Title: Tetrandrine Enhances H₂O₂-Induced Apoptotic Cell Death Through Caspase-dependent Pathway in Human Keratinocytes

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Abstract. Background: Tetrandrine, a bis-benzylisoquinoline alkaloid, induces apoptosis of many types of human cancer cell. Hydrogen peroxide (H₂O₂) is a reactive oxygen species inducer; however, there are no reports to show whether pre-treatment of tetrandrine with H₂O₂ induces more cell apoptosis than H₂O₂ alone. Thus, the present study investigated the effects of tetrandrine on H₂O₂-induced cell apoptosis of human keratinocytes, HaCaT, in vitro. Materials and Methods: HaCaT cells were pre-treated with and without tetrandrine for 1 h, and then treated with H₂O₂ for examining cell morphological changes and cell viability using contrast-phase microscopy and propidium iodide (PI) exclusion assay, respectively. Cells were measured apoptotic cell death by using annexin V/PI double staining and further analyzed by flow cytometer. Cells were further assessed for DNA condensation using 2-(4-amidinophenyl)-6-indolecarbamidine staining. Western blotting was used to measure expression of apoptosis-associated proteins and confocal laser microscopy was used to measure the protein expression and nuclear translocation from the cytoplasm to nuclei. Results: Pre-treatment of tetrandrine for 1 h and treatment with H₂O₂ enhanced H₂O₂-induced cell morphological changes and reduced cell viability, whilst increasing apoptotic cell death and DNA condensation. Furthermore, tetrandrine significantly increased expression of reactive oxygen species-associated proteins such as superoxide dismutase (Cu/Zn) and superoxide dismutase (Mn) but significantly reduced the level of catalase, which was also confirmed by confocal laser microscopy. It also increased expression of DNA repair-associated proteins ataxia telangiectasia mutated, ataxia-telangectasia and Rad3-related, phospho-P53, P53 and phosphorylated histone H2AX, and of pro-apoptotic proteins BCL2 apoptosis regulator-associated X-protein, caspase-3, caspase-8, caspase-9 and poly ADP ribose polymerase in HaCaT cells. Conclusion: These are the first and novel findings showing tetrandrine enhances H₂O₂-induced apoptotic cell death of HaCaT cells and may provide a potent approach for the treatment of proliferated malignant keratinocytes.

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Skin covers the human body's outer surface and is the first line of defense against microbial and chemical attacks (1). The skin is therefore exposed to various stressors (2, 3) such as UV light (4, 5), and air pollutants (6, 7), including heavy metals (Cu, Mn, Ni, Pb, and Ti) and polycyclic aromatic hydrocarbons (8). Prolonged exposure to such stresses may cause skin damage, leading to the induction of skin cancer (9). The majority of the epidermis consists of keratinocytes (10), which form a physical barrier, and they have diverse receptors for the stimulation of signaling transduction pathways to other layers of the skin (11, 12). However, epidermal keratinocytes are very susceptible to the effects of environmental pollutants, leading to oxidative stress, which induces cancer, aging, and inflammatory disorders (13, 14). Abnormalities of keratinocyte growth leads to skin disorders, such as psoriasis, inflammatory allergic diseases, and chronic wounds (15). Reactive oxygen species (ROS), including most free radicals, have been shown to damage cellular proteins, lipids, and DNA (16) which can result in skin disorders (17). Oxidative stress is an important cause of DNA damage (18) and may induce about 10,000 DNA alterations per cell per day (19). In the body, endogenous antioxidant enzymes, including nicotinamide adenine dinucleotide phosphate dehydrogenase (quinone) 1 and heme oxygenase-1, function to reduce ROS in order to maintain normal skin biology (20). ROS at low levels is essential for cells to avoid extracellular invaders and to maintain cellular signaling. Overproduction of ROS by oxidative stress can injure DNA, proteins and lipids (21), resulting in cancer induction, cardiovascular diseases, and neurodegenerative diseases (22). Therefore, approaches for protecting the skin against oxidative stress are needed.

Hydrogen peroxide (H$_2$O$_2$) is an unstable ROS, and induces oxidative stress (14). Exogenous H$_2$O$_2$ inhibited porcine trophoderm, reduced cell viability, arrested cells in S and G$_2$/M phases, and increased cell apoptosis and autophagy (23). Moreover, H$_2$O$_2$ was reported to freely pass through the cell membrane to damage cells, such as during replicative senescence (19). H$_2$O$_2$ also triggers apoptosis of keratinocytes via the release of cytochrome c, cleaved caspase activity, and pro-apoptotic gene expression (21).

Tetrandrine, a bis-benzylisoquinoline alkaloid, was extracted from the dried root of *Stephania tetrandra*. Tetrandrine has anti-allergenic (24) and anti-inflammatory (25) properties. It is clinically used for treating rheumatoid arthritis (26, 27), silicosis (28), and cardiovascular disease (29) in the Chinese population. Furthermore, tetrandrine exhibits anticancer activities via induction of apoptosis in human breast (30), gastric (31), lung (32), oral (33), and liver (34) cancer, nasopharyngeal (35) and epidermoid (36) carcinoma cells, and laryngeal cancer stem cells (37). However, the bioactivity of tetrandrine pre-treatment in skin cells subjected to oxidative stress has not yet been studied. Therefore, in the present study, the effects of tetrandrine on apoptosis of H$_2$O$_2$-treated HaCaT human keratinocytes were evaluated *in vitro*. Furthermore, the molecular mechanisms of apoptosis associated with the effect of tetrandrine were also investigated in HaCaT cells.

**Materials and Methods**

**Chemicals and reagents.** Tetrandrine, dimethyl sulfoxide, propidium iodide (PI), and trypsin-ethylenediamine tetra-acetic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, L-glutamine, and antibiotic (penicillin/streptomycin) were purchased from Gibco®/Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies to superoxide dismutase (SOD) (Cu/Zn), SOD (Mn), catalase, and β-actin were obtained from Santa Cruz Biotechnology; antibodies to caspase-3, BCL2 apoptosis regulator-associated X-protein (BAX), and BCL2 were from Cell Signaling Technology, Inc. (Beverly, MA, USA); and those against poly ADP ribose polymerase (PARP), ataxia telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3 related (ATR), P53, and phospho-P53 (p-P53) were from Calbiochem (San Diego, CA, USA); anti-phosphorylated histone H2AX (p-H2A.X) was from GeneTex Inc. (Irvine, CA, USA). The secondary antibody (anti-IgG) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Tetrandrine stock solutions were dissolved in dimethyl sulfoxide and further diluted in the culture medium.

**Cell culture.** HaCaT Human keratinocyte cells were kindly provided by Professor Huey-Chun Huang (China Medical University, Taiwan, ROC). HaCaT cells were cultured in DMEM supplemented with 1% antibiotic (100 units/ml penicillin and 100 μg/ml streptomycin), 10% fetal bovine serum, and 2 mM L-glutamine at 37°C in a humidified incubator with 5% CO$_2$.

**Examination of cell morphological changes and cell viability.** HaCaT cells were cultured to 80% confluence and harvested (passage 2). Cells (1×10$^6$ cells/ml) were cultured in 12-well plates with DMEM overnight. Cells were treated with DMSO (as control group), tetrandrine at 20 μM, H$_2$O$_2$ at 500 μM, or pre-treated with 20 μM tetrandrine for 1 h and then treated with 500 μM H$_2$O$_2$ for 3, 6, or 12 h. After treatment, cells were observed under phase-contrast microscopy, and cell viability examination was performed by PI exclusion assay as described previously (38).

**Annexin V/PI staining.** Annexin V/PI double staining assay was used to measure apoptotic cell death as described previously (38). HaCaT cells (1×10$^5$ cells/ml) were plated in 12-well plates for 24 h. The cells were treated with DMSO, 500 μM H$_2$O$_2$ or pre-treated with 20 μM tetrandrine for 1 h and then treated with 500 μM H$_2$O$_2$ for 12 h. After treatment, cells from individual wells were collected, resuspended in annexin V binding buffer, and incubated with annexin V/PI in the dark for 15 min. All cells from each treatment were collected and further analyzed using BD FACSCalibur (BD Biosciences, San Jose, CA, USA) for determining apoptotic cell numbers. Experiments were performed in triplicate.
with 20 μM tetrandrine for 1 h and then treated with 500 μM H\textsubscript{2}O\textsubscript{2} for 12 h. Subsequently, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and stained with DAPI solution (2 μg/ml) and examined and photographed under a fluorescence microscope as described previously (39).

**Western blotting analysis.** HaCaT cells (3×10\textsuperscript{6} cells/dish) were cultured onto 10-cm dishes overnight and treated with DMSO, 500 μM H\textsubscript{2}O\textsubscript{2} or pre-treated with 20 μM tetrandrine for 1 h and then treated with 500 μM H\textsubscript{2}O\textsubscript{2} for 12 h. At the end of incubation, cells were collected, total proteins were extracted, and their protein concentration was quantified by Bio-Rad protein assay kit (Hercules, CA, USA) (40-42). A defined amount of sample (30 μg) from each treatment was separated by 10% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (Millipore, Belford, MA, USA). Subsequently, the membranes were reacted with primary antibodies against SOD (Cu/Zn), SOD (Mn), catalase, ATM, ATR, p-H2A.X, p-P53, P53, BAX, BCL2, caspase-3, caspase-8, caspase-9, PARP, and β-actin at 4°C overnight. Membranes were then washed with PBS with Tween\textsuperscript{®} 20, incubated with peroxidase-conjugated anti-mouse IgG, and proteins bands were visualized by their chemiluminescence signals using enhanced chemiluminescent
detection kit (Amersham Biosciences ECLTM, Buckinghamshire, UK) as described previously (40, 43).

Confocal laser scanning microscopy for measuring protein expression. After plating on chambered coverslips at 1×10^{5} cells/ml, HaCaT cells were treated with DMSO, 500 μM H_{2}O_{2} or pre-treated with 20 μM tetrandrine for 1 h and then with H_{2}O_{2} for 12 h. Cells were then fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.1% Triton-X 100 in PBS for 15 min. All samples were then washed and stained with primary antibodies to SOD1, catalase, and glutathione. Cells were washed with PBS, stained by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (green fluorescence), and their nucleus was stained by PI (red fluorescence) as described previously (41). Individual samples were photographed for expression of associated proteins under a Leica TCS SP2 confocal microscope (Leica Microsystems, Bannockburn, IL, USA).

Statistical analysis. The results are presented as the mean±standard deviation. All samples were obtained three times independently. Statistical analyses were performed by one-way analysis of variance followed by Tukey test for determining significant differences among groups (p<0.05) using Sigma Plot 12 software (Systat Software, Inc., San Jose, CA, USA).

Results

Tetrandrine altered cell morphology and viability of HaCaT cells. HaCaT cells were treated with tetrandrine or H_{2}O_{2}, alone, or sequentially. Cells were monitored and photographed under phase-contrast microscopy. The cell morphology was unmistakably changed after 12-h treatment (Figure 1A). The total cell viability under each treatment was measured by PI exclusion assay. As shown in Figure 1B, pre-treatment of HaCaT cells with tetrandrine and then H_{2}O_{2} led to a reduction of cell viability (Figure 1B) when compared to cells treated with TET or H_{2}O_{2} alone.

Tetrandrine induced apoptotic cell death of HaCaT cells. As shown in Figure 2, when compared to the control group, H_{2}O_{2} (500 μM) treatment induced 7.2-fold annexin V-
positive cells (apoptotic cells), whilst pre-treatment with 20 μM of tetrandrine and then with \( H_2O_2 \) induced 9.0-fold annexin V-positive cells. These results indicated that \( H_2O_2 \) treated with tetrandrine increased apoptotic cell death and reduced the number of viable cells.

**Tetrandrine induced morphological changes and chromatin condensation in nuclei (apoptotic death) of HaCaT cells.** After HaCaT cells were pre-treated with tetrandrine and then with \( H_2O_2 \), cells were stained with DAPI and photographed under fluorescence microscopy. The results shown in Figure 3 indicate the brighter fluorescence of nuclei in HaCaT cells after pre-treatment with tetrandrine compared with that of \( H_2O_2 \) treatment alone. This bright fluorescence indicates that DNA was naked or chromatin was condensed. After calculating the difference in fluorescence (fold of control) between treated and untreated cells, the data indicated that cells pre-treated with tetrandrine had a 2.6-fold higher intensity than those under \( H_2O_2 \) treatment.

**Tetrandrine affects ROS production, DNA damage, and expression of apoptosis-associated proteins in HaCaT cells.** As shown in Figure 4, when compared to the control group, pre-treatment with tetrandrine then with \( H_2O_2 \) significantly increased the production of ROS-associated proteins such as SOD (Cu/Zn) and SOD (Mn) but significantly reduced the level of catalase (Figure 4A). It also increased the expression of DNA repair-associated proteins ATM, ATR, p-P53, P53, and p-H2A.X when compared to the control group (Figure 4B). Furthermore, such treatment increased the levels of pro-apoptotic proteins BAX, caspase-3, caspase-8, caspase-9, and PARP in HaCaT cells, whilst that of anti-apoptotic protein BCL2 did not decrease when compared to the control group (Figure 4C).

**Tetrandrine affects the expression and the nuclear translocation of SOD1, catalase, and glutathione in \( H_2O_2 \)-treated HaCaT cells.** The results from western blotting indicated that pre-treatment of tetrandrine then treatment with \( H_2O_2 \) significantly increased SOD (Cu/Zn) and SOD
but reduced catalase (Figure 4A) in HaCaT cells. In order to further confirm these associations of H$_2$O$_2$ treatment and their effects on SOD and glutathione expression, confocal laser microscopy was used to measure expression of both proteins in HaCaT cells. After cells were pre-treated with or without tetrandrine and treated with H$_2$O$_2$ (500 μM) for 12 h, cells were probed with SOD1, catalase, and glutathione antibodies, and then examined, observed and photographed under confocal laser microscopy. As shown in Figure 5, tetrandrine promoted the expression and nuclear translocation of SOD1 and glutathione but reduced the expression of catalase in HaCaT cells, indicating these observations are in agreement with the results from the western blotting assay.

**Discussion**

It is well documented that H$_2$O$_2$, an inducer of ROS (42, 44), also induces cytotoxic effects which cause cell death via induction of apoptosis, depending on the dose (39, 45). Cells produce ROS such as O$_2^-$, OH$^-$, and H$_2$O$_2$ in physiological intracellular reactions and functions in cellular metabolism,
Antioxidants are able to attenuate the damaging effects of ROS, and tetrandrine has been suggested to have potential as an antioxidant drug in (47). Studies have shown that tetrandrine induces cytotoxic effects through the induction of apoptosis of many types of human cancer cells in vitro (48, 49). The relationship between tetrandrine- and H$_2$O$_2$-induced cell death remains underexplored. Herein, we found that H$_2$O$_2$ induced morphological cell changes and reduced total viable HaCaT cells in vitro. However, HaCaT cells pretreated with tetrandrine and then treated with H$_2$O$_2$ led to increasing morphological changes and increased cell death.
Tetrandroline augmented the H$_2$O$_2$-induced cytotoxic effect. Another reason for selecting H$_2$O$_2$ for this study is that H$_2$O$_2$-induced apoptosis of retinal pigmented epithelial cells is a well-known study model for drug discovery (50-53), so our model system is similar.

Alone, tetrandroline and H$_2$O$_2$ both induce cancer cell apoptosis (30-32, 45, 46). Therefore, in order to further understand whether tetrandroline enhances H$_2$O$_2$ induced cytotoxic effects (reduced total viable cell number) in HaCaT cells through apoptotic cell death, annexin V/PI double staining assay was used to measure cell apoptosis. Annexin V/PI double staining assay indicated that H$_2$O$_2$ induced cell apoptosis (Figure 2A) and tetrandroline indeed enhanced H$_2$O$_2$-induced cell apoptosis (Figure 2B) in HaCaT cells. DAPI staining assay confirmed that tetrandroline also significantly enhanced H$_2$O$_2$-induced chromatin condensation (one of the characteristics of cell apoptosis) (Figure 3) in HaCaT cells. Both results are in agreement for tetrandroline enhancing H$_2$O$_2$-induced cell apoptosis in HaCaT cells in vitro.

We used western blotting assay for examining cellular protein expression in HaCaT cells in order to elucidate the possible molecular mechanisms involved in apoptosis induction. Pre-treatment of HaCaT cells with tetrandroline significantly enhanced H$_2$O$_2$ induced protein expression of pro-apoptotic BAX, caspase-3, caspase-8, caspase-9, and PARP but reduced that of anti-apoptotic protein BCL2 (Figure 4C). The anti-apoptotic BCL2-family proteins and apoptotic BAX protein play important roles in mitochondrion-dependent extrinsic and intrinsic cell death pathways (54, 55). BAX induces release of cytochrome c from mitochondria, resulting in activation of caspase-9, PARP and caspase-3 for the development of apoptosis (54, 55). Caspase-3, BAX, BCL2, and cytochrome c are well-known markers of apoptosis. Our results also showed that pre-treatment with tetrandroline significantly enhanced expression of H$_2$O$_2$-increased DNA repair-associated proteins, including ATM, ATR, p-P53, P53, and p-H2A.X (Figure 4B). Upon DNA damage, P53 expression and activation increases, resulting in repair of DNA damage or leading to cell death. The DNA repair-associated proteins such as ATM, ATR, and p-H2A.X that in these studies were increased (Figure 4B). ATR is closely related to two other DNA damage response kinases (ATM, and DNA-PK), these kinases responding to different DNA damage insults, that are primarily double-strand breaks for ATM and DNA-PK, and replication stress for ATR (56). γH2A.X expression was increased in HaCaT cells after pre-treatment with tetrandroline and treatment with H$_2$O$_2$. γH2A.X is a marker associated with DNA damage and repair (57).

Psoriasis is a skin disorder that shows a marked epidermal hyper-proliferation of keratinocytes, aberrant differentiation, and keratinocyte inflammation (58, 59). Herein, our results indicated that tetrandroline enhanced H$_2$O$_2$-induced apoptotic cell death of keratinocytes, thus, we suggest tetrandroline combined with H$_2$O$_2$ may be a potent approach for treating diseases involving hyper-proliferation of keratinocytes.

Numerous studies have shown that many diseases such as cancer, diabetes, and Parkinson's disease are associated with intracellular H$_2$O$_2$ generated via extracellular H$_2$O$_2$ (60-62). Antioxidants extracted from natural products can reduce skin injury, aging, and cancer risk based on their antioxidant activities (63, 64). Tetrandroline was reported to increase the expression of antioxidative enzymes (SOD and glutathione) in animal studies (65). Tetrandroline is one of the major components in traditional Chinese herbal medicine and has been shown to attenuate oxidative stress. Catalase, SOD, and glutathione peroxidase are major enzymes for effectively scavenging ROS and attenuating oxidative damage (66, 67). Catalase can break down H$_2$O$_2$ into its inert components, which has been associated with the determining the cancer-killing ability of natural products (68). Furthermore, H$_2$O$_2$ has been applied to different cell types, and can reduce cell viability, depending on a decrease in the concentration of catalase (69). Based on the results from our confocal laser microscopy study, tetrandroline pre-treatment and then treatment with H$_2$O$_2$ reduced H$_2$O$_2$-induced expression of catalase; however, it increased that of SOD(Cu/Zn) and SOD(Mn) (Figure 4A) in HaCaT cells. These results are in agreement with the results from confocal laser microscopy (Figure 5A).

In the present work, pre-treatment of HaCaT cells with tetrandroline significantly subsequently enhanced H$_2$O$_2$-induced cell apoptosis, through the molecular mechanism...
shown in Figure 6. These findings indicate a potential approach for the therapy of diseases associated with keratinocyte proliferation.

**Conflicts of Interest**

The Authors confirm that there are no conflicts of interest in regard to this study.

**Authors’ Contributions**

Study conception and design: YCC, FSC and WWH. Acquisition of data: YCC, CLK, SYH, TDW, CLC and JCC. Analysis and interpretation of data: YCC, KCL, SFP and WJH. Drafting of article: YCC, FSC and WWH. Critical revision: YCC and WWH.

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