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Cytoprotective Effects of Sulfuretin from *Rhus verniciflua* through Regulating of Heme Oxygenase-1 in Human Dental Pulp Cells

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Abstract – *Rhus verniciflua* Stokes (Anacadiaceae) is a plant that is native to East Asian countries, such as Korea, China, and Japan, and it has been found to exert various biological activities including antioxidative, anti-aggregatory, anti-inflammatory, anti-mutagenic, and apoptotic effects. Sulfuretin is one of the major flavonoid component isolated from the heartwood of *R. verniciflua*. Reactive oxygen species (ROS), produced via dental adhesive bleaching agents and pulpal disease, can cause oxidative stress. In the present study, we isolated sulfuretin from *R. verniciflua* and demonstrated that sulfuretin possesses cytoprotective effects against hydrogen peroxide (H_2O_2)-induced dental cell death. H_2O_2 is a representative ROS and causes cell death through necrosis in human dental pulp (HDP) cells. H_2O_2-induced cytotoxicity and production of ROS were blocked in the presence of sulfuretin, and these effects were dose dependent. Sulfuretin also increased heme oxygenase-1 (HO-1) protein expression. In addition, to determine whether sulfuretin-induced HO-1 expression mediated this cytoprotective effect, HDP cells were cotreated with sulfuretin in the absence or presence of SnPP, an inhibitor of HO activity. Sulfuretin-dependent HO-1 expression was required for suppression of H_2O_2-induced HDP cell death and ROS generation. These results indicate that sulfuretin-dependent HO-1 expression was required for the inhibition of H_2O_2-induced cell death and ROS generation. In addition, sulfuretin may be used to prevent functional dental cell death and thus may be useful as a pulpal disease agent.
Keywords – *Rhus verniciflua*, Sulfuretin, Human dental pulp cells, Heme oxygenase-1, Cytoprotective effect

Introduction

Rhus verniciflua Stokes (Anacadiaceae) is a plant that is native to East Asian countries, such as Korea, China, and Japan. *R. verniciflua* has been found to exert various biological activities including antioxidant activity (Lee *et al*., 2001; Lim *et al*., 2001), anti-aggregatory activity (Jeon *et al*., 2003), anti-inflammatory effects (Kim *et al*., 2004), anti-mutagenic activity (Park *et al*., 2004) and also apoptotic effects in human cancer cell lines (Lee *et al*., 2002; Son *et al*., 2005; Ko *et al*., 2005). Recent studies suggested that an extract of *R. verniciflua* protected against oxidative stress by scavenging reactive oxygen species (ROS) (Lim *et al*., 2001). The antioxidant properties of *R. verniciflua* are mainly attributable to their abundance of phenolics and flavonoids. Sulfuretin is one of the major flavonoid component isolated from the heartwood of *R. verniciflua.* In our previous study, sulfuretin exhibited anti-inflammatory effects against lipopolysaccharide (LPS)-induced inflammation in RAW264.7 and murine macrophages obtained from C57BL/6 mice (Lee *et al*., 2010) and we also suggested the effect of sulfuretin on an ovalbumin-induced airway inflammation model in mice (Song *et al*., 2010). However, to date, no studies have described the protective effects of sulfuretin in dental cells.

The human dental pulp (HDP) contains a heterogeneous population of cells. HDP is the living connective tissue in the center of a tooth made up of cells called odontoblasts.

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The primary function of the dental pulp is the formation of dentin by odontoblasts. The dentine-pulp complex is formed by differentiated and undifferentiated cell populations, including odontoblasts, odontoblast-like cells, and pulp stem cells (Sloan and Smith, 2007). Cells are damaged by imbalances in the systems generating and scavenging ROS. In addition, ROS, produced by dental adhesive bleaching agents and pulpal disease, can cause oxidative stress in dental cells (Min *et al*., 2008). Under mild oxidative stress, the odontoblast differentiated from the pulp usually can survive to produce dentin. However, continued severe stress, including oxidative stress, induces apoptosis of pulp cells resulting in a lack of ability to proliferate to replace the injured odontoblast. Therefore, pulp viability is important for tooth vitality. Among the various types of ROS, hydrogen peroxide (H_2O_2) is generated from most sources of oxidative stress and plays a key role in oxidative stress-mediated pulpal diseases (Matés and Sánchez-Jiménez, 1999). H_2O_2 has been used directly or indirectly to the tooth surface as a tooth bleaching agent (Gerlach *et al*., 2004).

The inducible protein heme oxygenase-1 (HO-1) is expressed in response to oxidative stress and exhibits various cytoprotective effects, which are mostly associated with the different by-products of heme catabolism, with the exception of its ability to remove pro-oxidant heme molecules. HO-1 degrades heme to generate carbon monoxide (CO), biliverdin, and free iron (Montellano, 2000). HO-1 and its enzymatic by-products play key roles in regulating biological responses, including oxidative stress and inflammation (Ryter *et al*., 2002). Previously, we reported that the HO-1 pathway is involved in the adaptation of dental cells, including HDP cells and periodontal ligament (PDL) cells, to stressful conditions and mediates their recovery from injurious events (Jeong *et al*., 2010). In the present study, we found that sulfuretin possesses cytoprotective effects on H_2O_2-induced dental cell death. Our data showed that sulfuretin inhibited H_2O_2-induced cell death and ROS production through regulation of HO-1 expression. These results indicate that sulfuretin, a natural compound isolated from *R. verniciflua*, may be used to prevent functional dental cell death and thus may be useful as a pulpal disease agent.

Experimental

Preparation of sulfuretin – Sulfuretin (> 96.8% pure) was isolated from the heartwood of *R. verniciflua* as described by Lee *et al*. (Lee *et al*., 2010). The heartwood of *Rhus verniciflua* Stokes (Anacardiaceae) was purchased from Dongbu Market, Iksan, Korea in March, 2009. The voucher specimen (WK-2009-42) was deposited at the Herbarium of College of Pharmacy, Wonkwang University (Korea). Sulfuretin (NNMBP009) was deposited at the New Natural Material Bank of the College of Pharmacy, Wonkwang University, Korea. Sulfuretin was dissolved in dimethyl sulfoxide (DMSO) to make a 30 mM solution (stock solution) and then diluted with medium. The final DMSO concentration in each experimental and control well was kept constant at 0.1%. This final DMSO concentration had no relevant effects on cellular growth or survival in our assay.

Chemicals and reagents – Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Gaithersburg, MD, USA). Tin protoporphyrin IX (SnPP IX), an inhibitor of HO activity, was obtained from Porphyrin Products (Logan, UT, USA). Primary antibodies, including HO-1, and secondary antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tissue culture plates (96-well) and other tissue culture dishes were obtained from Falcon (Biosciences, Oxford, UK). All other chemicals, including 3'-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO, were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise noted.

Cell culture – Immortalized HDP cell lines obtained by transfection with the telomerase catalytic subunit human telomerase reverse transcriptase gene were used for this study (Kitagawa *et al*., 2007). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 ºC.

MTT assay for cell viability – The effects of various experimental treatments on cell viability were evaluated by determining mitochondrial reductase functionality on the basis of the reduction of a tetrazolium salt, MTT, into formazan crystals (Mosmann, 1983). The formation of formazan is proportional to the number of functional mitochondria in living cells. After the treatment with hydrogen peroxide (H_2O_2) (500 μM) for 12 h, cells were washed twice with PBS, and the MTT solution was added at a final concentration of 50 μg/mL to each well. After 4 h incubation at 37 ºC, the medium was discarded and the formazan blue that formed in the cells was dissolved in 150 μL DMSO. The optical density was determined with a microplate reader at 570 nm, and the optical density of control (untreated) cells was taken as 100% viability.

Measurement of ROS – For the measurement of ROS,

HDP cells (2.5×10^4 cells/well in 24-well plates) were treated with 500 μM H_2O_2 in the presence or absence of sulfuretin, cobalt protoporphyrin (CoPP, an HO-1 inducer) or SnPP (an HO inhibitor) and incubated for 8 h. After washing with PBS, the cells were stained with 10 μM dichlorofluorescein diacetate (DCFDA) in Hanks' balanced salt solution for 30 min in the dark. Cells were then washed twice with PBS and extracted with 1% Triton X-100 in PBS for 10 min at 37 °C. Fluorescence was recorded with an excitation wavelength of 490 nm and an emission wavelength of 525 nm (Spectramax Gemini XS; Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis – Western blot analysis was performed by lysing cells in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 1 mg/mL chymostatin). Protein concentrations were determined using the Lowry protein assay kit (P5626; Sigma). An equal amount of protein for each sample was resolved using 7% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skim milk and sequentially incubated with anti-HO-1 or anti-actin at 4 °C overnight (all antibodies were used at a 1 : 1000 dilution and were purchased from Santa Cruz Biotechnology (Santa Curz, CA, USA). Immunoreactive bands were visualized by horseradish peroxidase-conjugated secondary antibodies (1 : 1000 dilution, Santa Cruz Biotechnology) followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ) and were quantified using an imaging program (Image Gauge *v*3.12 software, Fujifilm, Tokyo, Japan).

Statistical analysis – Data are expressed as the mean ± SD of at least 3 independent experiments. To compare 3 or more groups, one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test was used. Statistical analysis was performed with GraphPad Prism software, version 3.03 (GraphPad Software Inc, San Diego, CA, USA). Differences associated with a *p*-value of less than 0.05 were considered statistically significant.

Results and Discussion

Oxidative stress is a major underlying contributor to the development of various pathological dental diseases. ROS are produced by exposure to dental adhesive bleaching agents and pulpal disease, and there are known to cause oxidative stress in dental cells. Because of this, naturally occurring compounds that possess intrinsic antioxidant properties and can trigger intracellular cytoprotective signaling cascades in dental cells may offer a promising paradigm for therapeutic applications. The antioxidant properties of *R. verniciflua* are mainly attributable to the abundance of phenolics and flavonoids in this plant. Sulfuretin is one of the major flavonoid component isolated from the heartwood of *R. verniciflua.* Our previous studies have shown that sulfuretin has pharmacological effects, such as antioxidative and anti-inflammatory effects in murine macrophages (Lee *et al*., 2010) and inhibition effects of allergic airway inflammation (Song *et al*., 2010). In addition, previous other studies have shown that sulfuretin has several pharmacological effects, such as anti-apoptotic effect (Lee *et al*., 2012a) and preventing or delaying the progression of rheumatoid arthritis (Lee *et al*., 2012b). However, no studies have been published on the protective effects of sulfuretin *via* HO-1 expression in dental cells.

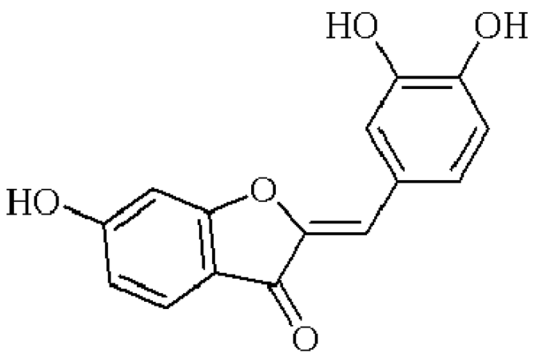

Fig. 1. Chemical structure of sulfuretin.

To examine the cytotoxic potential of sulfuretin (Fig. 1), its effect on the viability of HDP cells was measured by MTT assay. HDP cells were treated with sulfuretin and then incubated for 12 h and treated with sulfuretin and then incubated for various times. Although sulfuretin had no cytotoxic effects at 1 - 40 μM concentrations, we found that sulfuretin exhibited slight cytotoxicity on HDP cells at high concentrations (> 40 μM; Fig. 2A). In addition, sulfuretin for 40 μM had no cytotoxic effects up to 48 hours (Fig. 2B). H_2O_2, a representative ROS, causes cell death through necrosis in HDP cells (Min *et al*., 2008). Next, we tested the effects of sulfuretin on H_2O_2-induced cytotoxicity and ROS production in HDP cells. HDP cells were treated with sulfuretin and then incubated for 12 h with 500 μM H_2O_2. Exposure of HDP cells to 500 μM H_2O_2 for 12 h increased the production of ROS. Our data demonstrated that 500 μM H_2O_2 caused a

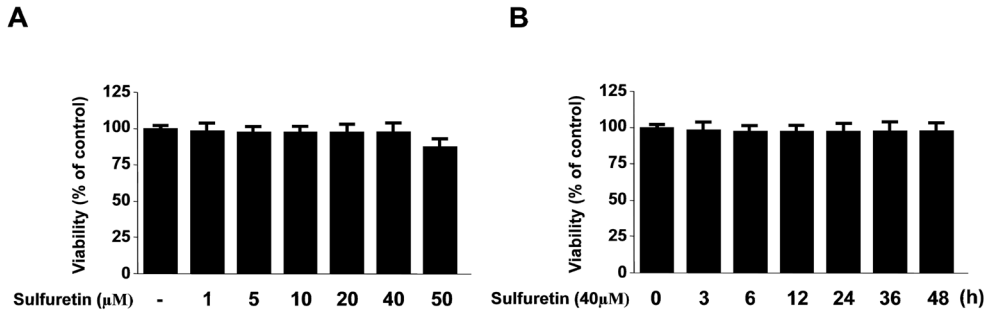

Fig. 2. Effects of sulfuretin on cell viability. Cells were treated with sulfuretin and then incubated for 12 h (A). Cells were treated with sulfuretin and then incubated for various times (B). Cell viability was then determined by MTT assay. Data represent the mean values ± SD of three experiments.

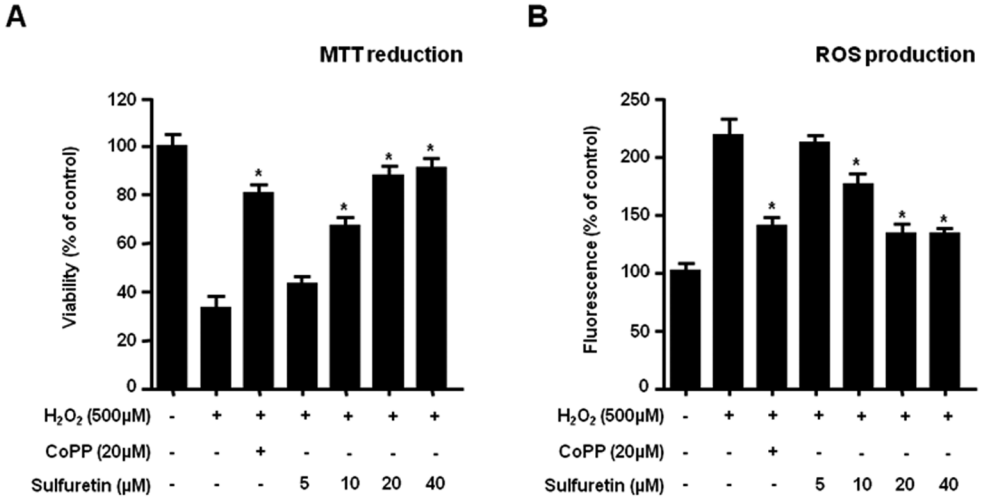

Fig. 3. Effects of sulfuretin on hydrogen peroxide-induced oxidative toxicity and inhibition of reactive oxygen species generation. Cells were treated with sulfuretin and then incubated for 12 h with hydrogen peroxide (500 μM) (A). Exposure of cells to 500 μM hydrogen peroxide for 12 h increased the production of reactive oxygen species (B). Cell viability was then determined by MTT assay. Data represent the mean values ± SD of three experiments. $^{*}p < 0.05$ vs. untreated control. (one-way ANOVA and Tukey's keyway AN comparison tests). CoPP (20 μM) was used as the positive control.

significant reduction in cell viability to 38.52 ± 1.32% of control levels and also induction in ROS production to 223 ± 8.78% of control levels, as indicated by MTT assay (Fig. 3A) and ROS measurements (Fig. 3B), respectively. H_2O_2-induced cytotoxicity and ROS production were significantly inhibited in the presence of CoPP, a HO-1 inducer and sulfuretin, and these effects were dose dependent. The pre-treatment of sulfuretin with 10, 20 and 40 μM caused a significant induction in cell viability to 68.21 ± 2.21%, 80.41 ± 1.5% and 88.15 ± 1.76% of control levels and also reduction in ROS production to 175.33 ± 11.01%, 142.11 ± 8.3% and 140.22 ± 4.32% of control levels. Generally, the cytoprotective properties of antioxidants have been partially attributed to their ability to induce cytoprotective enzymes.

HO-1 has various cytoprotective effects that are mostly associated with the different by-products of heme catabolism, with the exception of its ability to remove pro-oxidant heme molecules. HO-1 is a key cytoprotective and rate-limiting enzyme in the degradation of heme to bilirubin, carbon monoxide (CO), and iron. It is well known that the expression of HO-1, a critical cytoprotective enzyme that is induced in response to oxidative injury, is considered to be an adaptive and protective response against oxidative insults in a wide variety of cells (Maines, 1988; Alam *et al*., 1999; Otterbein *et al*., 2003). Previous other reports have demonstrated that the HO-1 pathway is a key mechanism for the adaptation of cells to stressful conditions and recovery from the injurious events of human pulp and HPDL cells (Min *et al*., 2008). At noncytotoxic concentrations (5 - 40 μM), we investigated whether sulfuretin affected HO-1 protein expression.

HDP cells were incubated for 12 h with 5 - 40 μM sulfuretin. Sulfuretin treatment increased HO-1 protein expression (Fig. 4) in HDP cells in a concentration-dependent manner. As a positive control, the HO-1 inducer, CoPP, caused an increase HO-1 protein expression. To determine whether sulfuretin-induced HO-1 expression mediated this cytoprotective effect and ROS reduction, HDP cells were co-treated with 5 - 40 μM sulfuretin for 12 h in the absence or presence of SnPP, an inhibitor of HO activity (Fig. 5). HDP cells were treated with 20 or 40 μM sulfuretin or 20 μM CoPP in the presence or absence of 50 μM SnPP and then exposed to 500 μM H_2O_2 for 12 h. While the HO-1 inducer CoPP showed comparable cytoprotection to sulfuretin alone, sulfuretin-dependent HO-1 expression was required for suppression of H_2O_2-induced HDP cell death (Fig. 5A) and ROS generation (Fig. 5B). A series of plant-derived chemicals have been reported to induce HO-1 expression in different cell types, and HO-1 was shown to mediate the antioxidant/ cytoprotective properties of these compounds (Chen and Regan, 2005). Therefore, we demonstrated, for the first time, that naturally occurring sulfuretin isolated from *R. verniciflua* suppressed cellular damage from oxidation caused by H_2O_2 in HDP cells, presumably through HO-1 expression.

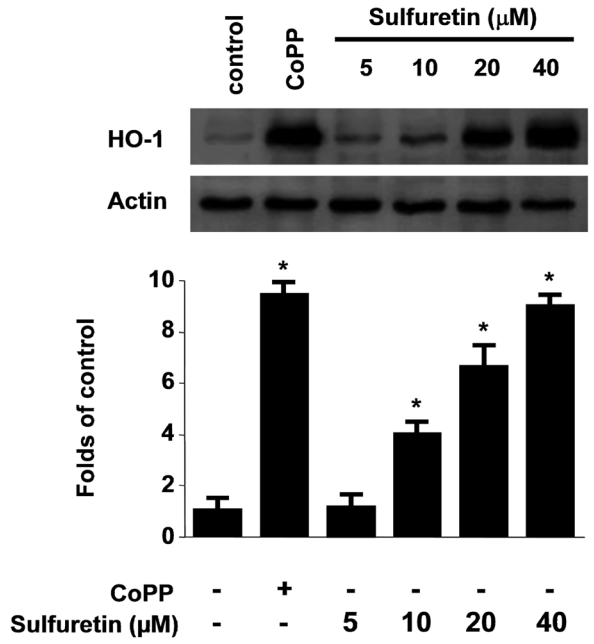

Fig. 4. Effects of sulfuretin on HO-1 expression in human dental pulp cells. Cells were incubated for 12 h with sulfuretin. Expression of HO-1 was determined by western blot analysis, and representative blots from 3 independent experiments are shown. Data represent the mean values ± SD of three experiments. $^{*}p < 0.05$ vs. untreated control. (one-way ANOVA and Tukey's multiple comparison tests).

Conclusion

Natural products have been recognized as invaluable source of the most active components of medicines for treating and preventing various human diseases. The importance of natural products in drug discovery and

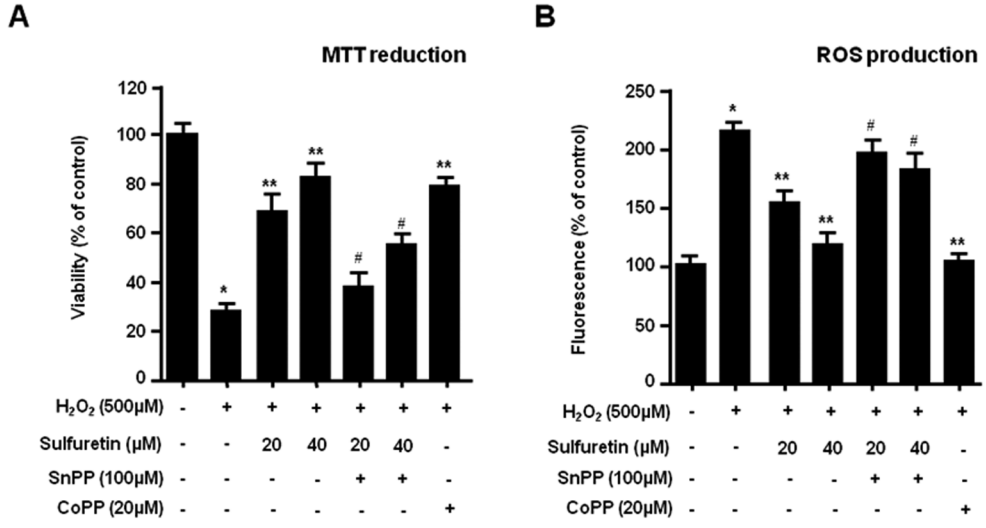

Fig. 5. Effects of sulfuretin-induced HO-1 on hydrogen peroxide-induced oxidative toxicity and inhibition of reactive oxygen species generation. Cells were treated with 20 or 40 μM sulfuretin or 20 μM CoPP in the presence or absence of 50 μM SnPP and then exposed to hydrogen peroxide (500 μM) for 12 h (A). Exposure of human dental pulp (HDP) cells to 500 μM hydrogen peroxide for 12 h increased reactive oxygen species production (B). Data represent the mean values ± SD of three experiments. $^{*}p < 0.05$ vs. untreated control. $^{**}p < 0.05$ vs. hydrogen peroxide (500 μM). $^{\#}p < 0.05$ vs. hydrogen peroxide with sulfuretin (20 or 40 μM). (one-way ANOVA and Tukey's multiple comparison tests). CoPP (20 μM) was used as the positive control.

development has been discussed in recent reviews and reports (Newman *et al*., 2003; Koehn and Carter, 2005; Paterson and Anderson, 2005). Oxidative stress is a major underlying contributor to the development of dental disease. In dental cells, ROS, produced by exposure to dental adhesive bleaching agents and pulpal disease, are known to cause oxidative stress. Because of this, naturally occurring compounds that possess intrinsic antioxidant properties and can trigger intracellular cytoprotective signaling cascades in dental cells may offer a promising paradigm for therapeutic applications. In addition, it is well known that HO-1 plays important roles in regulating biological responses, including oxidative stress. Taken together, this study found that sulfuretin possesses cytoprotective effects on H_2O_2-induced dental cell death. H_2O_2-induced cytotoxicity and ROS production were significantly inhibited in the presence of sulfuretin, and these effects were dose dependent. In addition, sulfuretin treatment increased HO-1 protein expression in a concentration-dependent manner, and sulfuretin-dependent HO-1 expression was required for suppression of H_2O_2-induced HDP cell death and ROS generation. Our data showed that sulfuretin isolated from *R. verniciflua* actively induced HO-1 expression in HDP cells, leading to the suppression of H_2O_2-induced oxidative cell death and ROS production. This study strongly suggests that sulfuretin may be a promising therapeutic agent for the treatment of various dental diseases.

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