BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 33(4), 716-727 (2025)

Albiflorin, a Monoterpene Glycoside, Protects Myoblasts against Hydrogen Peroxide-Induced Apoptosis by Activating the Nrf2/HO-1 Axis

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Abstract

Albiflorin, a key active compound in the roots of *Paeonia lactiflora* Pall, is known to have multiple health benefits. Although albiflorin has been shown to exert its major pharmacological effects via its antioxidant activity, its efficacy in the muscles has not been evaluated. In this study, we examined the protective activity of albiflorin against oxidative injury in C2C12 murine myoblasts. C2C12 cells were pretreated with nontoxic concentrations of albiflorin and exposed to hydrogen peroxide (H_2O_2) to mimic oxidative stress. Albiflorin pretreatment inhibited H_2O_2-mediated decrease in cell viability and extracellular release of lactate dehydrogenase, and reduced comet tail formation, 8-hydroxy-2'-deoxyguanosine production, and phosphorylated form of histone 2AX expression, which are representative biomarkers of DNA damage. Albiflorin also blocked H_2O_2-induced apoptosis by inhibiting the activation of caspase-3, which is associated with the maintenance of mitochondrial membrane stability by decreasing the Bax/Bcl-2 expression ratio. Additionally, albiflorin markedly suppressed H_2O_2-induced accumulation of reactive oxygen species (ROS) and decreased glutathione levels, while increasing the phosphorylation of nuclear factor erythroid 2-related factor 2 (Nrf2) and activating heme oxygenase-1 (HO-1) in the presence of H_2O_2. However, artificial inhibition of HO-1 activity using zinc protoporphyrin (ZnPP) markedly abrogated the protective effects of albiflorin against ROS production and mitochondrial damage in H_2O_2-treated cells. ZnPP significantly reversed the protective effects of albiflorin against H_2O_2-induced apoptosis and decreased cell viability. Taken together, these findings suggest that albiflorin protects myoblasts from oxidative stress-induced DNA damage and apoptosis by activating Nrf2/HO-1 signaling, thus highlighting its potential in the management of myofunctional homeostasis.

Key Words: Albiflorin, C2C12 myoblasts, Oxidative stress, Apoptosis, Nrf2/HO-1

INTRODUCTION

Albiflorin is a monoterpene glycoside and is the main active compound found in Paeoniae Radix, the roots of *Paeonia lactiflora* Pall. An herb used in many traditional medicine prescriptions in East Asia, including Korea, to treat dementia, inflammation, pain disorders, and traumatic injuries (Jiang *et al*., 2020; Ma *et al*., 2024; Xu *et al*., 2024). Recent studies

Open Access **https://doi.org/10.4062/biomolther.2025.020**

Received Feb 7, 2025 **Revised** Mar 25, 2025 **Accepted** May 1, 2025
Published Online Jun 10, 2025

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have shown that albiflorin is effective in treating numerous diseases because of its various pharmacological properties, including neuroprotective, anti-inflammatory, hepatoprotective, anti-osteoarthritis, and antidiabetic effects, without any side effects (Yang and Yang, 2023; Zhou *et al*., 2023; Li *et al*., 2024a; Lu *et al*., 2024; Ou *et al*., 2024; Song *et al*., 2024). These beneficial effects are closely related to the inhibition of oxidative stress by targeting excess reactive oxygen species (ROS) production. For example, albiflorin has been reported to reduce or prevent osteoblast degeneration in osteoporosis by decreasing ROS production through improved mitochondrial function to suppress oxidative damage (Suh *et al*., 2013). Albiflorin also reduced β-amyloid (Aβ)-induced toxicity and ROS generation in rat primary hippocampal cells (Ho *et al*., 2015), suppressed Aβ levels in the brain of mice, and inhibited ROS-mediated mitochondrial apoptotic pathway while preserving manganese superoxide dismutase (Mn-SOD) activity (Xu *et al*., 2019). In addition, albiflorin increased the survival of lipopolysaccharide-inhibited mouse primary hepatocytes, and reduced hepatocyte apoptosis and expression of inflammatory markers in a mouse sepsis model, which were associated with the inhibition of ROS production through increased SOD activity and decreased levels of malondialdehyde (MDA), a marker of oxidative stress (Liu *et al*., 2024). Another interesting result showed that Jakyakgamcho-Tang, a traditional medicine prescription, effectively inhibited hydrogen peroxide (H_2O_2)-induced apoptosis and intracellular ROS production in myoblasts, and one of the bioactive components contained in this prescription was albiflorin (Kim *et al*., 2021).

Recent findings indicate that albiflorin effectively inhibits nuclear factor-kB ligand-induced osteoclast differentiation by inhibiting intracellular ROS and mitochondrial ROS (mtROS) production (Wang *et al*., 2024). The blockade of ROS production occurred via increased expression of nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent antioxidant enzymes, including heme oxygenase 1 (HO-1). Consistent with these results, albiflorin exhibits anti-inflammatory effects by reducing ROS production through modulation of the Nrf2 pathway and increased cell viability in gingival fibroblasts exposed to advanced glycation end products (Gao *et al*., 2024). In addition, the paper reported the involvement of Nrf2, a transcription factor that regulates cellular defense against oxidative damage, in the antioxidant activity of albiflorin by demonstrating that Nrf2 activation-mediated induction of glutathione (GSH) synthesis is involved in the therapeutic effect of albiflorin on hepatitis with severe cholestasis (Ma *et al*., 2015). Similarly, albiflorin alleviates cerebral ischemia-reperfusion and spinal cord injury by activating Nrf2-mediated HO-1 (Fang *et al*., 2023; Zhu *et al*., 2023), suggesting that the activation of Nrf2 is crucial for the antioxidant activity of albiflorin. Albiflorin also shows potent antioxidant activity in muscle cells. Although research on albiflorin in muscle cells is limited, its potential therapeutic effects in muscle-related disorders warrant further investigation. Sarcopenia and degenerative muscle diseases are closely associated with increased oxidative stress and mitochondrial dysfunction (Zhang *et al*., 2020; Luo *et al*., 2025). Given that albiflorin exhibits strong antioxidant and anti-inflammatory properties, it may help alleviate these pathological features, thereby improving muscle function and preventing disease progression. A recent study reported that albiflorin suppressed oxidative stress induced by excessive exercise by reducing MDA production in myocardial tissues while enhancing the levels of GSH and SOD. In addition, the protective effect of albiflorin against myocardial injury was at least partially mediated by Nrf2/HO-1 signaling to limit ROS production (Tian and Li, 2024). Skeletal muscles consume a lot of energy during contraction, and oxidant species, such as ROS, are continuously generated during this process owing to high oxygen consumption. Oxidative stress plays a key role in the disruption of muscle homeostasis by inhibiting skeletal muscle differentiation and increasing muscle loss (Lian *et al*., 2022; Zhang *et al*., 2023). Despite increasing evidence that Nrf2/HO-1 signaling may be a potential therapeutic target in albiflorin-mediated attenuation of oxidative stress, the involvement of this signaling pathway in the alleviation of oxidative stress-induced skeletal muscle damage has not yet been adequately evaluated. Therefore, in this study, we aimed to establish the role of Nrf2/HO-1 signaling in albiflorin-mediated protection of skeletal muscle cells from oxidative stress. To this end, we used C2C12 mouse myoblasts and treated them with hydrogen peroxide (H_2O_2) to mimic an oxidative stress environment.

MATERIALS AND METHODS

Cell culture and albiflorin treatment

C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were cultured according to the method described in a previous study (Haddish and Yun, 2024). Albiflorin (Sigma-Aldrich Co., St. Louis, MO, USA) was solubilized in dimethyl sulfoxide (Sigma-Aldrich Co.) to prepare a stock solution (100 mM), which was then diluted to various concentrations with culture medium before treating the cells.

Cytotoxicity assay

Cells were cultured in medium containing H_2O_2 (Thermo Fisher Scientific, Waltham, MA, USA) or albiflorin at different concentrations for 48 h; pretreated with or without albiflorin, N-acetyl-L-cysteine (NAC, Sigma-Aldrich Co.), or zinc protoporphyrin (ZnPP, Sigma-Aldrich Co.) for 1 h, and then treated with H_2O_2 for 48 h. After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Co.) assay was performed according to a previously described method (Kang *et al*., 2024b). The lactate dehydrogenase (LDH) release assay was performed using an LDH Activity Assay Kit (Sigma-Aldrich Co.) according to the manufacturer's protocol.

Quantitative assessment of apoptosis

To quantitatively assess the extent of apoptotic induction, an Annexin V/Propidium iodide (PI) Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) was used. Briefly, Annexin V-FITC and PI buffer were added to cells suspended in binding buffer and allowed to react for 20 min, as per the manufacturer's protocol (Jeon *et al*., 2024b). The cell suspension was then analyzed using a flow cytometer (Millipore Corporation, Hayward, CA, USA).

Immunoblotting

Whole cell lysates were prepared from cells cultured under various conditions as previously described (Kang *et al*., 2024a). Mitochondrial and cytoplasmic fractions were isolated using a Mitochondria/Cytosol Fractionation Kit (Sigma-Aldrich Co.). Equal amounts of protein extracted from the cells in each

treatment group were fractionated by electrophoresis using sodium dodecyl sulfate-polyacrylamide gels, and transferred to immunoblot membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were hybridized with primary antibodies against the target proteins and then incubated with secondary antibodies conjugated to horseradish peroxidase. Antibodies against the target proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technology (Danvers, MA, USA), and Abcam Inc. (Cambridge, MA, UK). Proteins bands were detected using an Enhanced Chemiluminescence Detection Kit (Sigma-Aldrich Co.). β-actin was used as a loading control.

Caspase-3 activity

Caspase-3 is a key executioner of apoptosis, and its activation serves as a reliable marker of cell death. Measuring caspase-3 activity allowed us to assess the protective effects of albiflorin against oxidative stress-induced apoptosis. The activity of caspase-3 was measured using a Caspase-3 Assay Kit (Abcam, Inc.), which is based on the hydrolysis of fluorescent substrate peptides by activated caspase-3. Briefly, after resuspending the cells in the cell lysis buffer provided in the kit, the supernatants were reacted with the substrate according to the manufacturer's instructions. Finally, the concentration of *p*-nitroaniline released from the substrates was determined using a microplate reader (VERSA Max, Co., Sunnyvale, CA, USA) (Park *et al*., 2024).

Comet assay

To assess DNA damage, a comet assay was performed using a comet Assay Kit (Trevigen, Gaithersburg, MD, USA). Briefly, cells treated with H_2O_2 in the presence or absence of albiflorin were collected and the comet assay was performed according to the manufacturer's protocol. Random images were acquired using a fluorescence microscope (Carl Zeiss; Oberkochen, Germany).

Detection of phosphorylated form of histone 2AX (γH2AX) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels

To analyze the expression of γH2AX, a marker of double-strand breaks (DSBs) in DNA, immunostaining was performed as previously described (Hwangbo *et al*., 2024). Briefly, cells were labeled with antibodies against mouse anti-γH2AX (serine 139, Abcam Inc.) followed by Alexa Fluor™ goat anti-mouse IgG (Thermo Fisher Scientific). In addition, 4′,6′-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) staining was performed to counterstain intracellular DNA, and the cells were observed under a fluorescence microscope. The levels of 8-OHdG, an oxidized nucleoside form of DNA, were determined using an 8-OHdG ELISA kit (Abcam Inc.). The cells were mixed with the reaction buffer and then reacted with the 8-OHdG antibody provided in the kit according to the manufacturer's instructions. The cells were then washed with washing buffer and the absorbance was read using an ELISA reader (BioTek, Winooski, VT, USA) at 405 nm, as previously reported (Hwangbo *et al*., 2024).

ROS generation

To evaluate the antioxidant activity of albiflorin, intracellular ROS levels were analyzed using 2',7'-dichlorofluorescein diacetate (DCF-DA) staining. In brief, harvested cells were incubated with 10 μM DCF-DA solution (Thermo Fisher Scientific), and ROS levels were measured using flow cytometry (Jeon *et al*., 2024a). Cells stained with DCF-DA were observed under a fluorescence microscope to assess ROS production.

GSH/oxidized glutathione (GSSG) ratio

The antioxidant capacity of albiflorin was assayed using a GSH Assay Kit (Sigma-Aldrich Co.). Briefly, after reacting the cells under the conditions suggested by the manufacturer, the GSH/GSSG ratio was calculated using a standard curve of GSH and GSSG.

HO-1 activity

HO-1 activity was assayed using an HO-1 ELISA Kit (Abcam Inc.). In brief, after reacting the cells under the conditions suggested by the manufacturer, HO-1 activity was expressed as picomoles of bilirubin formed per milligram of cell lysate extracted per hour, using the difference in absorbance between 464 and 530 nm.

Mitochondrial membrane potential (MMP) assay

To analyze MMP, an indicator of mitochondrial membrane stability, 5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi-dazoylcarbocyanine iodide (JC-1) dye was used. JC-1 is a mitochondrial membrane potential indicator that reflects mitochondrial health and function. A shift from red to green fluorescence indicates mitochondrial depolarization, which is a hallmark of mitochondrial dysfunction. The collected cells were stained with 10 μM JC-1 (Abcam Inc.) for 30 min at room temperature. The frequency of JC-1 aggregates and monomers was immediately monitored using flow cytometry, as previously described (Ni *et al*., 2024).

Statistical analyses

All data were analyzed using GraphPad Prism 5.03 software (GraphPad Software Inc., La Jolla, CA, USA) using an unpaired two-tailed Student's *t*-test and one-way analysis of variance. All results are presented as the mean ± standard deviation (SD) of at least triplicate independent experiments. Statistical significance was set at $p<0.05$.

RESULTS

Albiflorin attenuated H_2O_2-induced cytotoxicity in C2C12 cells

The MTT assay revealed that H_2O_2 inhibited viability of C2C12 cells in a concentration-dependent manner, and albiflorin significantly inhibited cell viability starting at 100 μM concentration (Fig. 1A, 1B). Therefore, a concentration of 600 μM H_2O_2, which resulted in approximately 53% cell viability in the MTT assay, was used to mimic oxidative stress, whereas 50 μM (or lower) of albiflorin was used for pretreatment of cells. Next, we investigated whether albiflorin inhibits H_2O_2-induced cytotoxicity in C2C12 cells. We found that pretreatment of cells with albiflorin alleviated the H_2O_2-mediated decrease in cell viability in a concentration-dependent manner (Fig. 1C). Albiflorin also significantly blocked LDH leakage in H_2O_2-treated cells (Fig. 1D). In addition, NAC, a ROS scavenger, markedly blocked the H_2O_2-mediated cytotoxicity; when cells were simultaneously pretreated with albiflorin and NAC prior to H_2O_2 treatment, cell viability remained at the control level,

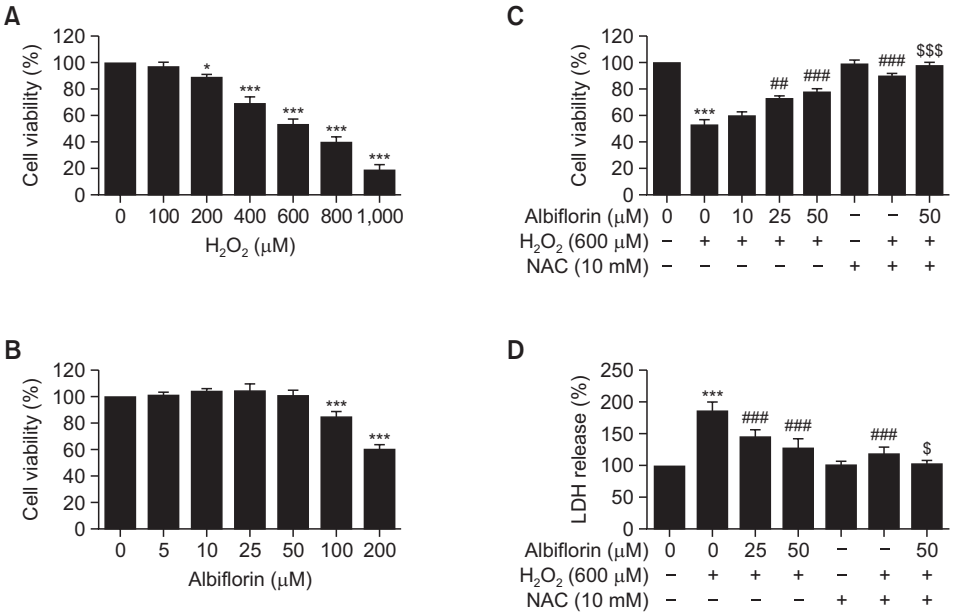

Fig. 1. Inhibition of H_2O_2-induced cytotoxicity by albiflorin in C2C12 cells. Cells were treated with various concentrations of H_2O_2 (A) or albiflorin (B) for 48 h or treated with the indicated concentration of albiflorin or NAC for 1 h, and then treated with H_2O_2 for 48 h (C, D). (A-C) Cell viability was analyzed by MTT assay (n=3), and the relative level of LDH released (D) was analyzed using a LDH Activity Assay Kit (n=3). *p<0.05 and ***p<0.001 *vs* control cells; $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 *vs* H_2O_2-treated cells; $^{\$}p$<0.05 and $^{\$\$\$}p$<0.001 *vs* albiflorin (50 μM) and H_2O_2-treated cells. H_2O_2, hydrogen peroxide; NAC, N-acetyl-L-cysteine; LDH, lactate dehydrogenase.

and LDH leakage was completely blocked (Fig. 1). These data demonstrated that albiflorin attenuates H_2O_2-induced cytotoxicity in C2C12 cells.

Albiflorin reduced H_2O_2-induced apoptosis

To determine whether albiflorin inhibits H_2O_2-induced cytotoxicity via inhibition of apoptosis, flow cytometric analysis was performed following Annexin V/PI staining. As shown in Fig. 2A and 2B, the frequency of Annexin-positive cells, indicating apoptosis, was significantly increased in H_2O_2-treated cells. However, this frequency was significantly reduced in cells pretreated with albiflorin. The expression of Bax protein, a pro-apoptotic protein, which increased following H_2O_2 treatment, and that of Bcl-2, an anti-apoptotic protein, which decreased following H_2O_2 treatment, were maintained at the control level after albiflorin pretreatment (Fig. 2C, 2D). In addition, the expression and enzymatic activity of the active form of caspase-3, a key effector of caspase-dependent apoptosis, and poly(ADP-ribose) polymerase (PARP, a caspase-3 substrate) cleavage, were upregulated in H_2O_2-treated cells (Fig. 2C-2E). However, neither caspase-3 activation nor PARP cleavage was observed in the presence of albiflorin. These results indicated that albiflorin attenuates apoptosis in the cells by inhibiting an increase in Bax/Bcl-2 expression ratio and inactivation of the caspase cascade caused by H_2O_2 stimulation.

Albiflorin alleviated H_2O_2-induced DNA damage

To evaluate whether the reduction in H_2O_2-induced cytotoxicity by albiflorin was associated with the inhibition of DNA damage, we performed comet assays and analyzed changes in 8-OHdG levels and γH2AX expression. As shown in Fig. 3A and 3B, the formation of comet tails, which indicates DSBs in DNA, and the level of 8-OHdG, a marker of oxidative DNA damage, were markedly increased in H_2O_2-treated cells. In addition, the expression of the γH2AX, whose phosphorylation increases when DSB in DNA occur, was also increased following H_2O_2 treatment (Fig. 3C-3E). However, the increase in the expression of these DNA damage markers was largely abrogated in the presence of albiflorin, indicating that albiflorin significantly blocked DNA damage caused by oxidative stress.

Albiflorin mitigated ROS production and activates Nrf2/HO-1 signaling in H_2O_2-treated cells

To determine whether the protective effect of albiflorin against H_2O_2-induced cytotoxicity was mediated through its antioxidant activity, we evaluated whether albiflorin prevents H_2O_2-induced increase in ROS production. DCF-DA staining of cells followed by flow cytometric analysis showed that the increased intracellular ROS levels in H_2O_2-treated C2C12 cells were significantly restored by albiflorin pretreatment (Fig. 4A, 4B), and similar results were observed by fluorescence microscopy (Fig. 4C). In addition, H_2O_2-exposed cells showed a decrease in GSH/GSSG ratio, a marker of oxidative stress, that was significantly ameliorated in the NAC and albiflorin pretreated groups (Fig. 4D). To investigate whether Nrf2/HO-1 signaling is involved in the ROS-scavenging effect of albiflorin, changes in Nrf2 and HO-1 expression were examined. Immunoblotting results showed a slight increase in the expression of phosphorylated Nrf2 (p-Nrf2), which indicates Nrf2 activation, in cells treated with albiflorin or H_2O_2 alone, and further increase in cells co-treated with albiflorin and H_2O_2 while the total protein levels remained unchanged (Fig. 4E, 4F). Concomitantly, the expression and activity of HO-1 increased, consistent with the change in the expression of Nrf2, whereas that of Kelch-like ECH-associated protein 1 (Keap1), a negative regulator of Nrf2, decreased (Fig. 4E-4G). These results

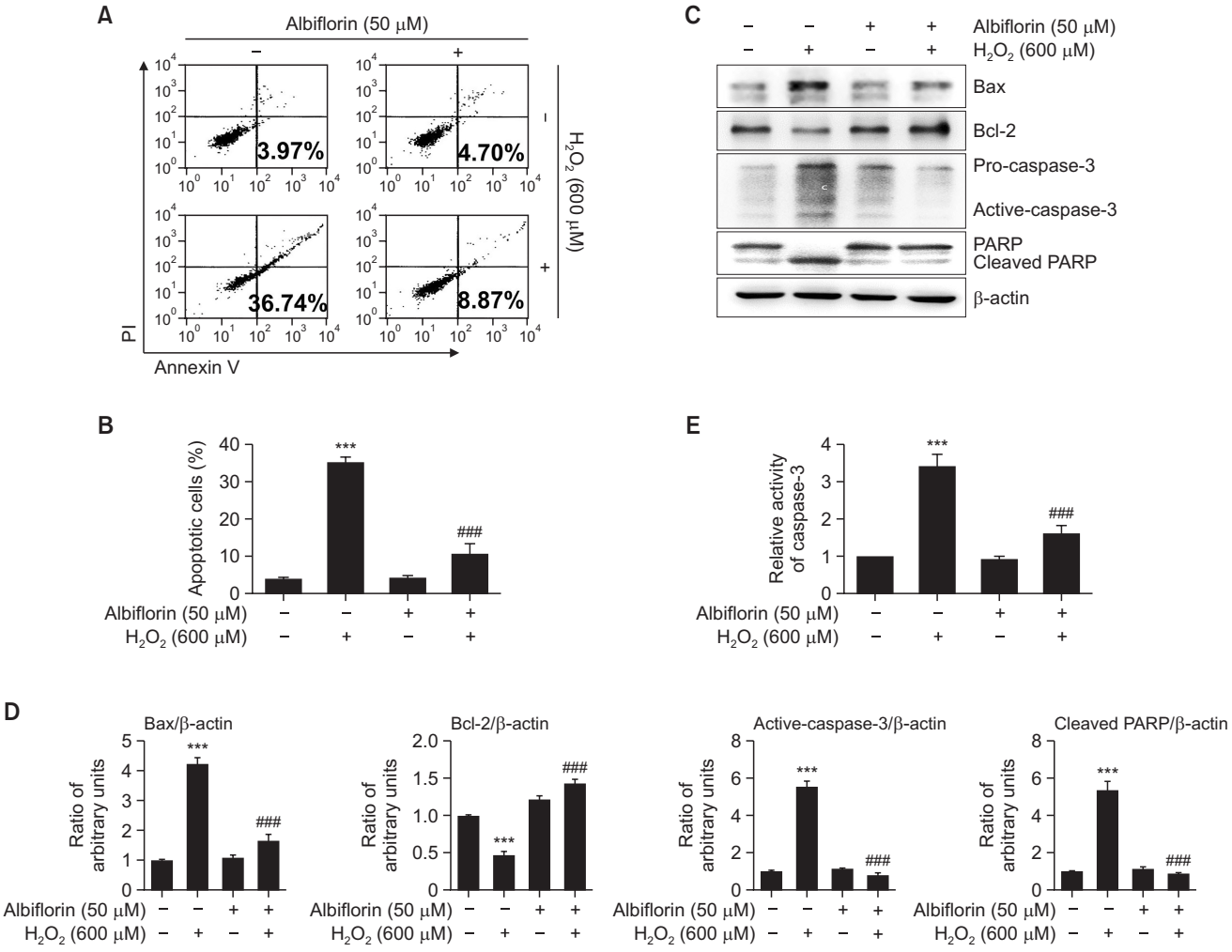

Fig. 2. Attenuation of H_2O_2-induced apoptosis by albiflorin in C2C12 cells. Cells were pretreated with or without 50 μM albiflorin for 1 h and then stimulated with 600 μM H_2O_2 for 48 h. (A, B) Apoptosis was analyzed by flow cytometry following Annexin V/PI staining, and the frequency of Annexin V-positive cells was shown (n=3). (C) Immunoblotting and (D) statistical analysis of the protein expression levels of key apoptosis-related regulators. (E) Caspase-3 activity was measured using a commercially available kit (n=3). ***p<0.001 *vs* control cells; ###p<0.001 *vs* H_2O_2-treated cells. H_2O_2, hydrogen peroxide; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase.

suggest that the antioxidant activity of albiflorin is associated with the activation of Nrf2/HO-1 signaling.

Inhibition of HO-1 activity abrogated albiflorin-mediated antioxidant activity and preservation of mitochondrial function in H_2O_2-treated C2C12 cells

Because the expression and activity of HO-1 increased when albiflorin was exposed to oxidative stimuli, we investigated whether the Nrf2-mediated increase in HO-1 activity plays a role in the antioxidant activity of albiflorin. Analysis of ROS production showed that inhibition of H_2O_2-induced ROS accumulation by albiflorin was significantly reduced in the presence of ZnPP, a pharmacological inhibitor of HO-1 (Fig. 5A, 5B). However, this counteracting effect of ZnPP was abrogated in the presence of NAC. To examine whether albiflorin-mediated protection against H_2O_2-induced cytotoxicity was because of the maintenance of mitochondrial homeostasis, we analyzed the changes in MMP, an indicator of mitochondrial stability. Flow cytometry results revealed an increase in the proportion of JC-1 monomers in H_2O_2-treated cells (Fig. 5C, 5D), indicating loss of MMP owing to mitochondrial depolarization, which was counteracted by pretreatment with albiflorin. However, the protective effect of albiflorin against H_2O_2-induced loss of mitochondrial membrane stability was abrogated by ZnPP treatment. These results indicate that mitochondrial damage in an oxidative environment is prevented by albiflorin via activation of HO-1.

Inhibition of HO-1 activity abrogated the protective effect of albiflorin against H_2O_2-induced cytotoxicity

Because the albiflorin-mediated antioxidant and mitochondrial protective effects were attenuated by the inactivation of HO-1 in H_2O_2-treated cells, we further analyzed the effects of HO-1 inactivation on albiflorin-mediated anti-cytotoxic activity. As shown in Fig. 6A, the ameliorating effect of albiflorin on H_2O_2-induced decrease in cell viability of C2C12 cells was abrogated in the presence of ZnPP. Additionally, the neutralizing effect of albiflorin on H_2O_2-induced apoptosis was abrogated

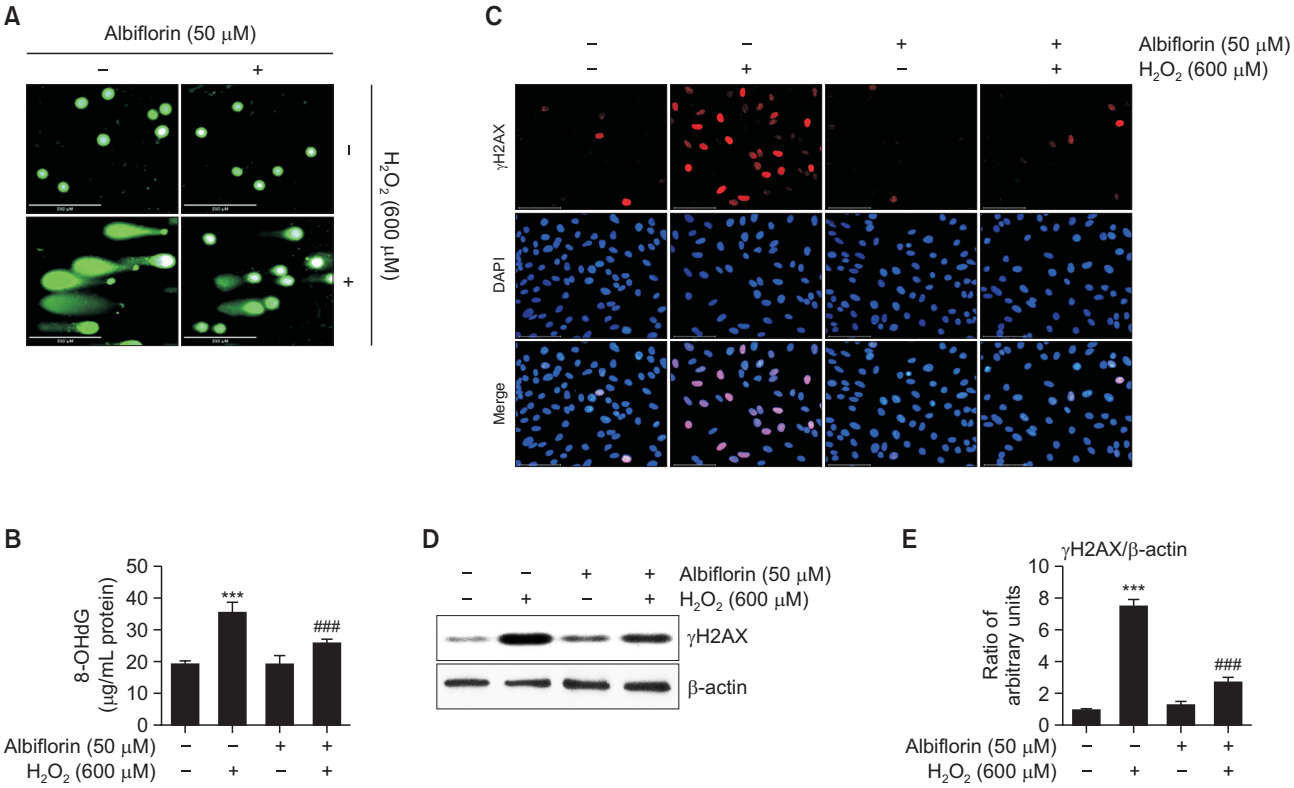

Fig. 3. Amelioration of H_2O_2-induced DNA damage by albiflorin in C2C12 cells. Cells were pretreated with or without 50 μM albiflorin for 1 h and then stimulated with 600 μM H_2O_2 for 48 h. (A) DNA damage was assessed by comet assay or (B) changes in 8-OHdG levels (n=3). ***p<0.001 *vs* control cells; $^{###}p$<0.001 *vs* H_2O_2-treated cells. (C) Changes in γH2AX expression were analyzed by immunostaining. (D) Immunoblotting and (E) statistical analysis of the protein expression levels of γH2AX. H_2O_2, hydrogen peroxide; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; γH2AX, phosphorylated form of histone 2AX; DAPI, 4′,6′-diamidino-2-phenylindole.

in the presence of ZnPP. by blocking caspase-3 activity (Fig. 6B-6D). These results demonstrate that the inhibitory potential of albiflorin against oxidative damage in C2C12 cells occurs via Nrf2-mediated HO-1 activation.

DISCUSSION

Oxidative stress, resulting from an imbalance between ROS production and the ability of cells to neutralize these reactive molecules, damages essential cellular components such as proteins, lipids, and DNA (Hajam *et al*., 2022; Averill-Bates, 2024). In skeletal muscles, ROS also induce adaptive mechanisms that improve muscle function and recovery; however, excessive ROS production causes cell damage and contributes to the development of muscle-related diseases. In addition, oxidative stress triggers muscular atrophy and inhibits skeletal muscle differentiation, contributing to muscle fatigue and aging, and decrease in muscle mass and strength (Foreman *et al*., 2021; Agrawal *et al*., 2023). In this study, we investigated whether albiflorin protects muscle cells against oxidative damage using myoblasts, the precursor cells that differentiate into myotubes. First, we performed the MTT assay to measure *in vitro* cell viability. As the MTT assay reflects intracellular mitochondrial activity in terms of the number of viable cells (Plumb, 2004), our finding that albiflorin restores H_2O_2-mediated decrease in cell viability may be associated with the maintenance of mitochondrial homeostasis. In addition, the increase in LDH, a cytosolic enzyme found culture supernatants, indicated that the cell membranes were damaged and that the cells were undergoing necrosis, apoptosis, or other forms of cell death (Kaja *et al*., 2017). The finding that albiflorin reduced H_2O_2-induced release of extracellular LDH provides evidence that it inhibits cell death. Overall, these results imply that albiflorin-mediated inhibition of apoptosis in C2C12 myoblasts induced by oxidative stimuli such as H_2O_2 occurs through preservation of mitochondrial function.

Most cellular injuries induced by oxidative stimuli are accompanied by DNA damage, and unrepaired DNA damage contributes to cell transformation and apoptosis (Wang *et al*., 2021; Li *et al*., 2024b). In this study, we investigated the effect of albiflorin on oxidative stress-induced DNA damage and found that DNA damage indicators such as comet tail formation, γH2AX expression, and 8-OHdG levels were increased in H_2O_2-treated C2C12 cells and attenuated in the presence of albiflorin. Among these DNA damage indicators, increase in 8-OHdG level is a strong indicator of oxidative DNA damage (Valavanidis *et al*., 2009). The comet assay and phosphorylation of γH2AX are commonly used to evaluate the presence of DNA strand breaks (Kopp *et al*., 2019; Cordelli *et al*., 2021). Because comet tail formation and increased expression of γH2AX are also used as indicators of apoptosis, our results demonstrate that blocking DNA damage with albiflorin blocks oxidative stress-induced apoptosis. Subsequent

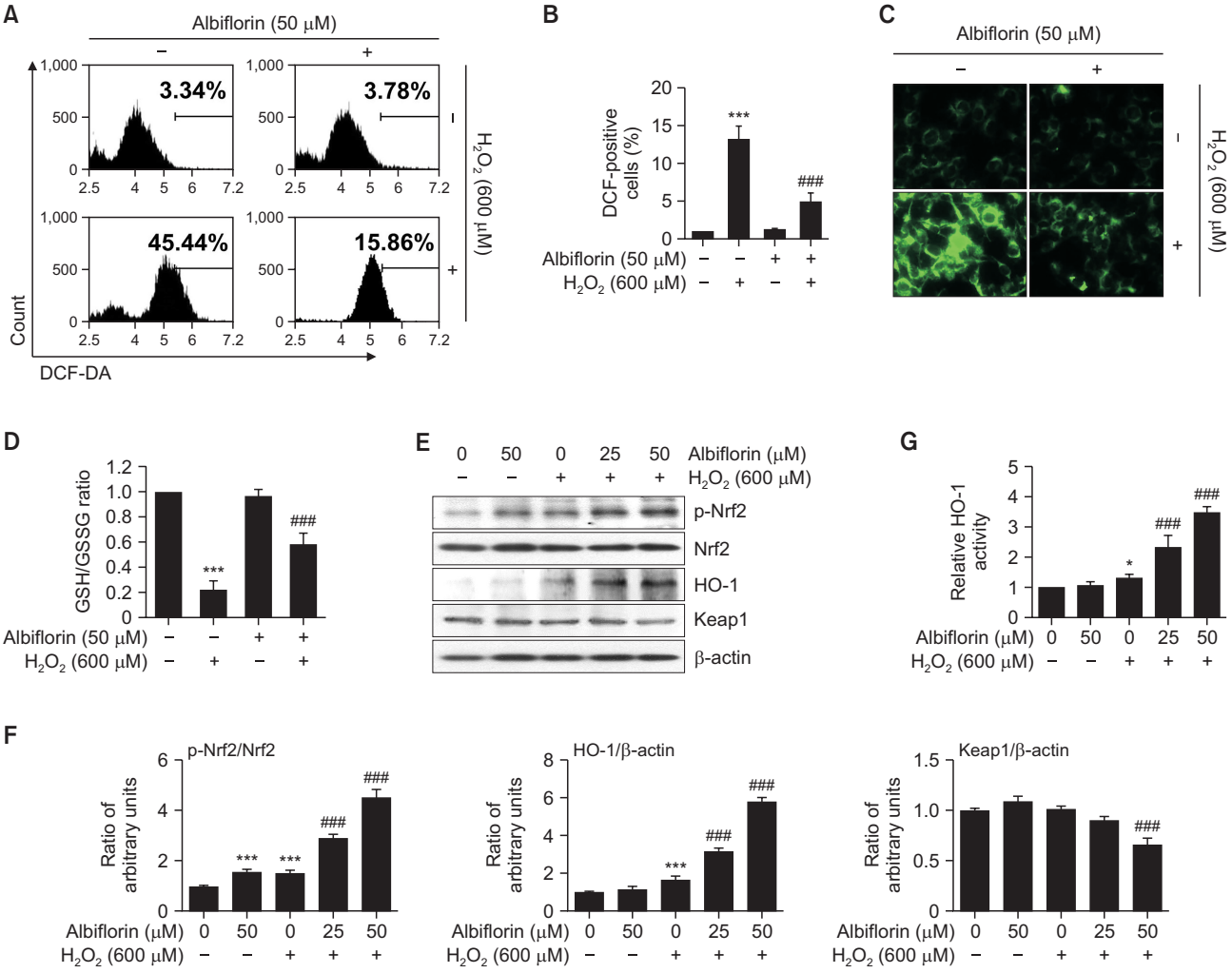

Fig. 4. Elimination of ROS production and activation of Nrf2-mediated HO-1 by albiflorin in H_2O_2-treated C2C12 cells. Cells were pretreated with or without albiflorin for 1 h and then stimulated with H_2O_2 for 1 h (A-C) or 48 h (D-F). (A) Changes in intracellular ROS levels were analyzed by flow cytometry after DCF-DA staining, (B) and the frequency of DCF-positive cells is presented (n=3). (C) Cells were stained with DCF-DA, and the fluorescence intensity (representing ROS production) was imaged under a fluorescence microscope. (D) Intracellular GSH/GSSG ratio was measured using a GSH Assay Kit (n=3). (E) Immunoblotting and (F) statistical analysis of the protein expression levels of p-Nrf2, Nrf2, HO-1, and Keap1. (G) Enzyme activity of HO-1 was measured using the corresponding assay kit (n=3). *p<0.05 and ***p<0.001 *vs* control cells; $^{\#\#\#}p$<0.001 *vs* H_2O_2-treated cells. H_2O_2, hydrogen peroxide; DCF-DA, 2',7'-dichlorofluorescein diacetate; GSH, glutathione; GSSG, oxidized glutathione; p-Nrf2, phosphorylated nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; Keap1, Kelch-like ECH-associated protein 1; ROS, reactive oxygen species.

experiments showed that albiflorin blocks the mitochondria-mediated apoptotic pathway in H_2O_2-treated C2C12 cells by neutralizing the increased Bax/Bcl-2 expression ratio, MMP loss, caspase-3 activation, and PARP cleavage. Albiflorin also significantly inhibited ROS production induced by H_2O_2 treatment, and restored GSH levels. These results suggest that albiflorin significantly protected C2C12 myoblasts from oxidative stress-mediated apoptosis, which also correlated with improved mitochondrial function, highlighting the importance of mitochondrial stability through ROS scavenging in albiflorin-mediated attenuation of oxidative stress-induced cytotoxicity.

Our results are similar to those of previous studies, which showed that the inhibition of ROS accumulation by albiflorin occurs via Nrf2-mediated HO-1 activation (Ma *et al*., 2015; Fang *et al*., 2023; Zhu *et al*., 2023; Ou *et al*., 2024; Tian and Li, 2024). Nrf2, a key redox-sensitive transcription factor, exists in the cytoplasm in a Keap1-bound form under physiological conditions, and is degraded via the ubiquitin-proteasome pathway (Saha *et al*., 2020; Ngo *et al*., 2023). However, when cells are exposed to oxidative stress or Nrf2 inducers, phosphorylation of Nrf2 liberates it from Keap1. The phosphorylated Nrf1 then enters the nucleus and transcriptionally activates antioxidant genes, including HO-1 (Liu *et al*., 2021; Ngo *et al*., 2023). Our results showed that the expression and activity of HO-1 were significantly enhanced in cells treated with H_2O_2 in the presence of albiflorin compared to those treated with albiflorin or H_2O_2 alone, which was associated with the phosphorylation of Nrf2 and downregulation of Keap1 protein, suggesting that albiflorin enhanced the activation of the Nrf2/HO-1 axis under oxidative conditions. In addition to redox bal-

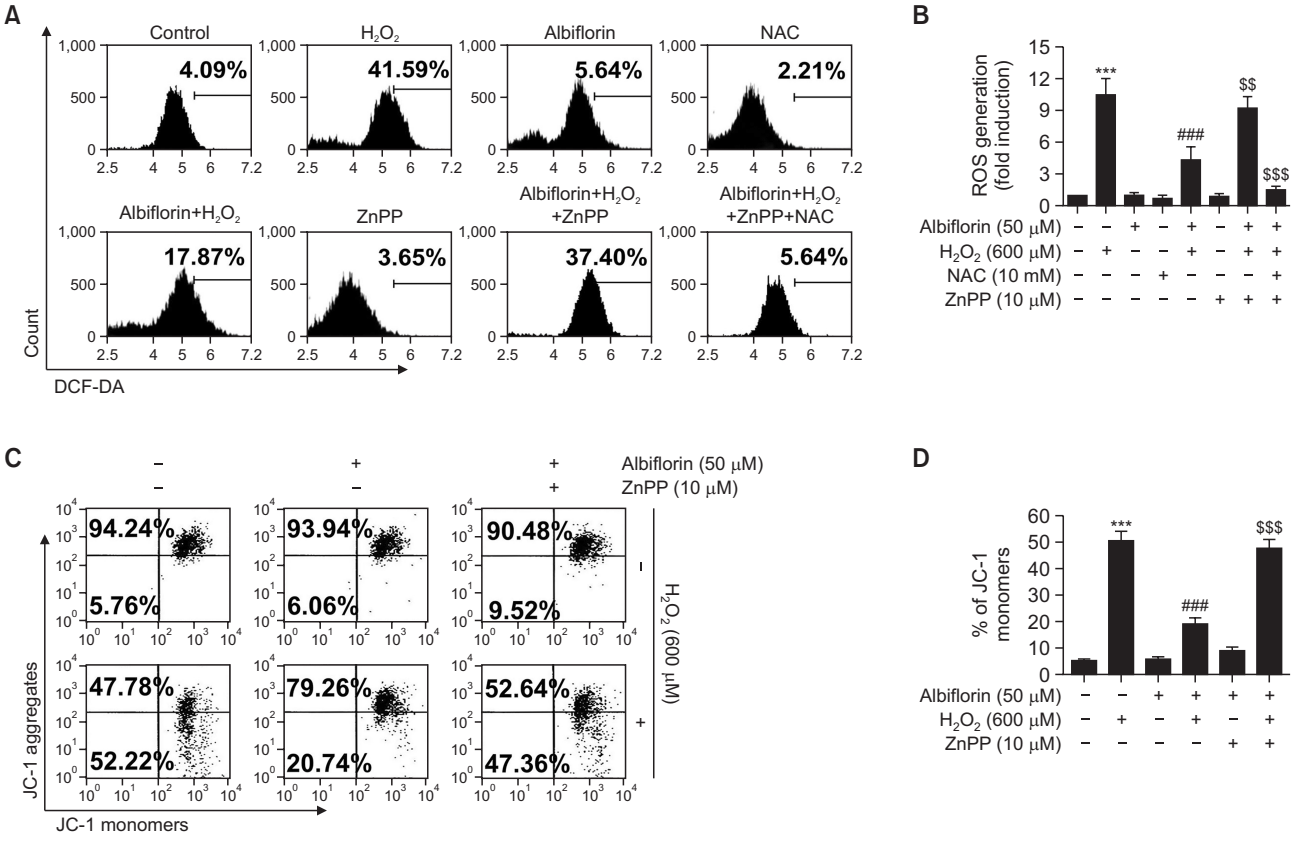

Fig. 5. Loss of ROS scavenging and mitochondrial protective effects of albiflorin following inhibition of HO-1 activity in H_2O_2-treated C2C12 cells. Cells were pretreated with or without albiflorin, NAC or ZnPP alone or together for 1 h and then stimulated with H_2O_2 for 1 h (A, B) or 48 h (C, D). (A, B) Changes in intracellular ROS levels were examined by flow cytometry following DCF-DA staining representative histograms (A) and averages (B) are shown (n=3). (C) Flow cytometric analysis following JC-1 staining , and (D) changes in the JC-1 monomer ratio are presented as histograms (n=3). ***p<0.001 *vs* control cells; $^{###}p$<0.001 *vs* H_2O_2-treated cells; $^{\$\$}p$<0.01 and $^{\$\$\$}p$<0.001 *vs* H_2O_2 and albiflorin and H_2O_2-treated cells. H_2O_2, hydrogen peroxide; NAC, N-acetyl-L-cysteine; ZnPP, zinc protoporphyrin; ROS, reactive oxygen species; JC-1, 5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi-dazoylcarbocyanine iodide; HO-1, heme oxygenase 1.

ance, this transcription factor has been shown to regulate a number of genes involved in a variety of physiological processes required for cell survival and proliferation, including mitochondrial biogenesis and maintenance of cellular metabolic homeostasis (van der Horst *et al*., 2022; Bhat *et al*., 2024; Luchkova *et al*., 2024). For example, impaired myocyte differentiation and skeletal muscle contractility in Nrf2-deficient mice compared to those in wild-type controls are associated with increased ROS production and decreased mitochondrial respiratory capacity (Whitman *et al*., 2013; Crilly *et al*., 2016). In contrast, Nrf2 activators such as sulforaphane, a potent ROS scavenger, restored Nrf2 activity and its target gene expression, including HO-1, in senescent C2C12 cells and old mice, thereby maintaining mitochondrial function and improving sarcopenia (Yan *et al*., 2022; Kasai *et al*., 2023). In this study, by including the albiflorin+NAC group, we further investigated whether the combined treatment had a synergistic or additive effect in reducing oxidative stress and improving mitochondrial function. Our results showed that the protective effect of the co-treatment was greater than that of either treatment alone, suggesting a potential synergistic interaction between the direct ROS-scavenging action of NAC and the Nrf2/HO-1-mediated endogenous defense enhancement by albiflorin. HO-1 is an enzyme that catabolizes heme into free iron, carbon monoxide, and biliverdin, which is then metabolized to bilirubin, a potent antioxidant, by bilirubin reductase (Chiang *et al*., 2021; Consoli *et al*., 2021). The importance of HO-1 activation in albiflorin-mediated protection of C2C12 cells in an oxidative environment was confirmed by pretreating cells with ZnPP, an HO-1 activity inhibitor. The inhibitory effect of albiflorin on H_2O_2-induced ROS production was lost in cell treated with ZnPP, leading to mitochondrial dysfunction and loss of the inhibitory effect of albiflorin on H_2O_2-induced apoptosis and cytotoxicity. These results suggest the importance of activation of the Nrf-2/HO-1 axis in the inhibitory effect of albiflorin on oxidative stress-induced cellular damage.

In this study, we demonstrate that the protective effect of albiflorin on C2C12 myoblasts in an oxidative environment mimicked by H_2O_2 treatment is mediated by maintenance of mitochondrial homeostasis and inhibition of ROS generation. The ROS-scavenging effect of albiflorin may contribute to the suppression of DNA damage and apoptosis during myoblast survival in an oxidative environment through Nrf-2-mediated HO-1 activation (Fig. 7). However, various intracellular signaling pathways other than the Nrf-2/HO-1 signaling pathway may be involved in the antioxidant activity of albiflorin. Therefore,

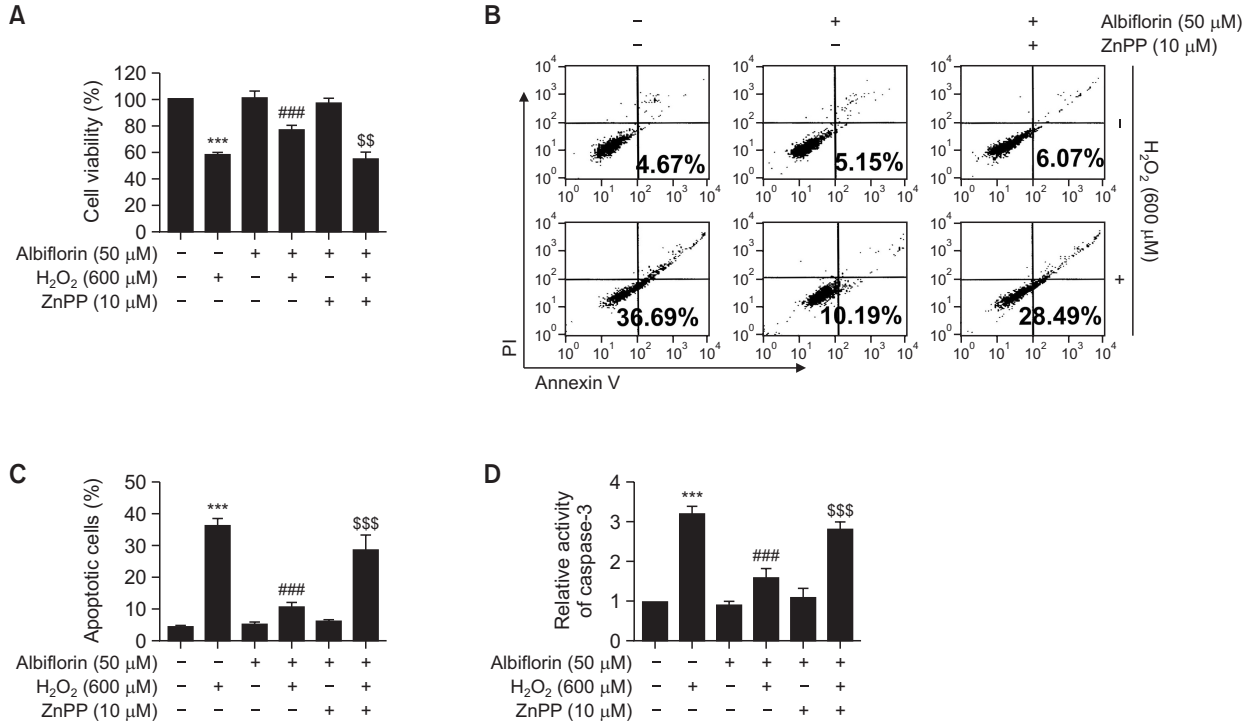

Fig. 6. Attenuation of the protective effect of albiflorin against H_2O_2-induced cytotoxicity following inhibition of HO-1 activity in C2C12 cells. Cells were pretreated with or without albiflorin or ZnPP alone or together for 1 h and then stimulated with H_2O_2 for 48 h. (A) Cell viability was analyzed by MTT assay (n=3). (B, C) Apoptosis was analyzed by Annexin V/PI staining, and the frequency of Annexin V-positive cells is presented (n=3). (D) Caspase-3 activity was assessed using the corresponding assay kit (n=3). ***p<0.001 *vs* control cells; $^{###}p$<0.001 *vs* H_2O_2-treated cells; $^{\$\$}p$<0.01 and $^{\$\$\$}p$<0.001 *vs* H_2O_2 and albiflorin and H_2O_2-treated cells. H_2O_2, hydrogen peroxide; ZnPP, zinc protoporphyrin; PI, propidium iodide; HO-1, heme oxygenase 1.

further studies are needed to identify the upstream kinases involved in albiflorin-induced Nrf-2 activation and determine how Nrf-2/HO-1 signaling is associated with various signaling pathways involved in redox regulation and mitochondrial homeostasis. Representative upstream kinases that regulate the activity of Nrf-2 may include protein kinase C, mitogen-activated protein kinases, and glycogen synthase kinase-3, as well as AP-1 proteins (Zhao *et al*., 2017a; Aliyu *et al*., 2018; Han *et al*., 2022; Simu *et al*., 2022; Liu *et al*., 2023). Moreover, Nrf2 is a transcription factor that regulates the expression of antioxidant enzymes such as SOD, CAT, and GPx (Truong *et al*., 2018; Gupta *et al*., 2020; Rashidi *et al*., 2022), and it extensively regulates the phosphorylation of Nrf-2 and the binding to antioxidant response elements for transcriptional activation of downstream Nrf2 genes translocated to the nucleus (Zhao *et al*., 2017b; Zhang *et al*., 2020).

In conclusion, the results of the present study demonstrate that albiflorin significantly suppresses cytotoxicity, DNA damage, and apoptosis in H_2O_2-treated C2C12 myoblasts, and that its apoptosis-blocking effect is related to the blockade of caspase-3 activation and PARP cleavage. Albiflorin also maintained the integrity of mitochondrial membranes in H_2O_2-treated cells, as evidenced by improved MMP and inhibition of the increase in Bax/Bcl-2 ratio, which is associated with attenuated ROS production. Moreover, Keap1 expression was further downregulated in cells co-treated with albiflorin and H_2O_2, compared to that in cells treated with albiflorin alone, whereas Nrf2 phosphorylation and HO-1 expression and activity were enhanced, revealing that Nrf2/HO-1 signaling is activated by albiflorin under oxidative conditions. However, when the activity of HO-1 was artificially reduced, the ROS-scavenging, anti-apoptotic, and cytotoxicity inhibitory effects of albiflorin on H_2O_2 were abrogated, emphasizing the importance of Nrf-2/HO-1 signaling in the antioxidant activity of albiflorin. Therefore, the results of this study indicate that albiflorin, as an Nrf-2 activator, has potential as a therapeutic agent for protecting muscle cells from oxidative damage. These results indicate that albiflorin could be a promising candidate for developing novel therapeutic strategies targeting muscle-related disorders. Further *in vivo* and clinical studies are needed to validate its efficacy and explore its potential as a muscle-protective agent.

CONFLICT OF INTEREST

The authors have no conflicts of interest relevant to this study to disclose.

ACKNOWLEDGMENTS

This study was supported by the Basic Science Research Program grant (RS-2022-NR070862, RS-2023-00217899, RS-2024-00336900) from the National Research Foundation (NRF) of the Republic of Korea. The authors would like to

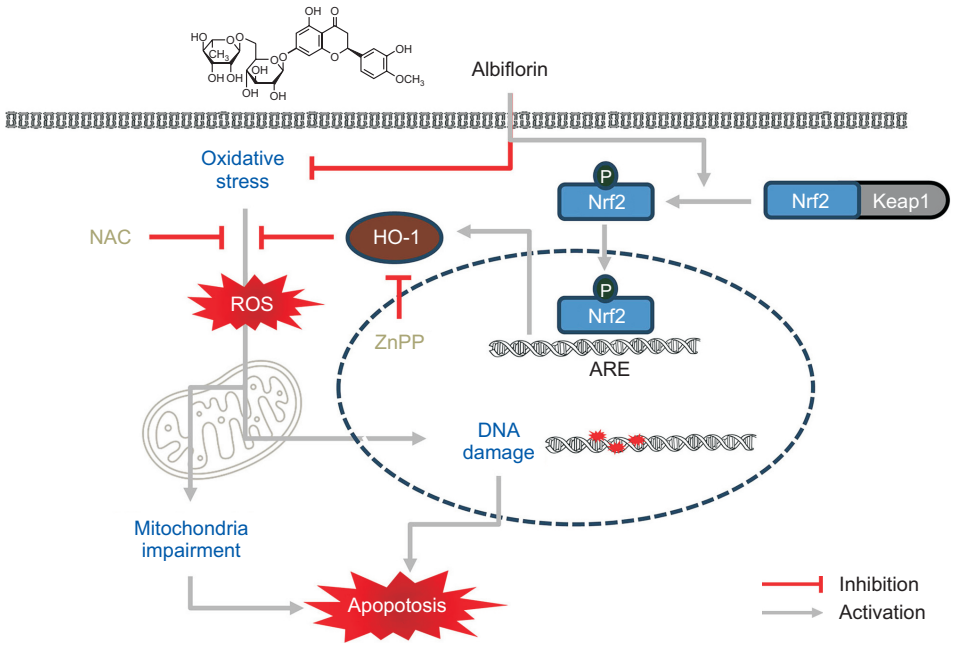

Fig. 7. Schematic diagram showing the protective activity of albiflorin against oxidative stress-induced cytotoxicity in C2C12 myoblasts. Albiflorin attenuated mitochondrial impairment and apoptosis while blocking ROS production in H_2O_2-stimulated C2C12 cells. Albiflorin also markedly augmented the HO-1 expression and its enzymatic activity, along with phosphorylation and translocation of Nrf2 to the nucleus. However, these beneficial effects of albiflorin were abolished when the activity of HO-1 was blocked. Therefore, our results provide evidence that albiflorin as an ROS scavenger can rescue C2C12 cells from oxidative damage *via* Nrf2/HO-1 axis activation. p-Nrf2, phosphorylated nuclear factor erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; NAC, N-acetyl-L-cysteine; HO-1, heme oxygenase 1; ROS, reactive oxygen species; ZnPP, zinc protoporphyrin; ARE, antioxidant response element.

thank Core-Facility Center for Tissue Regeneration, Dong-eui University (Busan, Republic of Korea), for letting us use flow cytometer and fluorescence microscope.

AUTHOR CONTRIBUTIONS

Cheol Park: Conceptualization, Investigation and Original Draft. Hee-Jae Cha: Methodology, Investigation and Formal analysis. Dong-Gu Kim: Investigation and Validation. Su Hyun Hong: Validation and Formal analysis. Sung-Kwon Moon: Methodology and Resources. Cheng-Yun Jin: Resources and Data Curation. Gi Young Kim: Methodology and Data Curation. Heui-Soo Kim: Formal analysis and Validation. Na Yeong Lee: Validation and Review & Editing. Jung-Hyun Shim: Supervision, Review & Editing and Funding acquisition. Yung Hyun Choi: Supervision, Project administration and Funding acquisition.

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