Protective Effects of γ-Oryzanol against Oxidative Damage Induced by Heat Stress in Skin Fibroblasts

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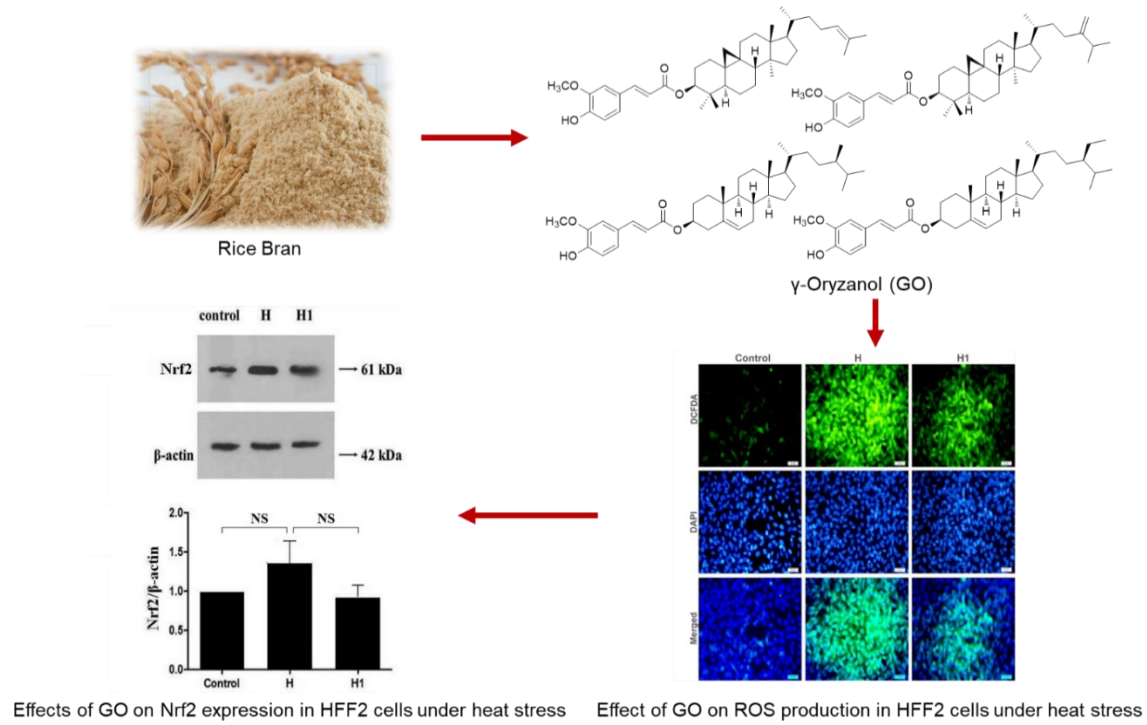
GRAPHICAL ABSTRACT



Effects of GO on Nrf2 expression in HFF2 cells under heat stress

Effect of GO on ROS production in HFF2 cells under heat stress

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γ-Oryzanol (GO), a steryl ferulate derived from rice bran oil, exhibits a broad range of biological activities. However, the mechanisms underlying GO's protective role against heat-induced cellular stress remain largely unexplored. This study aimed to investigate the protective effects of GO against heat stress in human foreskin fibroblast (HFF2) cells. HFF2 cells were exposed to heat stress (43 °C) in the presence or absence of 100 µg/mL GO, and cell viability, reactive oxygen species (ROS), glutathione (GSH), and nuclear factor erythroid 2–related factor 2 (Nrf2) expression were evaluated. Heat stress significantly increased intracellular ROS levels (~2.8-fold, $p < 0.01$), decreased GSH levels ($p < 0.01$), and upregulated Nrf2 expression. Treatment with 100 µg/mL GO significantly improved cell viability ($p < 0.05$), reduced ROS levels ($p < 0.01$), increased GSH content by approximately 1.35-fold ($p < 0.05$), and reduced Nrf2 expression by approximately 40% compared with heat-stressed cells. These findings suggest that GO protects skin fibroblasts against heat-induced oxidative damage by restoring cellular redox homeostasis and modulating antioxidant defense mechanisms.

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Keywords: γ-Oryzanol; Nrf2; Glutathione; Fibroblast; Oxidative stress

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INTRODUCTION

The skin, as the first line of defense against external stressors, undergoes inevitable aging over time. Skin aging is attributed in part to the formation of free radicals, particularly reactive oxygen species (ROS), which alter gene expression and result in the degradation of collagen and elastin (Kammeyer and Luiten 2015). Heat stress, a significant environmental stressor for humans and animals, influences comfort and performance (Wu *et al.* 2019; Saadeldin *et al.* 2020). It indirectly enhances free radical production by inhibiting the electron transfer chain and ATP production, while also directly stimulating the production of free radicals through the induction of enzymes like NADPH oxidase (Emami *et al.* 2021).

In the pursuit of preventing lifestyle-related diseases and aging, natural antioxidants have garnered attention (Choi *et al.* 2024; Kim *et al.* 2025). These antioxidants, containing phytochemicals, aid the body in combating oxidative stress (Rungratanawanich *et al.* 2018). γ-Oryzanol (gamma-oryzanol, GO), a steryl ferulate derived from rice bran oil, has been reported to exhibit antioxidant properties akin to tocopherols (Patel and Naik 2004).

Rice (*Oryza sativa*) contains several bioactive compounds, including phenolic acids, tocopherols, tocotrienols, carotenoids, and γ-oryzanol (GO) (Minatel *et al.* 2014). Some rice cultivars contain 8 to 10 times more GO than vitamin E, one of the most effective antioxidants due to its bioavailability (Minatel *et al.* 2014). The antioxidant activity of GO has been attributed to multiple complementary mechanisms (Patel and Naik 2004). As a ferulic acid ester, GO can directly scavenge reactive oxygen species (ROS) by donating hydrogen atoms from its phenolic hydroxyl group, thereby interrupting free radical chain reactions. In addition, GO has been reported to reduce lipid peroxidation and enhance endogenous antioxidant defense by modulating antioxidant enzymes, including superoxide dismutase, catalase, and glutathione-related enzymes. Furthermore, GO has been shown to regulate redox-sensitive signaling pathways, particularly the nuclear factor erythroid 2–related factor 2 (Nrf2) pathway, thereby contributing to the maintenance of cellular redox homeostasis. In a *Drosophila melanogaster* model of Parkinson's disease induced by rotenone, GO enhanced antioxidant defenses, reduced oxidative stress, and mitigated mitochondrial dysfunction (Araujo *et al.* 2015). These benefits were accompanied by a significant increase in antioxidant enzymes, such as catalase, superoxide dismutase, and glutathione-S-transferase, which helped abrogate harmful malondialdehyde (MDA) and reactive oxygen species (ROS) produced by rotenone. These benefits are likely due to the inhibition of free radical generation and the prevention of inflammation. The GO also exhibits a variety of biological effects, including antidiabetic (Ghatak and Panchal 2012) and anti-inflammatory activities (Islam *et al.* 2008). It reduces endoplasmic reticulum (ER) stress in pancreatic β-cells, enhancing insulin production and adipocyte production of adiponectin. Higher levels of insulin and adiponectin activate 5′-AMP-activated protein kinase (AMPK), reducing the expression of phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase, thus inhibiting gluconeo-genesis (Minatel *et al.* 2016). The GO also regulates proinflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8, as demonstrated in a colitis model, where significant reductions in these cytokines' mRNA expression and tissue infiltration of inflammatory cells were observed (Gilmore 2006).

Recently, GO's hypolipidemic and anti-obesity effects have been the most extensively studied (Wang *et al.* 2015). These health benefits are often associated with diets high in brown rice (BR) or germinated BR (GBR) (Shimabukuro *et al*. 2014). The GO and its major metabolite, ferulic acid (FA), have been shown to improve lipid metabolism, hypertension, and glucose tolerance. They reduce triglyceride and cholesterol levels by suppressing hepatic lipogenesis and enhancing fecal lipid excretion. In 3T3-L1 cells differentiating into adipocytes, GBR and GO decrease the mRNA expression of fatty acid synthase (FAS), demonstrating anti-adipogenic activities (Son *et al.* 2010). Known for reducing plasma cholesterol and platelet aggregation, GO is approved for use in both animals and humans with minimal side effects, serving as a dietary supplement in the United States (Ghatak and Panchal 2011).

Recent research highlights GO's cell-protective (Rungratanawanich *et al.* 2018; Huang *et al.* 2020) and skin anti-aging effects (Yodkeeree *et al.* 2018) against oxidants. However, the protective mechanism of GO against heat stress has remained unexplored. Fibroblasts play a crucial role in the synthesis and degradation of skin proteins, making them pivotal for studying the molecular mechanisms influencing skin aging (Shin *et al.* 2019). Cell culturing provides a valuable tool to investigate the molecular mechanisms regulating fibroblast function in both normal and stressful conditions (Rittié and Fisher 2005). Despite these reported antioxidant mechanisms, it remains unclear whether GO protects skin fibroblasts against heat-induced oxidative stress through modulation of

intracellular redox homeostasis. Therefore, this study aimed to assess the effects of GO on cell viability, ROS formation, glutathione (GSH) synthesis, and Nrf2 expression following heat stress in HFF2 cells. This study is the first to demonstrate the skin-protective properties of γ-oryzanol (GO) against oxidative damage induced by heat stress in skin fibroblasts.

EXPERIMENTAL

Materials

γ-Oryzanol (GO) powder (purity ≥98%), a mixture of ferulic acid esters of phytosterols and triterpene alcohols, was purchased from Tsuno Food Industrial Co., Ltd. (Wakayama, Japan). The human skin fibroblasts (HFF2) were obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). All reagents and chemicals used in the present study were procured from Sigma Aldrich (Hamburg, Germany).

Liquid Chromatography-mass Spectrometry (LC-MS) Analysis of GO

Liquid chromatography–mass spectrometry (LC–MS) analysis of γ-oryzanol was performed using an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector and a 6130 Series electrospray ionization (ESI) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) (Lee *et al.* 2024; Bridget *et al.* 2025). The mass spectrometer was operated in the negative electrospray ionization mode. Separation was carried out at 40 °C on an Agilent Zorbax Eclipse XDB-C18 column (4.6 × 150 mm^2, 5 μm) using a mobile phase of acetonitrile/ methanol/isopropanol (25:70:5, v/v) at a flow rate of 1.0 mL/min. The injection volume was 20 μL, and the constituents of GO were detected by UV at 298 and 325 nm and identified based on previously reported data (Wongwaiwech *et al.* 2019).

Cell Culture and Study Design

Human skin fibroblasts (HFF2) were obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were cultured in RPMI-1640 medium (Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, New York, NY, USA) and 1% Pen-Strep antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) (Gibco, New York, NY, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced daily, and cells were passaged using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) solution when they reached 80 to 90% confluence. Before seeding, cells were counted using a hemocytometer under an inverted microscope, and cell viability was determined by the trypan blue exclusion assay. The cell suspension was then adjusted to a final density of 3 × 10^4 cells/mL using only viable cells. Cells were seeded into 96-well tissue culture plates at a density of 3 × 10^4 cells/mL (200 μL/well) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h prior to treatment. For heat stress experiments, cells were transferred to an incubator set at 43 °C under the same atmospheric conditions for 12 h. The cells were divided into three groups, each with three replicates. The normal control group (NC) was maintained at 37 °C, whereas the heat stress group (H) and the heat stress plus γ-oryzanol group (H1) were incubated at 43 °C. The H1 group was treated with 100 μg/mL GO based on previous studies showed non-toxic and protective effects in fibroblasts and other cell types (Rungratanawanich *et al.* 2018; Yodkeeree *et al.* 2018).

Evaluation of Cell Viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HFF2 cells were seeded into 96-well tissue culture plates at a density of 3×10^4 cells/mL (200 µL/well) and incubated at 37 °C for 24 h. Subsequently, the culture medium was completely replaced with fresh RPMI-1640 medium containing the appropriate treatment (100 µg/mL GO for the H1 group or medium alone for the NC and H groups). The plates were then incubated under their respective temperature conditions for 12 h. After heat treatment, the supernatants were removed, and the cells were washed with phosphate-buffered saline (PBS). Then, 200 µL of MTT solution (Sigma, Germany) at a concentration of 2 mg/mL in culture medium was added to each well, followed by incubation at 37 °C for an additional 4 h. Supernatants were removed, and 100 µL of dimethyl sulfoxide (DMSO) (Applichem, Germany) was added. After a 30-min incubation, absorbance was measured at 570 nm using a microplate reader (Tecan SunRise, Austria). Cell viability was expressed as a percentage of the control.

Measurement of Reactive Oxygen Species (ROS)

The production of reactive oxygen species (ROS) in HFF2 cells under heat stress, with or without the presence of GO (100 µg/mL), was quantified using the 2′,7′-dichlorofluorescein diacetate (DCFDA) fluorescent dye and a cellular ROS assay kit following the manufacturer's guidelines (Aurogene, Italy). After 12 h of incubation, cells from each experimental group were treated with DCFDA for 10 min at 5% CO_2 and 37 °C. Subsequently, the levels of intracellular ROS were assessed using flow cytometry (Eruslanov *et al.* 2010).

Measurement of Glutathione (GSH)

To assess the effects of GO (100 µg/mL) on glutathione (GSH) synthesis in heat-treated cells, intracellular glutathione levels were measured using a colorimetric assay with a commercial glutathione assay kit (Sigma-Aldrich, St. Louis, MO, USA; Catalog No. CS0260). Cells were lysed and deproteinized, and the resulting supernatants were reacted with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). Absorbance was measured at 412 nm using a microplate reader, and glutathione levels were calculated according to the manufacturer's calibration procedure (Lu 2013).

Measurement of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2) Expression

Western blotting was employed to assess the expression of Nrf2. Cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing a phosphatase inhibitor cocktail, followed by centrifugation at 12,000 g for 4 min at 4 °C. Total protein concentrations were determined using the Bradford method (Pickering *et al.* 2012). Subsequently, 50 µg of protein was separated *via* sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred to a nitrocellulose membrane (Sigma-Aldrich, Germany). The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature and then immunoblotted at 4 °C for 18 h with mouse anti-Nrf2 and mouse anti-β-actin primary antibodies (Santa Cruz, CA, USA). After three washes with TBS-T, the membrane was incubated with a goat anti-mouse horseradish peroxidase (HRP)-conjugated IgG (ElabScience, USA) at a dilution of 1:1000 in TBS-T for 1 h at room temperature. Subsequent to three additional washes with TBS-T,

protein bands were detected using an Enhanced Chemiluminescence (ECL) reagent kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Protein expressions of Nrf2 and β-actin (utilized as a loading control) were quantified by densitometry and analyzed using ImageJ software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 9. One-way analysis of variance (ANOVA) was utilized to compare the results of different tests between groups. The results were expressed as the mean ± standard deviation. A p-value of < 0.05 was considered indicative of a significant difference.

RESULTS AND DISCUSSION

Analysis of γ-Oryzanol Contents

γ-Oryzanol is a mixture of ferulic acid esters of various phytosterols and triterpene alcohols. First identified in rice bran about 60 years ago by Kaneko Ryohei and Tsuchiya Chitaro (Krishna *et al.* 2001), more than ten different GO species have been identified in rice and other cereals, *e.g.* wheat, barley, and corn (Sawada *et al.* 2021). Recently, there has been increasing evidence of beneficial effects of these GO species (Sawada *et al.* 2021).

In this study, GO was sourced from Tsuno Food Industrial Co., Ltd. (Wakayama, Japan), and its content was analyzed using previously reported liquid chromatography-mass spectrometry (LC-MS) methods (Wongwaiwech *et al.* 2019). The LC-MS analysis in negative ion-mode identified peaks corresponding to cycloartenyl ferulate (*m/z* 601.3), 24-methylene cycloartenyl ferulate (*m/z* 615.3), campesteryl ferulate (*m/z* 575.3), and β-sitosteryl ferulate (*m/z* 589.3) (Fig. 1), consistent with previous findings (Wongwaiwech *et al.* 2019). Generally, GO is primarily composed of esters of *trans*-ferulic acid (hydroxycinnamic acid) with phytosterols, including cycloartenol, β-sitosterol, 24-methylenecycloartenol, and campesterol (Wongwaiwech *et al.* 2019).

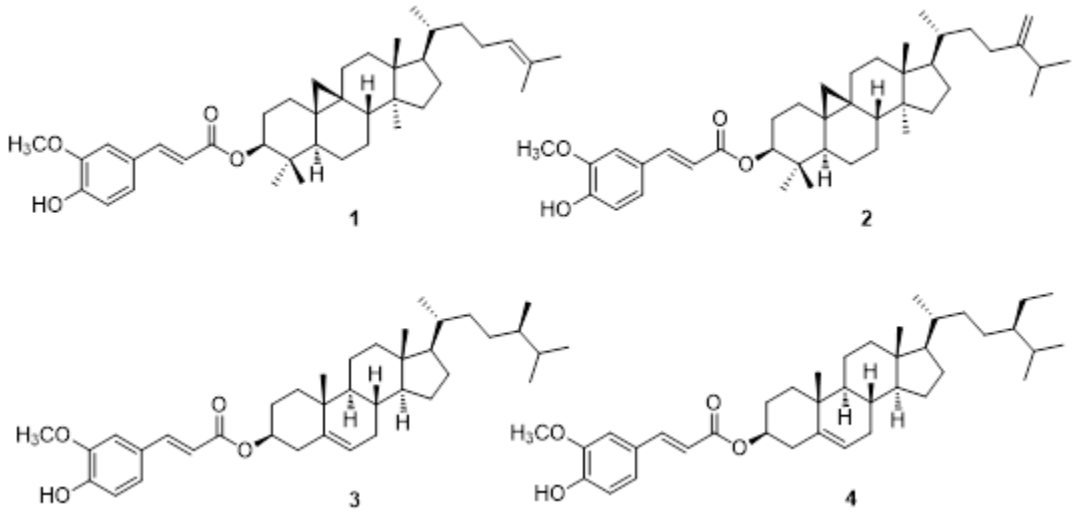

Fig. 1. Structures of cycloartenyl ferulate (**1**), 24-methylene cycloartenyl ferulate (**2**), campesteryl ferulate (**3**), and β-sitosteryl ferulate (**4**)

Effect of GO on Cell Viability of HFF2 Cells under Heat Stress

To investigate the protective effects of GO against heat stress, its effect on the cell viability of HFF2 (human skin fibroblasts) following the induction of heat stress was initially assessed. HFF2 skin fibroblast cells were cultured at 43 °C, and cell viability was evaluated using the MTT assay. The results of the MTT test (Fig. 2) indicated that the addition of different concentrations (0.01 to 100 µg/mL) of GO to the HFF2 cell medium did not significantly reduce cell viability following the induction of heat stress for 12 h. Consequently, a concentration of 100 µg/mL GO was selected for further tests assessing ROS, glutathione, and Nrf2 expression. The cell viability in the heat-stress group (H) significantly decreased compared to the normal control (NC) group ($p < 0.01$), while the group treated with GO (H1) showed a notable improvement in viability ($p < 0.05$), though not reaching the NC level.

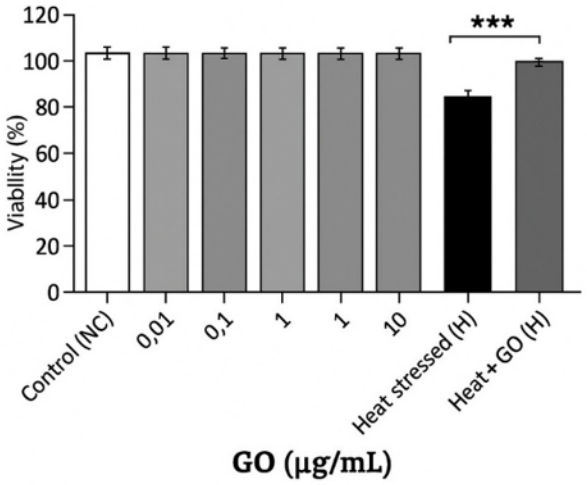

Fig. 2. The effect of GO on viability of HFF2 cells following the induction of heat stress for 12 h as assayed by MTT. Data are presented as mean ± standard deviation from three independent experiments; ***$p < 0.001$, compared to the heat-stressed group (H)

Effect of GO on ROS Production in HFF2 Cells under Heat Stress

After selecting the tested concentration of GO, the production of reactive oxygen species (ROS) in HFF2 cells under heat stress was measured, both with and without the presence of GO (100 µg/mL). The experimental conditions included the normal control group cultured at 37 °C and the high-temperature group cultured at 43 °C. The assessment, utilizing the DCFDA fluorescent dye and a cellular ROS assay kit, provided results for ROS measurement in HFF2 cells (Fig. 3). The green and blue colors in Fig. 3 represent the presence of ROS after DCFDA and DAPI (4′,6-diamidino-2-phenylindole) staining, respectively. Generally, the fluorescent intensity is proportional to the ROS level.

The fluorescence intensity in the high-temperature group (H) without GO was 2.8 times higher than that of the control group ($p < 0.01$), indicating an increase in ROS levels due to the heat treatment (Fig. 4). In the high-temperature group (H1) with GO (100 µg/mL), the fluorescence intensity decreased significantly ($p < 0.01$) compared to the H group, reaching levels similar to the control group.

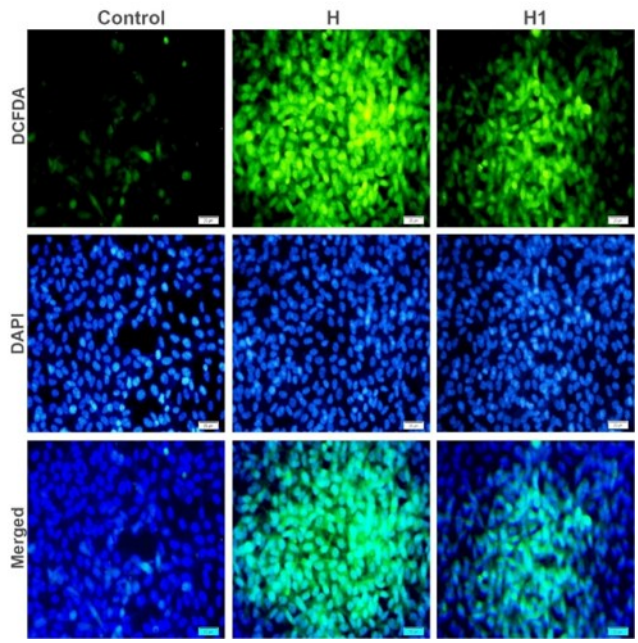

Fig. 3. Fluorometric images for the effects of GO on ROS production in HFF2 cells following the induction of heat stress for 12 h. From top to bottom, the cells are stained by DCFDA, DAPI, and merged images of the two methods, respectively. Control: the normal control group cultured at 37 °C, H: the high-temperature group cultured at 43 °C without GO, and H1: the high-temperature group cultured at 43 °C with GO

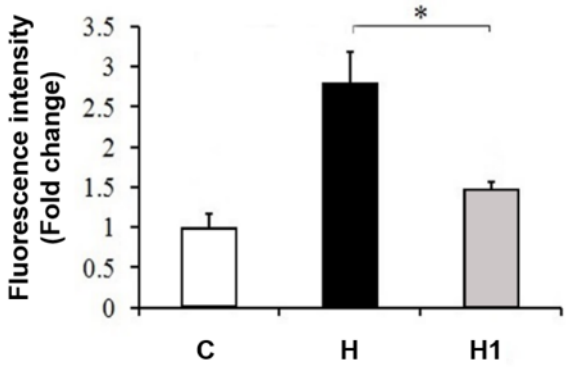

Fig. 4. The changes in fluorescence intensity for the effects of GO on ROS production in HFF2 cells following the induction of heat stress for 12 h. Control: the normal control group cultured at 37 °C, H: the high-temperature group cultured at 43 °C without GO, and H1: the high-temperature group cultured at 43 °C with GO

Effect of GO on GSH Level in HFF2 cells under Heat Stress

Glutathione (GSH) is a tripeptide, γ-L-glutamyl-L-cysteinylglycine, found in all mammalian tissues at concentrations ranging from 1 to 10 mM, with the highest concentration observed in the liver. It serves as the most abundant non-protein thiol, playing a crucial role in defending against oxidative stress (Lu 2013). Beyond its antioxidant function, GSH is a key player in redox signaling, critical for detoxification of xenobiotics, and it modulates cell proliferation, apoptosis, immune function, and fibrogenesis. The effect of GO on GSH synthesis following the induction of heat stress in skin fibroblasts was assessed to evaluate its antioxidant function. As depicted in Fig. 5, the GSH level exhibited a significant decrease in the high-temperature group (H) without GO

compared to the control group ($p < 0.01$). Conversely, in the high-temperature group (H1) with GO (100 μg/mL), the GSH level significantly increased compared to group H ($p < 0.05$), although it did not reach the level observed in the control group.

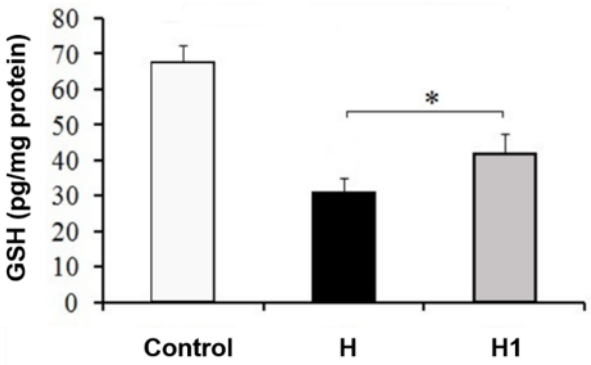

Fig. 5. The changes in intracellular GSH levels in HFF2 cells under heat stress with or without GO treatment (100 μg/mL). Control: the normal control group cultured at 37 °C, H: the high-temperature group cultured at 43 °C without GO, and H1: the high-temperature group cultured at 43 °C with GO. Data are presented as mean ± standard deviation from three independent experiments; *p < 0.01

Effect of GO on Nrf2 Expression in HFF2 Cells under Heat Stress

Reactive oxidants are intricately managed by a sophisticated network of antioxidant defense systems, orchestrated through a complex web of pathways to ensure a response tailored to the body's needs. A central aspect of oxidant signaling and antioxidant defense involves reactive cysteine thiol–based redox signaling.

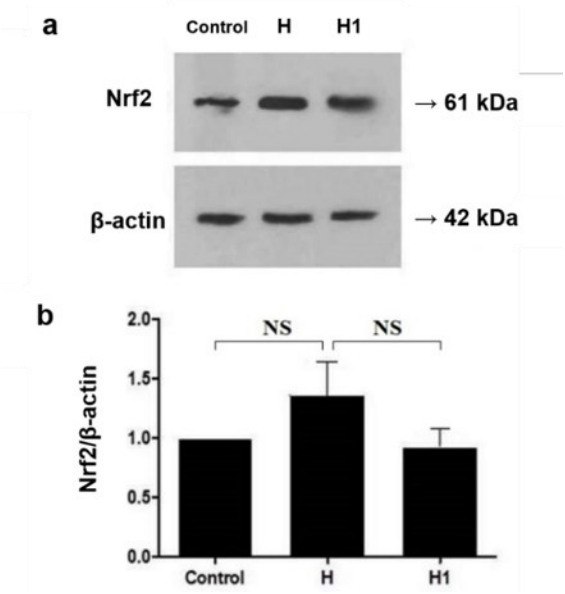

Fig. 6. The effects of GO on protein expression of Nrf2 in HFF2 cells following the induction of heat stress for 12 h. (A) The protein bands of Nrf2 and β-actin among different groups. (B) The band density of Nrf2 was normalized to that of β-actin. Control: the normal control group cultured at 37 °C, H: the high-temperature group cultured at 43 °C without GO, and H1: the high-temperature group cultured at 43 °C with GO. Data are presented as mean ± standard deviation from three independent experiments.

The nuclear factor erythroid 2–related factor 2 (Nrf2) emerges as a pivotal regulator of cellular resistance to oxidants. Nrf2 governs the basal and induced expression of an array of antioxidant response element–dependent genes, thereby influencing the physiological and pathophysiological outcomes of oxidant exposure (Zimta *et al.* 2019). The effect of GO on the protein expression of Nrf2 following the induction of heat stress in skin fibroblasts was assessed through Western blotting, utilizing β-actin as a control (Pickering *et al.* 2012). The expression of Nrf2 increased in the high-temperature group (H) without GO compared to the control group. Conversely, in the high-temperature group (H1) with GO (100 µg/mL), the expression of Nrf2 decreased compared to group H, nearly reaching the control level (Fig. 6).

During heat stress, various biochemical events alter the expression of genes and metabolic pathways, resulting in the production of free radicals (Kammeyer and Luiten 2015). Natural compounds, including melatonin (Zhao 2016) and curcumin (Kammeyer and Luiten 2015), have demonstrated protective effects against oxidative damage caused by heat stress due to their antioxidant properties. Additionally, rice bran extract has been shown to protect skin fibroblasts from aging by preventing the breakdown of collagen and hyaluronic acid (HA) (Binic *et al.* 2013; Menaa *et al.* 2014). The findings of the present study indicate that GO at a concentration of 100 µg/mL protects human skin fibroblasts against heat stress by reducing the production of free radicals, increasing glutathione production, and regulating the transcription factor Nrf2.

A study investigating the antioxidant effects of GO revealed that γ-oryzanol, at concentrations ranging from 1 to 20 µg/mL in the culture medium, exhibited no toxic effects on human embryonic stem cells (HEK-293) and provided protection against hydrogen peroxide (Rungratanawanich *et al.* 2018). Another evaluation focusing on the anti-aging effects of various compounds in rice bran oil found that GO, even at concentrations up to 100 µg/mL in the culture medium, had no toxic effects on human fibroblasts, and its IC50 exceeded 200 µg/mL (Yodkeeree *et al.* 2018). In the current study, treatment with 100 µg/mL of GO did not compromise the viability of human skin fibroblast cells. The GO demonstrated its potential to enhance cell survival through various mechanisms, including fortifying antioxidant responses, reducing oxidative stress, and inhibiting apoptosis (Rungratanawanich *et al.* 2018; Wang *et al.* 2020).

GO has been reported to exhibit antioxidant effects by directly eliminating free radicals or activating antioxidant pathways. The antioxidant properties of GO have been demonstrated in various living models of oxidative stress, including the *Drosophila melanogaster* model of Parkinson's (Araujo *et al.* 2015), diabetic rats (Ghatak and Panchal 2012), and a rat model of alcoholic liver injury (Chotimarkorn and Ushio 2008). Studies on NIH3T3 fibroblasts (mouse embryonic fibroblasts) (Islam *et al.* 2009), HEK-293 cells (human kidney embryonic cells) (Rungratanawanich *et al.* 2018), and B16F10 cells (mouse melanoma cells) (Zeinali *et al.* 2021) have shown that GO inhibits hydrogen peroxide-induced production of free radicals.

Heat stress of various durations has been shown to induce oxidative stress in cells. Storage of chicken embryonic fibroblast cells at 43 and 44 °C for 6 to 24 h increases ROS production and lipid peroxidation and decreases catalase activity (Ibtisham *et al.* 2018). Similarly, storage of bovine skin fibroblasts at 44 °C for 3 h (Singh *et al.* 2014), human skin fibroblasts (Hs68) at 43 °C for 30 min (Liu *et al.* 2015), and chicken fibroblasts at 43 °C for 12 h (Kammeyer and Luiten 2015) has been reported to increase the production of free radicals in cells. In the current study, exposure of human skin fibroblast cells to 43 °C for 12 h resulted in a more than 2.8-fold increase in cellular free radicals compared to the

control, consistent with previous reports on the ability of heat stress to induce oxidative stress. Natural compounds with antioxidant properties, such as aloin from *Aloe vera* and curcumin, have been shown to reduce free radicals and combat oxidative stress in response to heat stress in various cell types. Aloin exhibited antioxidant effects at concentrations of 150 and 300 μM, reducing lipid peroxidation and ROS production in human skin fibroblasts (Hs68) subjected to heat stress at 43 °C for 30 min (Liu *et al.* 2015). Similarly, curcumin, at concentrations of 40 and 20 μmol/L, was shown to reduce ROS production induced by heat stress (43 °C for 12 h) in chicken fibroblasts (Kammeyer and Luiten 2015). GO, in numerous studies, has demonstrated its ability to prevent oxidative damage by inhibiting free radical formation and lipid peroxidation. Oral administration of GO to diabetic rats reduced oxidative damage and lipid peroxidation in liver tissue (Ghatak and Panchal 2012). Additionally, GO reduced the production of free radicals in human renal embryonic cells (HEK-293) (Rungratanawanich *et al.* 2018) and inhibited ROS production while reducing lipid peroxidation in H2O2-treated human liver L02 cells (Huang *et al.* 2020). In the present study, the addition of 100 μg/mL of GO to human skin fibroblast cell culture medium effectively reduced ROS production. Investigations into the antioxidant properties of GO against various types of radicals *in vitro* have demonstrated its role as a free radicals scavenger, directly neutralizing them (Juliano *et al.* 2005; Saenjum *et al.* 2012). Moreover, GO counters the increase in free radicals under oxidative stress by enhancing the activity of antioxidant enzymes (Ghatak and Panchal 2012; Zhu *et al.* 2019; Huang *et al.* 2020).

Heat stress has been observed to compromise cellular antioxidant defense mechanisms by depleting cellular glutathione content. A recent study demonstrated that heat stress reduces glutathione levels in human skin fibroblasts (Hs68) (Liu *et al.* 2015). Consistent with these findings, the present study revealed that treatment of human skin fibroblast cells at 43 °C for 12 h led to a reduction in cellular glutathione content to less than half. Glutathione, a small peptide, plays a crucial role in the antioxidant system, and increasing its levels is known to protect cells against oxidative stress (Liu *et al.* 2020). Various studies have highlighted the potential of certain compounds to modulate glutathione levels and confer antioxidant protection. For instance, the addition of rice bran to the diet of mice for one week protected liver and testicular tissues against oxidative damage induced by carbon tetrachloride by increasing glutathione levels (GunaRti *et al.* 2021). Additionally, oral administration of GO to streptozotocin-induced diabetic rats increased glutathione levels in liver tissue (Ghatak and Panchal 2012). Earlier research has also suggested that increased non-protein thiol compounds mediate the protective effects of GO against cadmium-induced liver damage (Spiazzi *et al.* 2013). Another recent study demonstrated that GO increased glutathione content in the liver and reduced ethanol-induced oxidative stress in mice (Zhu *et al.* 2019). In line with these prior reports, the results of the present study showed a 1.35-fold increase in glutathione levels in human skin fibroblast cells treated with 100 μg/mL of GO under heat stress. This finding suggests that GO may enhance GSH synthesis or regeneration, or alternatively decrease its consumption by reducing the level of free radicals.

The antioxidant properties of GO are closely linked to its ability to regulate genes involved in redox homeostasis and cellular survival. The nuclear factor erythroid 2–related factor 2 (Nrf2) protein serves as a transcription factor, orchestrating the expression of genes that are crucial for the antioxidant system and cellular detoxification. Under oxidative stress, Nrf2 levels increase, prompting the activation of antioxidant genes upon translocation to the nucleus (Sies *et al.* 2017). In the current study, heat treatment at 43 °C

led to an upregulation of Nrf2 in human skin fibroblast cells, confirming the occurrence of oxidative stress induced by heat. Similar findings have been observed in response to H_2O_2-induced oxidative stress in human renal embryonic stem cells (HEK-293), where Nrf2 gene and protein expression were increased (Rungratanawanich *et al.* 2018). It is worth mentioning that in the present study, the addition of 100 μg/mL GO to the culture medium of HFF2 cells resulted in a reduction of Nrf2 expression of approximately 40%, bringing it close to the control level. This aligns with previous research demonstrating that GO treatment reduced Nrf2 expression in human kidney embryonic cells (HEK-293) exposed to hydrogen peroxide (Rungratanawanich *et al.* 2018). Under normal conditions, Kelch-like Nrf2-related protein 1 (Keap1) binds to Nrf2, leading to ubiquitination, degradation of Nrf2, and suppression of its transcriptional activity (Sies *et al.* 2017). However, under oxidative stress, Keap1 loses its binding capacity to Nrf2 due to the oxidation of its cysteine residues. The observed decrease in Nrf2 expression in the presence of GO (H1 group) may be attributed to the antioxidant effects of GO. In the presence of GO, the reduction in free radicals allows more Keap1 to bind to Nrf2. Moreover, GO itself has been shown to have inductive effects on Nrf2 (Rungratanawanich *et al.* 2018). The hydroxyl groups in the phenolic ring of GO can transform into phenoxyl radicals in the presence of free radicals, potentially participating in the oxidation of thiol groups in Keap1 and facilitating its separation from Nrf2. The observed higher expression of Nrf2 in the H1 group compared to the control group can be attributed to the induction effects of GO on Nrf2. Although Nrf2 is a cellular protective factor, its overactivity can be harmful, for example, by increasing the survival of cancer cells and their resistance to chemotherapy (Sies *et al.* 2017; Tsuji *et al.* 2019).

CONCLUSIONS

1. Heat stress induces oxidative stress through various biochemical mechanisms, altering metabolic pathways, increasing free radical formation in cells, and disrupting redox balance. In the current study, heat stress in HFF2 cells resulted in reduced glutathione (GSH) levels and increased reactive oxygen species (ROS) production, indicative of oxidative stress.
2. Notably, the addition of 100 μg/mL of γ-oryzanol (GO) to the culture medium of human skin fibroblasts mitigated ROS production, elevated GSH levels, and modulated the activity of the transcription factor Nrf2. These findings highlight the potential of GO as an effective compound for protecting skin fibroblasts against oxidative damage induced by heat stress.
3. Despite these promising results, this study was limited to *in vitro* experiments. Future research should focus on investigating the protective effects of GO in *in vivo* models and clinical applications. Further exploration of the molecular mechanisms underlying GO's antioxidant properties could also provide valuable insights into its broader therapeutic potential in dermatology and oxidative stress-related conditions.

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Conflict of Interest

No potential conflict of interest was reported by the authors.

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