HIF-1α protects osteoblasts from ROS-induced apoptosis

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ABSTRACT
The regulatory mechanism of hypoxia-inducible factor-1α (HIF-1α) is complex. HIF-1α may inhibit or promote apoptosis in osteoblasts under different physiological conditions, and induce bone regeneration and repair injury in coordination with angiogenesis. The relationship between H2O2 and HIFs is complex, and this study aimed to explore the role of HIF-1α in H2O2-induced apoptosis. Dimethylxallyl glycine (DMOG) and 2-Methoxyestradiol (2ME) were used to stabilize and inhibit HIFs, respectively. Cell viability was assessed with CCK8. Apoptosis and ROS levels were detected by flow cytometry, and HIF mRNA expression was assessed by reverse transcription-polymerase chain reaction (RT-PCR). Western blot was performed to detect HIF-1α, HIF-2α, Bax, Bak, Bcl-2, Bcl-XL, caspase-9, and PCNA protein amounts. Our data suggest that both HIF-1α and HIF-2α play a protective role in oxidative stress. HIF-1α reduces H2O2-induced apoptosis by upregulating Bcl-2 and Bcl-XL, downregulating Bax, Bak, and caspase-9, stabilizing intracellular ROS levels, and promoting the repair of H2O2-induced DNA damage to reduce apoptosis.

Introduction
Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor consisting of the α and β subunits [1,2]. Previous studies have identified three α subunits (HIF-1α, HIF-2α, and HIF-3α) and two β subunits (Arnt and ARNT2) [3–5]. In normoxic conditions, PHD is activated to hydroxylate proline at specific sites on HIF-α, which in turn binds the VHL ligase complex to HIF-1α, ultimately leading to HIF-1α ubiquitination and proteasomal degradation [6–13]. In hypoxia, PHD is inhibited, and HIF-α stabilizes and heterodimerizes with HIF-β in the nucleus, binding to the target gene hypoxia response element (HRE) and activating related gene expression [14–18].

Studies have shown that high levels of ROS could damage cellular compounds and cause cell apoptosis. However, at low levels, ROS act as cellular signaling molecules and may play important protective roles under destructive conditions [19,20], which may be related to the activation of HIF-1α [21]. As a common exogenous ROS, 

In mice, overexpression of HIF-1α leads to enhanced activity and function of mouse osteoblasts, and promotes osteoblast formation through non-autonomous angiogenesis of cells, showing a phenotype similar to ossification [25,26]. However, it was also reported that partial impairment of HIF-1α inhibits osteoblast apoptosis and promotes bone regeneration [27]. However, studies assessing cultured osteoblasts showed that HIF-1α expression upregulation and VHL loss do not affect osteoblast proliferation and apoptosis [25]. In addition, Wang et al. [25] showed that HIF-2α has compensatory activity in the absence of HIF-1α, which may partially replace the function of HIF-1α. These findings suggest that the regulatory mechanism of HIF-1α is complex; indeed, HIF-1α may inhibit apoptosis or promote apoptosis in osteoblasts under different physiological conditions, and induce bone regeneration and injury repair in coordination with angiogenesis. Osteoblasts, as a component of the hematopoietic stem cell (HSC) niche, play an important role in HSC regulation.

Human osteoblasts produce a variety of hematopoietic cytokines, such as G-CSF, GM-CSF, and LIF [28,29]. In vivo experiments in mice showed that increased number of osteoblasts results in a parallel increase in...
the amount of HSC, and loss of osteoblasts would lead to significantly decreased number of bone marrow cells and altered hematopoietic mode [30,31]. During HSC mobilization, ROS levels increase and induce osteoblast apoptosis, and ROS signal transduction pathway inhibition reduces G-CSF induced HSPC mobilization [32]. In addition, HSC mobilization leads to hypoxia and increases HIF-1α expression in the bone marrow [33], and HIF-1α is essential for the response of HSC mobilization in G-CSF and Pulisafor [34]. Our previous study demonstrated that osteoclasts and lymphocytes participate in the process of G-CSF mobilization as well as the inhibition of osteoblasts; meanwhile, extensive inhibition of osteoblasts is one of the key mechanisms of G-CSF mobilization in HSC [35,36]. However, the changes of osteoblasts under the effect of simple osteoclast and lymphocyte inhibition are more likely to be focal rather than extensive, and extensive inhibition of osteoblasts is earlier than the induction of osteoclast proliferation [36]. Therefore, we believe that HIF-1α, which regulates osteoblast apoptosis, may be involved in the process of extensive osteoblast inhibition, and the effect and mechanism of HIF-1α synergism with ROS in osteoblast proliferation and apoptosis was investigated experimentally.

In this study, H2O2 was used as an exogenous ROS, and HIF stabilizer A [37] and HIF inhibitor B [38] were used to alter the expression level of HIFs. This study demonstrated that HIF-1α and HIF-2α have significant protective effects on H2O2-induced oxidative stress-related apoptosis.

Methods and materials

Reagents

MEM-α and fetal bovine serum (FBS) were purchased from Gibco (Waltham, MA). DMOG, 2ME, and H2O2 were purchased from Sigma. Cell Proliferation and Toxicity Detection Kit, Reactive Oxygen Species Detection Kit, One Step TUNEL Apoptosis Assay Kit and Protease inhibitor mixture were purchased from Meilunbio (Dalian, China). Hydrogen Peroxide Quantitative Analysis Kit (Sangon Biotech, Shanghai, China), PrimeScript™ RT reagent Kit with gDNA Eraser and TB Green™ Premix Ex Taq™ II were purchased from Takara (Kusatsu, Japan).

Antibodies targeting Bax, caspase-9, Bcl-XL, PCNA, cyclin D1, and β-actin were purchased from Cell Signaling Technologies (Danvers, MA). Antibodies against HIF-1α, BCL-2, and PCNA were purchased from Abmart (Shanghai, China). Anti-HIF-2α antibodies were purchased from Abcam (Waltham, MA), and goat anti-rabbit IgG-HRP was from Absin (Shanghai, China).

Cell culture

In this study, Murine osteoblastic cell line MC3T3-E1 was purchased from Cell Bank of the Chinese Academy of Sciences. The cells were maintained at 37 °C in a 5% CO2 incubator with α-MEM containing 10% FBS, and at the confluence of 70–90%, the cells were sub-cultured. To analyze ROS levels, apoptosis rates, mRNA, and target proteins, cells in each experimental group were cultured at 37 °C in a 5% CO2 incubator with α-MEM containing 5% FBS and exposed to H2O2 after 12 h of drug stabilization or inhibition of HIFs.

CCK-8 assay

MC3T3-E1 cells were seeded in 96-well culture plates and cultured in α-MEM containing 10% FBS. At the specified time after H2O2, DMOG, and 2ME treatment, the cytotoxicity test was conducted according to the manufacturer’s instructions (Meilun, Fujian, China). Absorbance was measured at 450 nm on a microplate spectrophotometer. The relative cell survival rate was expressed as the percentage of the experimental group vs. control cells.

Determination of hydrogen peroxide concentration

After the cells were cultured in 6-well plates at 2.5 × 10^5/well for 24 h, hydrogen peroxide was added into the medium containing 10%, 5%, and 0% FBS, respectively, and hydrogen peroxide concentration was measured in the supernatant at 5, 10, 15, 30, 45, and 60 min, respectively, as required by the Hydrogen Peroxide Quantitative Analysis Kit.

ROS determination by flow cytometry

Intracellular ROS levels were detected with the oxygen-free radical sensitive probe DCFH-DA (Meilunbio, Dalian, China). After osteoblast treatment with H2O2, the culture medium was completely discarded, and the basal medium containing 5 μM DCFH-DA was added to osteoblasts for 30 min, and cells were digested with trypsin. Relative fluorescence intensity was measured by flow cytometry (excitation and emission at 488 nm and 530 nm, respectively).

Apoptosis determination by flow cytometry

The apoptotic rate of cells was measured with the apoptosis detection kit (BD Pharmingen, San Diego, CA). Cells were digested with trypsin and 5 μl Annexin
The TUNEL assay

After drug treatment, cells were fixed with 4% paraformaldehyde for 30 min. Then, they were treated with proteinase K and washed. The TUNEL detection solution was added and incubated at 37°C for 1 h, shielded from light. The TUNEL detection solution was removed and anti-fluorescence attenuating tablets containing DAPI were added for mounting before fluorescence microscopy.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

TRIzol reagent was used to extract total RNA. RNA was quantified and assessed for purity on a UV spectrophotometer. Total RNA was then reverse-transcribed according to the manufacturer’s instructions using a reverse transcription kit. All primer sequences are listed as follows: HIF-1α, forward 5’CAGCAACCCAGGT GACTGTGC3’ and reverse 5’AGTCTGATCGT AAATCGGAGG3’; HIF-2α, forward 5’GAAACATGG CCCCCGATGAAT3’ and reverse 5’CCCCCTAGCGCTC TGTTAGAT-3’; β-actin, forward 5’CTGCCCAGATCCTCTT CCTC3’ and reverse 5’GCCACAGGATTCCATACCCA3’.

Data were normalized to β-actin and assessed by the delta-delta cycle threshold (CT) method.

Western blot

The collected cells were lysed with the RIPA buffer containing a 1% protease inhibitor mixture and 1% PMSF. Protein concentration was determined by the BCA method. Equal amounts of total protein were separated by SDS-PAGE and transferred onto a PVDF membrane. Membranes were then blocked with 5% nonfat dry milk in TBST for 1 h at room temperature and incubated with appropriate monoclonal antibodies (HIF-1α, HIF-2α, Bax, Bak, Bcl-2, Bcl-xl, PCNA, caspase-9, and β-actin) at 4°C overnight. This was followed by incubation with goat antirabbit IgG-HRP for 1 h at room temperature. ECL detection reagents were used for development, and β-actin expression was used to normalize the sample values.

Data analysis

All data were presented as mean ± standard deviation (mean ± SD) from at least three independent tests.

Results

Effects of H$_2$O$_2$, DMOG, and 2ME on MC3T3 cells

In order to assess the effect of hydrogen peroxide on cells, we first confirmed its effect on cell viability. As shown in Figure 1(A), hydrogen peroxide at 0.5 mM and above significantly reduced cell viability. Therefore, we selected hydrogen peroxide at 0.5 mM for subsequent experiments, and assessed changes in hydrogen peroxide concentration under experimental conditions. Considering that protein components in serum may affect the degradation of H$_2$O$_2$, we seeded cells at $2.5 \times 10^5$ well into six-well plates. Twenty-four hours later, we added 0.5 mM H$_2$O$_2$ final concentration into the culture medium containing different serum concentrations, and measured hydrogen peroxide concentration in the culture medium at each time point. After 1 h, H$_2$O$_2$ concentrations in the culture medium in various groups were reduced to less than 100 μM, which is considered the minimal concentration commonly used to induce oxidative stress [39–41]. The medium containing 10% FBS significantly increased the degradation of H$_2$O$_2$; Figure 1(B) considering cell growth and protein synthesis, we chose the medium containing 5% FBS for further assays. Then, we detected the changes of intracellular ROS levels under the action of H$_2$O$_2$, and found that intracellular ROS levels decreased with decreasing hydrogen peroxide concentration in the culture medium, and returned to normal within 1 h (Figure 1(C,D)). These results showed that hydrogen peroxide decreased cell viability in a concentration-dependent manner. At the same time, intracellular ROS levels gradually returned to normal with decreasing extracellular H$_2$O$_2$ concentration.

Under experimental conditions, 0.1–1 mM DMOG had no significant effect on cell viability, and 2ME had a concentration-dependent inhibitory effect on cell viability (Figure 2(A,B)). Then, we treated cells with 0.5 mM DMOG and 20 μM 2ME for 12 h, and confirmed that DMOG significantly stabilized HIF-1α and HIF-2α mRNAs, but did not significantly change HIF-2α mRNA amounts. Meanwhile, 2ME inhibited both HIF-1α and HIF-2α protein and mRNA levels (Figure 3). Flow cytometry assays showed (Figure 4) that DMOG alone stabilized HIFs, while 2ME inhibited HIF expression without affecting cell apoptosis.
In order to verify the protective effect of HIFs on cell apoptosis under oxidative stress, cells were seeded at $2.5 \times 10^5$/well in six-well plates containing 5% FBS medium. At 70–80% confluence, DMOG (0.5 mM) and 2ME (20 $\mu$M) were added for 12 h. Then, added the final concentration of 0.5 mM H$_2$O$_2$ into the medium and incubated for 1 h. Flow cytometry was used to detect the cell apoptotic rate of each experimental group (Figure 4). H$_2$O$_2$ significantly increased cell apoptosis. H$_2$O$_2$-induced apoptosis was significantly reduced by DMOG, while 2ME inhibition of HIFs significantly increased the pro-apoptotic
Figure 3. DMOG can effectively stabilize HIFs and inhibit HIFs under normoxia. MC3T3-E1 cells were added with DMOG or 2ME and incubated for 12 h before total protein or total RNA was extracted. (A,B) Western blot analysis: HIF-1α, HIF-2α, and β-actin were detected, HIF-1α, HIF-2α were normalized to β-actin as an internal control. (C) mRNA expression of the HIF-1α, HIF-2α, and β-actin were quantified by real-time PCR. HIF-1α and HIF-2α cDNA was normalized to β-actin cDNA as an internal control. The data shown are the means of three independent experiments. The error bars represent standard error. Data were analyzed with one-way analysis of variance (*p < .05; **p < .01, vs. control).

Figure 4. Apoptosis of MC3T3-E1 cells was detected by flow cytometry. The data shown are the means of three independent experiments. The error bars represent standard error. Data were analyzed with one-way analysis of variance (*p < .05, **p < .01, vs. control; ##p < .01, vs. H2O2).
The effect of H$_2$O$_2$. The experimental results showed that HIF stability could protect cells from oxidative stress-related apoptosis caused by H$_2$O$_2$ attack, while HIF inhibition could render cells more vulnerable to oxidative stress injury and thus increase apoptosis.

HIF protein and mRNA amounts are shown in Figure 5. Under normoxic conditions, H$_2$O$_2$ effectively stabilized HIF-1$\alpha$ protein, which is consistent with previous studies [42,43]. In the case of HIF stabilization with DMOG, HIF-1$\alpha$ increase was more obvious after addition of H$_2$O$_2$, while in the case of HIF inhibition with 2ME, HIF-1$\alpha$ expression was still lower than that of the control group after addition of H$_2$O$_2$, suggesting that HIF-1$\alpha$ increase had a protective effect on ROS-induced apoptosis. H$_2$O$_2$ did not stabilize HIF-2$\alpha$, but instead decreased its protein and mRNA levels. DMOG antagonized HIF-2$\alpha$ inhibition by H$_2$O$_2$, and HIF-2$\alpha$ returned to normal levels. When 2ME was used in combination with H$_2$O$_2$, HIF-2$\alpha$ decrease was more obvious. These results suggest that ROS-induced apoptosis is promoted by HIF-2$\alpha$ inhibition. The imbalance between HIF-1$\alpha$ and HIF-2$\alpha$ may be one of the factors causing ROS-induced apoptosis, and HIF-1$\alpha$ increase could significantly inhibit ROS-induced apoptosis. At the same time, HIF-2$\alpha$ decrease could promote apoptosis.

**HIF-1$\alpha$ stabilizes intracellular ROS levels and upregulates PCNA**

To examine the changes of intracellular ROS levels under HIF-1$\alpha$ modification, flow cytometry was performed. Experimental data showed that H$_2$O$_2$ treatment for 0.5 h exerted, intracellular ROS levels were detected at 0.5 h (A) and 1 h (B), respectively. When 2ME was used in combination with H$_2$O$_2$, HIF-2$\alpha$ decrease was more obvious. These results suggest that ROS-induced apoptosis is promoted by HIF-2$\alpha$ inhibition. The imbalance between HIF-1$\alpha$ and HIF-2$\alpha$ may be one of the factors causing ROS-induced apoptosis, and HIF-1$\alpha$ increase could significantly inhibit ROS-induced apoptosis. At the same time, HIF-2$\alpha$ decrease could promote apoptosis.
stabilize intracellular ROS levels. However, ROS levels in each group basically returned to normal at 1 h (Figure 6), which may be related to the degradation of H$_2$O$_2$ in the medium.

Previous studies have confirmed that H$_2$O$_2$, as an exogenous ROS, can damage DNA, and PCNA is involved in repairing H$_2$O$_2$-damaged DNA [44,45]. In this study, we also detected PCNA (Figure 7), which was significantly increased in cells with HIF-1$\alpha$ overexpression. The expression of PCNA was downregulated under HIF-1$\alpha$ inhibition. Therefore, HIF-1$\alpha$ may prevent DNA damage induced by H$_2$O$_2$ by upregulating PCNA, thus reducing the apoptotic rate of cells. Then, the detection of DNA broken in apoptotic cells with the TUNEL kit also confirmed the above findings; as shown in Figure 8, H$_2$O$_2$ significantly increased the amounts of apoptotic cells with DNA breaks, and this effect could be significantly antagonized after HIF stabilization, while the inhibition of HIF made DNA breaks in apoptotic cells under oxidative stress more obvious.

**Figure 7.** HIF-1$\alpha$ up-regulated PCNA expression. 1: control; 2: H$_2$O$_2$; 3: DMOG + H$_2$O$_2$; 4: 2ME + H$_2$O$_2$. Western blot analysis: PCNA and $\beta$-actin were detected, PCNA was normalized to $\beta$-actin as an internal control. The data shown are the means of three independent experiments. The error bars represent standard error. Data were analyzed with one-way analysis of variance (*$p < .05$, **$p < .01$, vs. control; ***$p < .01$, vs. H$_2$O$_2$).

**Figure 8.** HIF-1$\alpha$ reduced ROS-induced cell damage. TUNEL staining was used to detect apoptosis of cells treated with H$_2$O$_2$ for 1 h. The data shown are the means of three independent experiments. The error bars represent standard error. Data were analyzed with one-way analysis of variance (**$p < .01$, vs. control; ***$p < .01$, vs. H$_2$O$_2$).

**HIF-1$\alpha$ decreases apoptosis by inhibiting pro-apoptotic proteins and promoting anti-apoptotic proteins**

We further detected the expression levels of apoptosis-related proteins and anti-apoptosis-related proteins, and results are shown in Figure 9. Compared with the control group, the expression levels of the pro-apoptotic proteins Bax, Bak, and caspase-9 were increased after addition of H$_2$O$_2$, while the anti-apoptotic proteins
Bcl-2 and Bcl-xl were downregulated. HIF-1α increase antagonized the suppression of Bax, Bak, and caspase-9 by hydrogen peroxide but increased the amounts of Bcl-2 and Bcl-xl. After inhibiting HIF-1α, H2O2 increased Bax, Bak, caspase-9, Bcl-2, and Bcl-xl. These results suggested that HIF-1α could reduce H2O2 oxidative stress-related injury by increasing Bcl-2 and Bcl-xl and decreasing Bax, Bak, and caspase-9.

**Discussion**

H2O2 is one of the most common forms of ROS because it can easily penetrate the plasma membrane and affect neighboring cells, altering intracellular ROS levels [46]. Excessive ROS can be used as a second messenger to trigger apoptotic signals and regulate apoptosis by controlling the expression of pro-apoptotic genes and activating nuclear transcription factors [47]. We used DMOG and 2ME to stabilize and inhibit HIF-1α and HIF-2α, respectively, and then stimulated cells with high concentrations of H2O2 as an exogenous ROS. Our data suggest that high concentration of H2O2 can induce apoptosis obviously, HIF-1α and HIF-2α have significant protective effects on H2O2-induced oxidative stress-related osteoblast apoptosis.

As shown above, exogenous H2O2 regulated HIF-1α and HIF-2α with duality, and H2O2 significantly increased the expression of HIF-1α, which is consistent with previous reports [22,42,43], but inhibited the expression of HIF-2α. This finding is consistent with the concept that reducing environments are favorable to HIF-2α accumulation [23]. ROS are known to upregulate HIF-1α by inhibiting PHD [22,42], but their effect on HIF-2α remains controversial. Chen et al. showed that H2O2 significantly inhibits HIF-2α, while Guzy et al. [24] showed that H2O2 stabilizes HIF-2α. Coincidentally, low concentrations of H2O2 (20 and 50 μM) in Guzy et al.’s study had a particularly significant effect on HIF-2α stabilization, unlike higher concentrations (100 μM), which inhibited HIF-2α accumulation in Chen et al.’s report.

Considering the inhibitory effect of high H2O2 concentration on HIF-2α in this study, we speculated that H2O2 has a dual regulatory effect on HIF-2α, that is, low H2O2 concentration stabilizes HIF-2α and high amounts inhibit HIF-2α. This may be related to changes in intracellular ROS levels. This study demonstrated that ROS in osteoblasts returned to normal when H2O2 concentration in the culture medium decreased to less than 100 μM, indicating that low H2O2 concentration does not increase intracellular ROS levels, while high H2O2 concentration significantly increases intracellular ROS levels and downregulates HIF-2α. This also supports the notion that reducing environments promote HIF-2α accumulation [23]. However, it is certain that when HIF-1α and HIF-2α are stabilized, the killing effect of H2O2 on cells is significantly reduced. HIF-1α and HIF-2α level decreases significantly increased the apoptotic effect of H2O2. These results suggest that both HIF-1α and HIF-2α play a protective role in oxidative stress, but the difference is that HIF-1α is upregulated, while HIF-2α is downregulated under normoxia. However, this was...
only the result under short-term oxidative stress. Unfortunately, this experiment did not explore the protective effect and mechanism of HIFs on osteoblasts under long-term oxidative stress, and the physiological/pathological changes of osteoblasts treated with constant flow of H₂O₂ and HIFs for a long time were not clear.

The regulation and control mechanism of HIFs on osteoblasts is extremely complex. On the one hand, overexpression of HIF-1α can enhance the activity and function of osteoblasts and promote osteogenesis [25,26,48]. However, partial impairment of HIF-1α can also promote bone regeneration by inhibiting osteoblast apoptosis. Considering that HIF-2α compensatory increases in the absence of HIF-1α and may partially replace the function of HIF-1α, this abnormal performance may be related to the increased compensated activity of HIF-2α [25,27]. However, recent studies have also shown that HIF-2α is a negative regulator of osteogenesis [49]. In summary, it is generally believed that HIF-1α overexpression enhances osteoblast activity, promotes osteoblast proliferation and bone repair, and this physiology is consistent with HIF-1α’s protective role in oxidative stress.

Previous studies have confirmed that ROS can damage DNA in cells, and PCNA can repair the damaged DNA in a variety of ways to alleviate cell apoptosis caused by DNA damage [44,45,50]. On the one hand, HIF-1α upregulation can significantly stabilize intracellular ROS, while HIF-1α level reduction can significantly enhance H₂O₂-induced intracellular ROS levels. On the other hand, HIF-1α upregulates PCNA, and the reduction of ROS-induced DNA breaks may be related to DNA damage repair, which is also indicated by reduced PCNA amounts and enhanced apoptosis caused by HIF-1α downregulation. Therefore, we believe that HIF-1α can promote the repair of H₂O₂-induced DNA damage to reduce apoptosis by increasing PCNA expression. However, the degree of DNA damage and the binding and repair of DNA by PCNA were not detected in this study, which requires further investigation in future studies.

The Bcl-2 protein family is a group of proteins closely related to cell apoptosis, and can be divided into two groups: one group inhibits cell apoptosis, including Bcl-2 and Bcl-xl, while the other promotes apoptosis, including Bax and Bak. Anti-apoptotic Bcl-2 and Bcl-xl inhibit apoptosis by binding to Bax or Bak [51,52]. In this study, H₂O₂ treatment downregulated Bcl-2 and Bcl-xl and upregulated Bax and Bak, at the protein level. HIF-1α upregulation antagonized these changes, while low HIF-1α amounts promoted them. These results suggest that HIF-1α can improve cell survival under oxidative stress by regulating the above-mentioned members of the Bcl-2 protein family. Previous findings have shown that ROS-mediated activation of caspase-9 increases cell apoptosis, and inhibition of caspase-9 effectively reduces cell apoptosis [53–55]. Our experiment also confirmed this notion, and we demonstrated that caspase-9 alteration is regulated by HIF-1α.

In conclusion, H₂O₂ significantly increases HIF-1α expression, while inhibiting HIF-2α expression. The imbalance between HIF-1α and HIF-2α may be a cause of ROS-induced apoptosis. Both HIF-1α and HIF-2α play a protective role in oxidative stress. HIF-1α can reduce H₂O₂-induced apoptosis by upregulating Bcl-2 and Bcl-xl, downregulating Bax, Bak, and Caspase-9 and stabilizing intracellular ROS levels. It may also increase the expression of PCNA, promoting the repair of H₂O₂-induced DNA damage to reduced apoptosis.

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All the authors have no financial conflict of interest to disclose. The authors are responsible for the content and writing of the article.

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