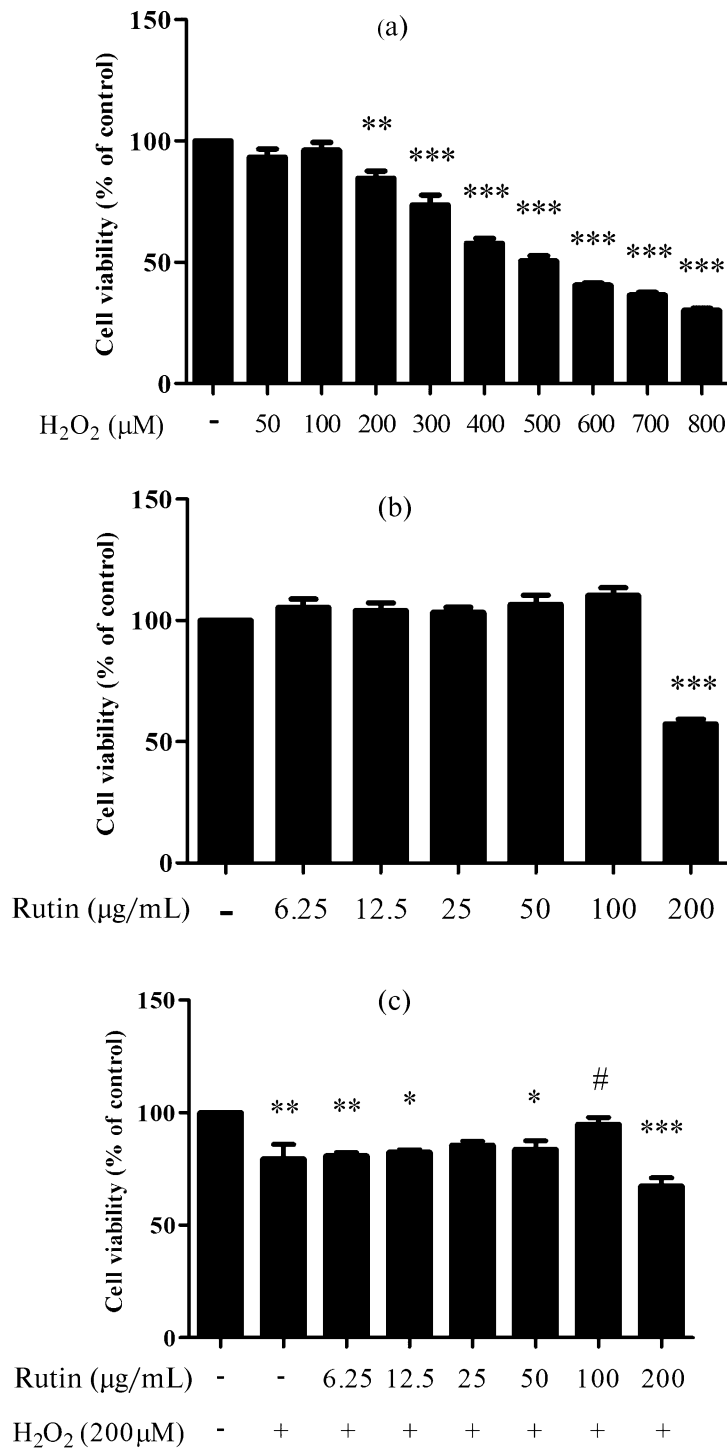


Fig. 1. Effects of rutin on cell viability of HaCaT cells treated with H₂O₂. (a) Viability of HaCaT cells after incubation with H₂O₂ (0, 50, 100, 200, 300, 400, 500, 600, 700 and 800 μM) for 24 h. (b) Viability of HaCaT cells after treatment with rutin (0, 6.25, 12.5, 25, 50, 100 and 200 μg/mL) for 1 hour. (c) Effects of rutin pretreatment on the viability of HaCaT cells treated with 200 μM H₂O₂ for 24 h. The viability of HaCaT cells was assessed by the CCK8 kit, and absorbance was examined by a microplate reader at 450 nm. Data were analyzed by one-way analysis of variance followed by Tukey's post-hoc test and expressed as the mean ± SEM. *—*p* < 0.05, **—*p* < 0.01, ***—*p* < 0.001, compared to the control group, #—*p* < 0.05, compared to the H₂O₂-treated group, *n* = 4.

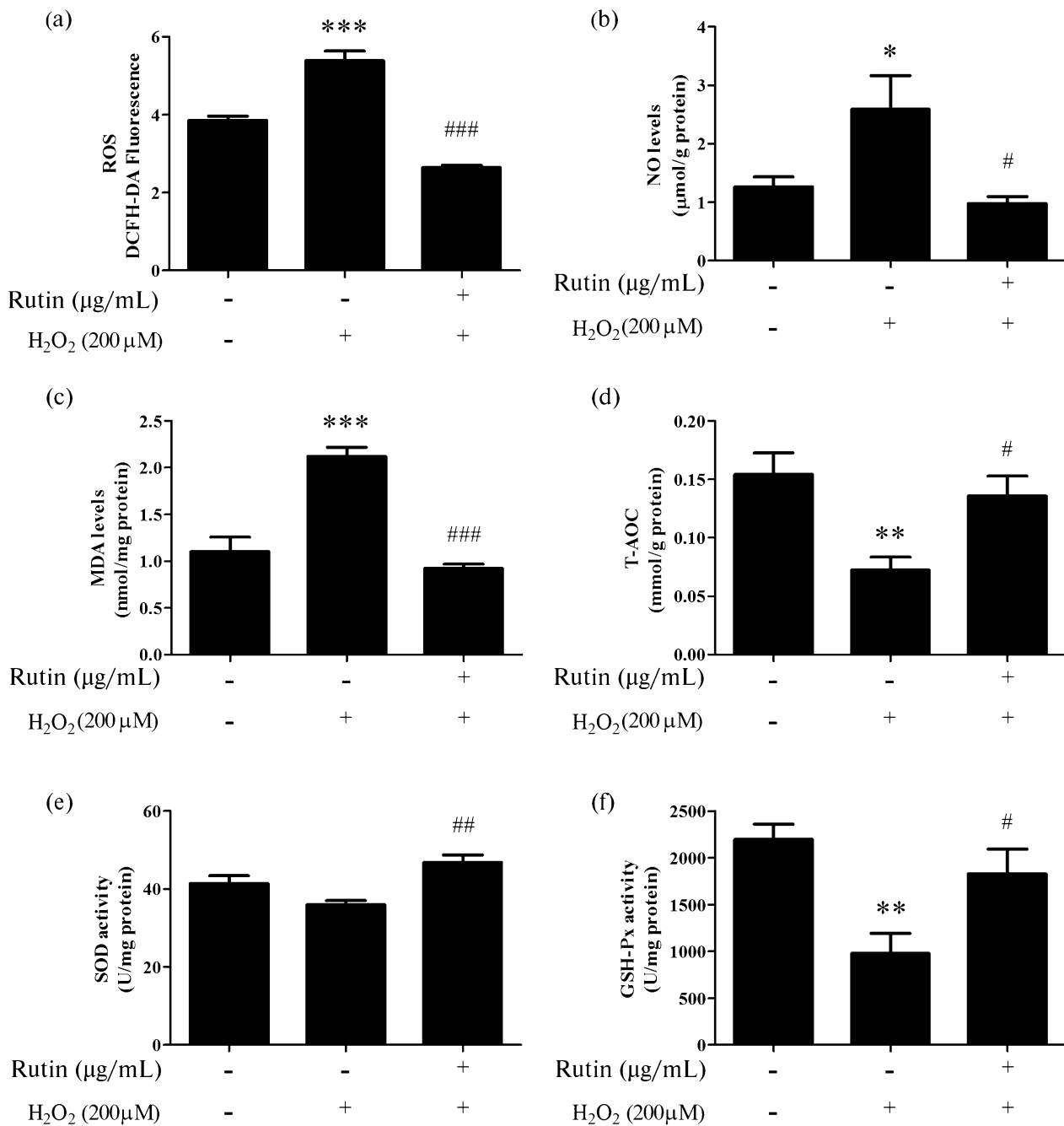


Fig. 2. Rutin attenuated H₂O₂-induced oxidative stress in HaCaT cells. HaCaT cells were treated with 100 μg/mL rutin for 1 h and further exposed to 200 μM H₂O₂ for 24 h. Oxidative stress biochemical indicators were measured by commercial kits. (a) ROS production, (b) NO levels, (c) MDA levels, (d) T-AOC, (e) SOD and (f) GSH-Px activity in HaCaT cells. Data are presented as the mean ± SEM. *—*p* < 0.05, **—*p* < 0.01, ***—*p* < 0.001, compared to the control group; #—*p* < 0.05, ##—*p* < 0.01, ###—*p* < 0.001, compared to the H₂O₂-treated group, *n* = 6.

ECH-associated protein 1), Nrf2 (nuclear factor erythroid 2-related factor 2), NQO1 (NAD(P)H quinone oxidoreductase 1), HO-1 (heme oxygenase-1) and GCLC (glutamate-cysteine ligase cat-

alytic subunit) were determined by Western blotting. The results showed that Nrf2 and NQO1 protein expression levels were decreased by H₂O₂ treatment, whereas rutin significantly reversed

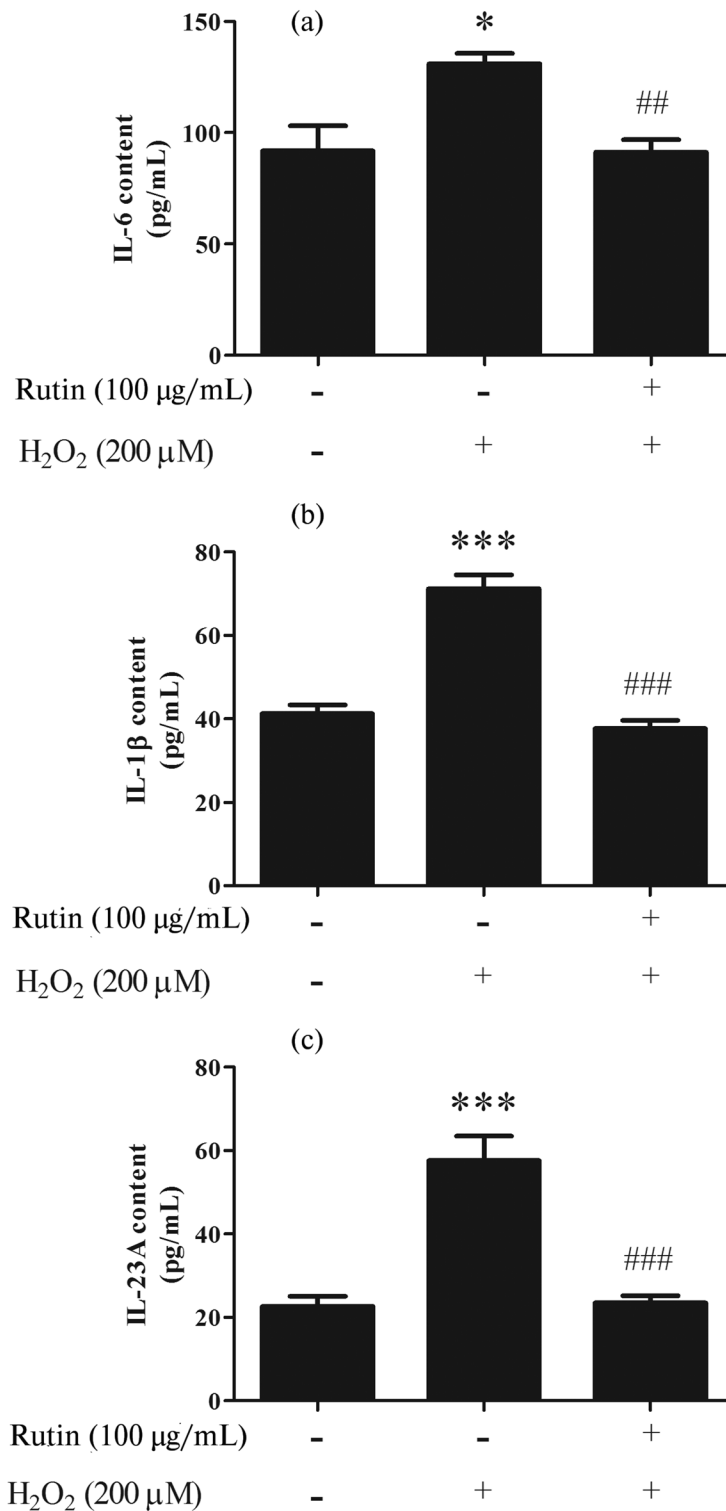


Fig. 3. Rutin inhibited cytokine production in H₂O₂-induced HaCaT cells. HaCaT cells were pretreated with 100 µg/mL rutin for 1 h followed by exposure to 200 µM H₂O₂ for 24 h. IL-6 (a), IL-1β (b), and IL-23A (c) content in cell supernatants were determined using commercial ELISA kits. The final absorbance values were measured at 450 nm using a plate reader. Data are presented as the mean ± SEM. *— $p < 0.05$, ***— $p < 0.001$, compared to the control group; ##— $p < 0.01$, ###— $p < 0.001$, compared to the H₂O₂-treated group, $n = 5$.

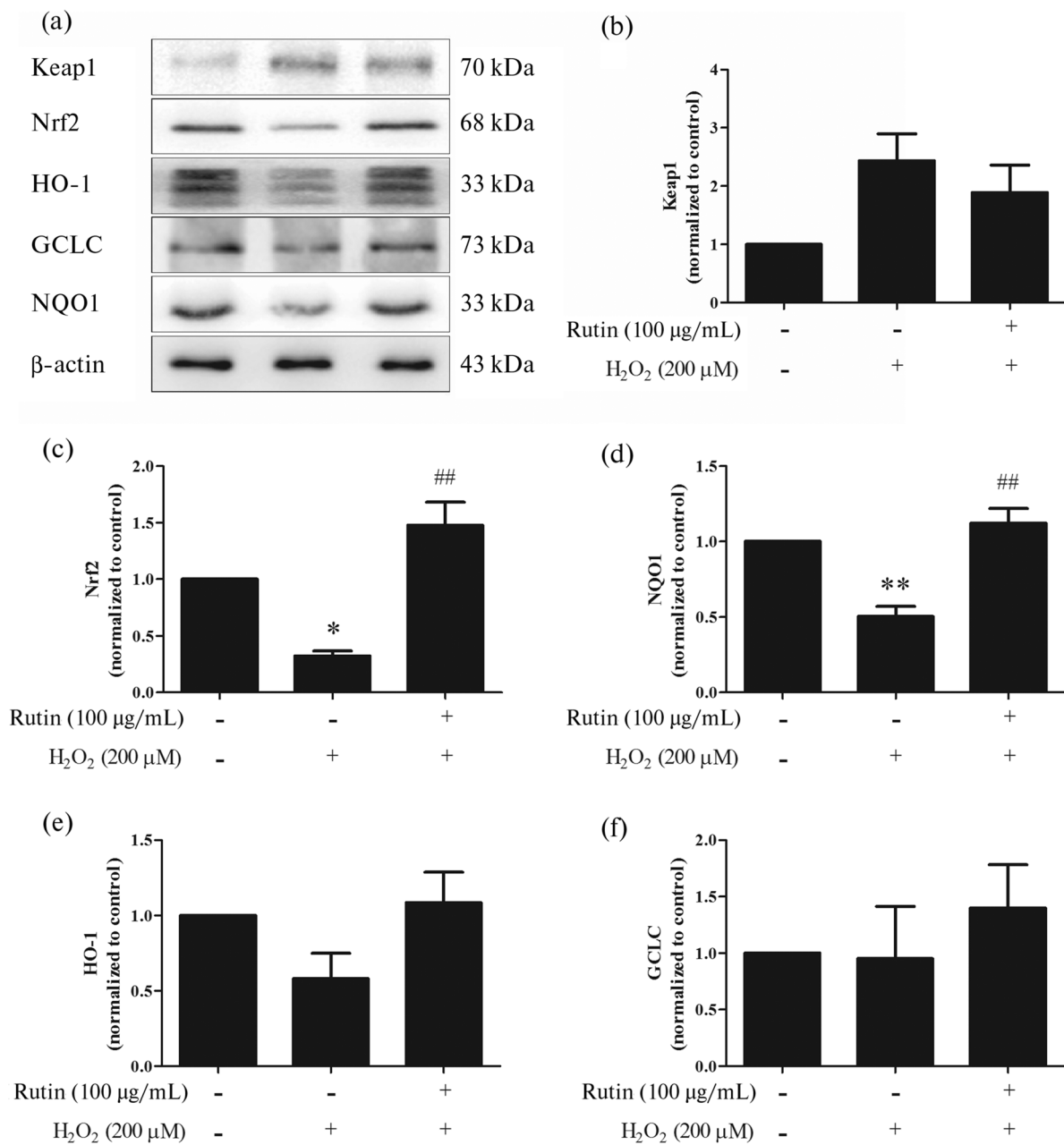


Fig. 4. Effect of rutin on protein levels of Nrf2 and antioxidant system proteins in H₂O₂-induced HaCaT cells. HaCaT cells were treated with 100 μ g/mL rutin for 1 h and further exposed to 200 μ M H₂O₂ for 24 h. Total protein levels from HaCaT cells were determined by BCA assay, and protein expression was measured by Western blotting. (a) Representative Western blotting images. (b–f) Quantitative protein analysis of Keap1, Nrf2, NQO1, HO-1 and GCLC expression in HaCaT cells. Data are presented as the mean \pm SEM. *— $p < 0.05$, **— $p < 0.01$, compared to the control group; ##— $p < 0.01$, compared to the H₂O₂-treated group, $n = 3$.

this downregulation in HaCaT cells (Figs. 4c, 4d). The protein expression of Keap1 and HO-1 showed an increasing or decreasing trend, but there was no statistically significant difference.

DISCUSSION

Increasing evidence shows that oxidative stress may aggravate the pathogenesis of psoriasis [4,

17]. Due to environmental problems such as heavy metal pollution, ionizing radiation, and ultraviolet radiation, the skin is constantly exposed to internal and external oxidizing conditions, which accelerate the production of ROS in the body [18]. Overgenerated ROS can upregulate the expression of proinflammatory cytokines and chemokines through signaling pathways such as MAPK/AP-1, NF- κ B, and JAK-STAT or by increasing DNA modification and lipid peroxidation reaction products [19]. As a result, this causes the occurrence of psoriasis or leads to aggravation of the disease. Meanwhile, studies have shown that the antioxidant system in the skin lesions of patients with psoriasis is unbalanced, with reduced expression of antioxidant enzymes, which leads to weakened antioxidant capacity of cells [20]. The above findings have proven that oxidative stress damage is closely related to the occurrence of psoriasis. H₂O₂ is a reliable agent for modelling oxidative stress, and the *in vitro* oxidative stress model jointly constructed with HaCaT cells is more cost-effective and easy to reproduce and is suitable for the pathological study of a variety of oxidative stress-related skin diseases. In our study, to evaluate the sensitivity of HaCaT cells to oxidative stress, cells were treated with H₂O₂ [21].

The antioxidant protection system of the cell includes antioxidant molecules and antioxidant enzymes [22]. Under physiological conditions, the formation of peroxide and antioxidant substances is in a dynamic balance, which depends on the effectiveness of the elimination of ROS by antioxidant substances [23]. In addition, the contents of MDA and the activities of SOD and GSH-Px are usually used to provide a comprehensive assessment of the antioxidant capacity [24]. MDA is a final product of lipid peroxidation, which is the hallmark of ROS-induced injury and reflects the ROS content and cell injury [25]. In contrast to ROS, NO and MDA, GSH-Px and SOD are considered as the key antioxidant enzymes of the antioxidant defense system [26]. Studies have shown that rutin can activate antioxidant enzymes, including catalase, to minimize the formation of free radicals while inhibiting oxidative stress by enhancing the activities of antioxi-

dant enzymes such as SOD, CAT, and GSH-Px [27, 28]. The present results revealed that the released ROS, NO and MDA of injured HaCaT cells increased and the antioxidant enzymes GSH-Px and SOD decreased, indicating that H₂O₂ induced cell oxidative damage in the model group. After pretreatment with rutin, the contents of ROS, NO and MDA declined significantly when compared with those in H₂O₂-treated HaCaT cells. The present investigation has demonstrated that rutin had a significant antioxidant capacity *in vitro*.

In addition, the pathogenesis of psoriasis is related to the excessive proliferation of epidermal keratinocytes and small proteins such as cytokines play an important role in this process. During the development of psoriasis, the increased expression of inflammatory cytokines such as TNF- α , IL-6, and IL-23 triggers inflammation and activates keratinocyte proliferation [29]. Our data showed that rutin inhibited H₂O₂-induced increases in IL-6, IL-1 β and IL-23A levels, thus exerting an anti-inflammatory effect.

The key components of endogenous antioxidant systems are controlled by the transcription factor Nrf2 [30]. When oxidative stress occurs, Nrf2 translocates into the nucleus and heterodimerizes with small Maf proteins to transactivate genes containing antioxidant response element (ARE) and promote the transcription of genes involved in defenses against oxidative stress injury, such as HO-1, NQO1, and GCLC [31, 32]. As expected, our results suggested that rutin pretreatment up-regulates Nrf2 protein expression and promotes the downstream NQO1 expression.

In summary, our study demonstrates that rutin reduced the inflammatory response and enhances the antioxidant system in human keratinocytes via an Nrf2-dependent mechanism.

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AUTHORS' CONTRIBUTION

GPL: conducting experiments; GPL, YYH: experimental data analysis, statistical data processing, preparing graphical material, writing and editing a manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

All ethical standards set forth by the Zunyi Medical University and local laws were complied with.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest, both evident and potential, that would be associated with the publication of this article.

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