

# Effect of vitamin C administration on hydrogen peroxide-induced cytotoxicity in periodontal ligament cells

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**Abstract.** Periodontitis is a disease, which is associated with chronic inflammation and leads to significant destruction of periodontal tissues. Periodontal ligament cells (PDLs) constitute the largest cell population in PDL tissues and a considerable body of evidence has demonstrated an association between oxidative stress and the progression of periodontitis. However, the effects on PDLs exposed to hydrogen peroxide ( $H_2O_2$ ) and the molecular mechanisms by which  $H_2O_2$  affects periodontitis remain to be elucidated. In the present study, the potential cytotoxic effect of  $H_2O_2$  and the antioxidative function of vitamin C (Vc) in PDLs were investigated. The results demonstrated that  $H_2O_2$  treatment decreased the viability of PDLs. The decreased PDL viability was primarily induced by apoptosis, which was evidenced by cleaved caspases-3, caspases-9 and poly (ADP-ribose) polymerase. Following optimal Vc addition, the proapoptotic effects of  $H_2O_2$  were partially antagonized. Taken together, the present study demonstrated that  $H_2O_2$  primarily induced the apoptosis of PDLs and that these adverse effects were partially rescued following treatment with Vc. These results revealed how  $H_2O_2$  promotes the progression of periodontitis and provide an improved understanding of the reversal effect of antioxidant treatment. Therefore, optimal Vc administration may provide a potentially effective technique in periodontal therapy.

## Introduction

Periodontitis is a chronic inflammatory disease, which is characterized by bleeding, destruction of connective tissues and

alveolar bone loss (1-3). In addition, periodontitis is reported to be implicated in the onset of a variety of diseases, including diabetes, rheumatoid arthritis and cardiovascular disease (4). The occurrence of periodontitis is ascribed to multiple factors. Of these, with the exception of bacterial infection and host contributing factors, the role of oxidative stress in the development and pathogenesis of periodontitis has been elucidated (5). Therefore, detailed investigation of the association between oxidative stress and periodontitis is of crucial importance.

Reactive oxygen species (ROS), which markedly contribute to cellular oxidative stress, are important signaling mediators in several biological processes (6). Living organisms have adapted to the efflux of ROS, in which antioxidants, including vitamin C (Vc) are important in counteracting the oxidative effects (7). These reactive species are generated from molecular oxygen and, if they are not cleared by the antioxidative system, these highly reactive species induce significant damage in the cellular environment (2). An imbalance between the upregulation of pro-oxidants and antioxidants can lead to severe impairment of critical cellular structures, membrane dysfunction and cell death by necrosis or apoptosis (8). Subtle alterations in the intracellular redox state initiates cellular events associated with altered gene expression and may eventually lead to destruction of or damage to the tooth supporting tissues secondary to the induction of proinflammatory processes.

The periodontal ligament (PDL) is an assembly of specialized tooth supporting tissue fibers that attach root cement to alveolar bone (9). The PDL is composed of diverse cell subpopulations, including fibroblasts and osteoblasts, with PDL cells (PDLs) constituting the majority. PDLs have fibroblast-like features and are able to produce collagen whilst simultaneously retaining osteoblastic features (10). The secreted collagens build up the PDL to ensure the attachment of root cement to alveolar bone and tissue recovery following injury. Human PDLs also produce cytokines and chemokines in conditions of stress, which are characteristic of leucocytes and macrophages (9). In addition, the abnormal reduction or structural destruction of PDLs increase the likelihood of pathogenesis in the periodontal tissues implying that PDLs may be important in initiating periodontal inflammation.

Given the proinflammatory status of periodontal tissues, ROS can also be linked to the pathogenesis of periodontitis.

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Hydrogen peroxide ( $H_2O_2$ ) is a type of ROS, which is generated by almost all types of oxidative stress and infiltrates cells through their membrane.  $H_2O_2$  has a broad spectrum of biological effects, while the cellular responses to  $H_2O_2$  may differ in a cell type and concentration-specific manner (11,12).  $H_2O_2$  may induce growth inhibitory effects, whereas in a similar setting it may also promote proliferation in other cell types (13). In addition a dual association between apoptosis and the concentration of  $H_2O_2$  has been demonstrated, which further complicates the exact functionality of  $H_2O_2$  exposure to cellular events (14).

## Materials and methods

**Cell culture.** The study was approved by the Ethics Committee of the Institute and Hospital of Stomatology, Nanjing University Medical School (Nanjing, China). Primary human PDLs were obtained from premolars, which were extracted from healthy patients aged between 10 and 18 years with no evidence of gingivitis, periodontitis or caries. All patients provided informed consent prior to sample collection. In brief, the human PDL tissues were obtained from the central tooth root surface using a surgical scalpel. The obtained tissues were then cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (50 g/ml streptomycin and 5,000 U/ml penicillin; Sigma Aldrich, St. Louis, MO, USA) at 37°C and 5%  $CO_2$  in a humidified atmosphere. The medium was replaced every 2-3 days. When the cultured PDLs reached confluence, they were trypsinized and divided at a ratio of 1:2 with 0.25% trypsin solution (Sigma Aldrich). Cells at passages 4-5 were used in the present study.

**MTT assay.** The PDLs were seeded into a 96-well-plate overnight at 37°C and were then washed with phosphate-buffered saline (PBS; Kangchen Biotech, Shanghai, China). The cells were treated with different concentrations of  $H_2O_2$  (600, 800 or 1,000  $\mu M$ ) for the indicated time periods between 0 and 48 h. MTT solution (20  $\mu l$ ; 5 mg/ml; Kangchen Biotech) was then added to each well. After 4 h incubation at 37°C, the media was removed and 100  $\mu l$  dimethyl sulfoxide (Kangchen Biotech) was added to each well in the plate to dissolve the purple formazan crystals. The plate was then agitated for 10 min for solubilization and the spectrophotometric absorbance at 540 nm was calculated using a Multilabel Counter (Safire; Tecan Austria GmbH, Grödig, Austria).

**Flow cytometry.** The cells were collected at specific time points (3 or 6 h) following  $H_2O_2$  treatment, washed in PBS and fixed using 75% ethanol overnight at -20°C. The fixed PDLs were then washed with cold PBS and stained using 5  $\mu l$  annexin V-fluorescein isothiocyanate (Kangchen Biotech) and propidium iodide (PI) solutions (0.1% Triton X-100, 100 mg/ml PI and 0.01 mg/ml RNase; Kangchen Biotech) for 30 min at 4°C in the dark. The successfully stained PDLs were further analyzed using a FACScan laser flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

**Western blot analysis.** The PDLs were incubated following treatment with various concentrations of  $H_2O_2$  and Vc for either

3 or 6 h. Following incubation, the PDLs were harvested and lysed. The proteins were then quantified using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions. The proteins were electrophoresed on a 12% SDS-PAGE gel for western blot analysis, followed by immunoblotting on a polyvinylidene difluoride membrane (Amersham Biosciences, Pittsburgh, PA, USA). Caspase-3 antibodies were purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA; cat no. BS1518). Mouse monoclonal antibodies for caspase-9 and PARP were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA; cat nos. 9508 and 9532, respectively). The proteins were incubated with the antibodies overnight at 4°C and subsequently with peroxidase-labeled anti-rabbit or anti-mouse antibodies (Kangchen Biotech) for 1 h at room temperature. Quantification of the blots was performed using a chemiluminescent method kit from Sino-American Biotechnology (Henan, China).

**Statistical analysis.** Statistical comparisons were performed using Welch's t-test or analysis of variance followed by Dunnett's test.  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$  were considered to indicate statistically significant differences.

## Results

**Identifying the optimal  $H_2O_2$  concentration for decreased PDL viability.** ROS have been suggested to induce marked destruction in periodontal tissues. To identify whether  $H_2O_2$  exerts a toxic effect on PDLs and to determine which concentration of  $H_2O_2$  causes a significant decrease in PDL viability, the PDLs were treated with different concentrations of  $H_2O_2$  for the indicated time periods (Fig. 1). The results demonstrated a significant decrease in PDL viability 3 h post- $H_2O_2$  treatment at concentrations  $> 600 \mu M$  (Fig. 1A). At 600  $\mu M$   $H_2O_2$ , ~50% of the PDLs were lost and the toxic effects of  $H_2O_2$  were relatively stable at higher levels of  $H_2O_2$  (Fig. 1A). Extending the treatment time to 6 h led to similar results (Fig. 1B) demonstrating that  $> 600 \mu M$   $H_2O_2$  caused PDL death within at least 6 h. Therefore,  $H_2O_2$  concentrations of 600, 800 and 1,000  $\mu M$  were selected for further investigation.

**High levels of  $H_2O_2$  result in morphological alterations and apoptosis in PDLs.** To evaluate the cytotoxic effect of  $H_2O_2$  on cell morphology, the PDLs were treated with different concentrations of  $H_2O_2$  (600-1,000  $\mu M$ ) for 3 or 6 h. Subsequently, morphological alterations in the PDLs were monitored. The untreated cells remained intact (Fig. 2A), however, when the PDLs were treated with high levels of  $H_2O_2$  (800 and 1,000  $\mu M$ ), the basic structure of the cells was lost, particularly at 1,000  $\mu M$   $H_2O_2$  (Fig. 2A). The cytotoxic effects on morphology were more evident 6 h post-stimulation (Fig. 2B) and the proportion of cells exhibiting shrinkage and loss of intact structure generally increased with increased  $H_2O_2$  levels (Fig. 2A and B). Taken together, these results suggested that  $H_2O_2$  induced PDL death through apoptosis in a dose- and time-dependent manner.

**Optimal concentrations of Vc antagonizes  $H_2O_2$ -induced cell death.** Several studies have suggested that the high degree

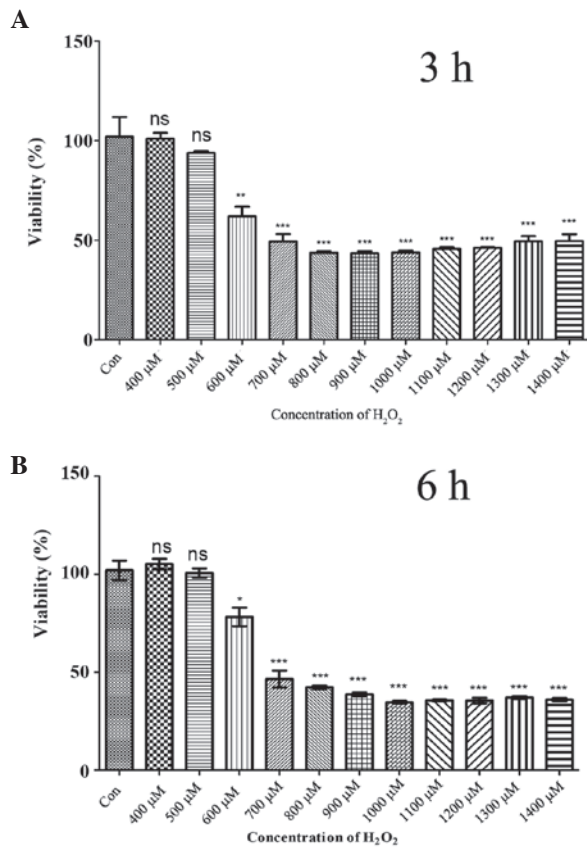


Figure 1. Superthreshold concentrations of H<sub>2</sub>O<sub>2</sub> decrease the viability of PDLCs. PDLCs were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> for (A) 3 and (B) 6 h and cell viability was evaluated using an MTT assay. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared with the control group. PDLCs, periodontal ligament cells; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; Con, control; ns, not significant.

of free radicals generated by bacterial stimulation function as an integrated part of host defense to infection and result in marked oxidative damage of periodontal tissues (2,3,11). The damage mediated by free radicals can be mitigated by the antioxidant defense system. To gain further insights into the potential protective effect of antioxidants, PDLCs were treated with different concentrations of Vc and the viability of cells was evaluated (Fig. 3). Relatively high levels of Vc (50-200 μM) were found to be cytotoxic, even without additional H<sub>2</sub>O<sub>2</sub> treatment, with the exception of 10 μM Vc (Fig. 3). These results suggested that Vc exerted pro-survival effects on H<sub>2</sub>O<sub>2</sub>-treated cells at specific concentrations only.

*Optimal Vc antagonizes the adverse effect of H<sub>2</sub>O<sub>2</sub>.* To understand the role of Vc, PDLCs were treated with different concentrations of Vc together with 800 or 1,000 μM H<sub>2</sub>O<sub>2</sub>. The viability of the PDLCs was monitored at 3 and 6 h. PDLCs did not retain their intact structure when treated with H<sub>2</sub>O<sub>2</sub> for 3 or 6 h (Fig. 4A and B). However, Vc administration partially reversed the adverse effect of H<sub>2</sub>O<sub>2</sub> treatment and this effect was more marked when the cells were treated with 1,000 μM H<sub>2</sub>O<sub>2</sub> (Fig. 4A and B). The antioxidative effect of Vc was also evaluated using an MTT assay. The results demonstrated a clear cytoprotective effect of Vc even at <600 μM H<sub>2</sub>O<sub>2</sub> for 3 h. (Fig. 4C). This antioxidative effect was even more clear when the PDLCs were treated with 1,000 μM H<sub>2</sub>O<sub>2</sub> (Fig. 4C).

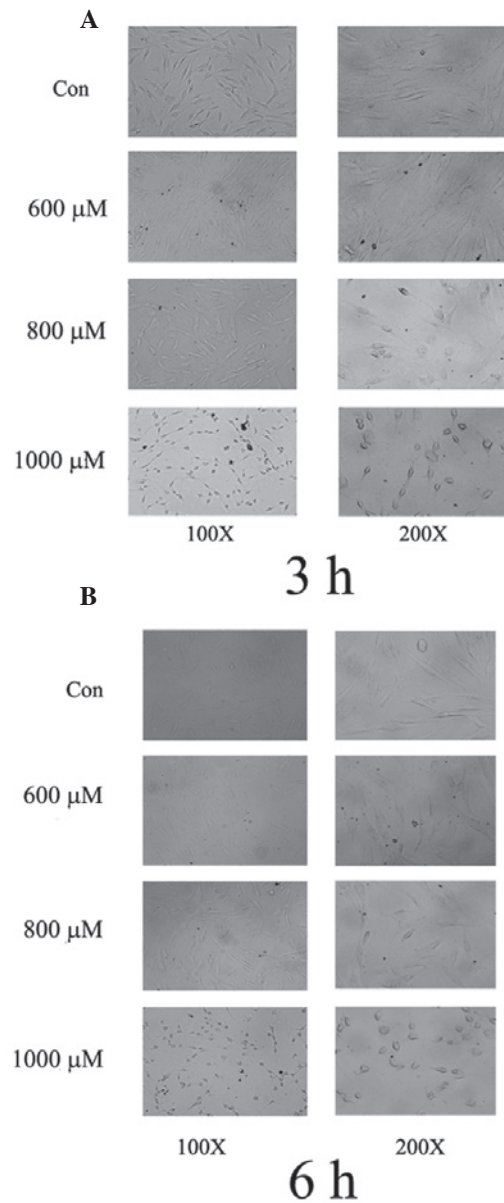


Figure 2. Effect of different concentrations of H<sub>2</sub>O<sub>2</sub> on the morphological alterations of periodontal ligament cells. Morphology was monitored at (A) 3 and (B) 6 h post-H<sub>2</sub>O<sub>2</sub> treatment using phase contrast microscopy (magnification, x100 and x200, respectively). H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; Con, control.

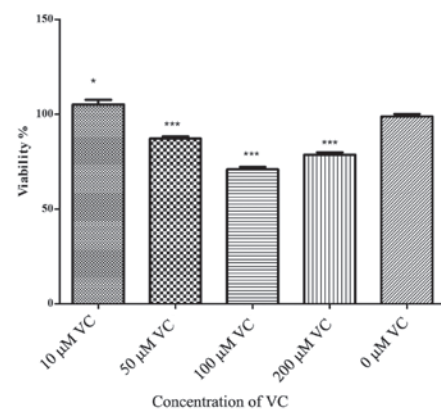


Figure 3. Effect of Vc on the viability of PDLCs. PDLCs were administered with varying concentrations of Vc and the viability was quantified using an MTT assay. \*P<0.05 and \*\*\*P<0.001 compared with the 0 μM Vc group. PDLCs, periodontal ligament cells; Vc, vitamin C.



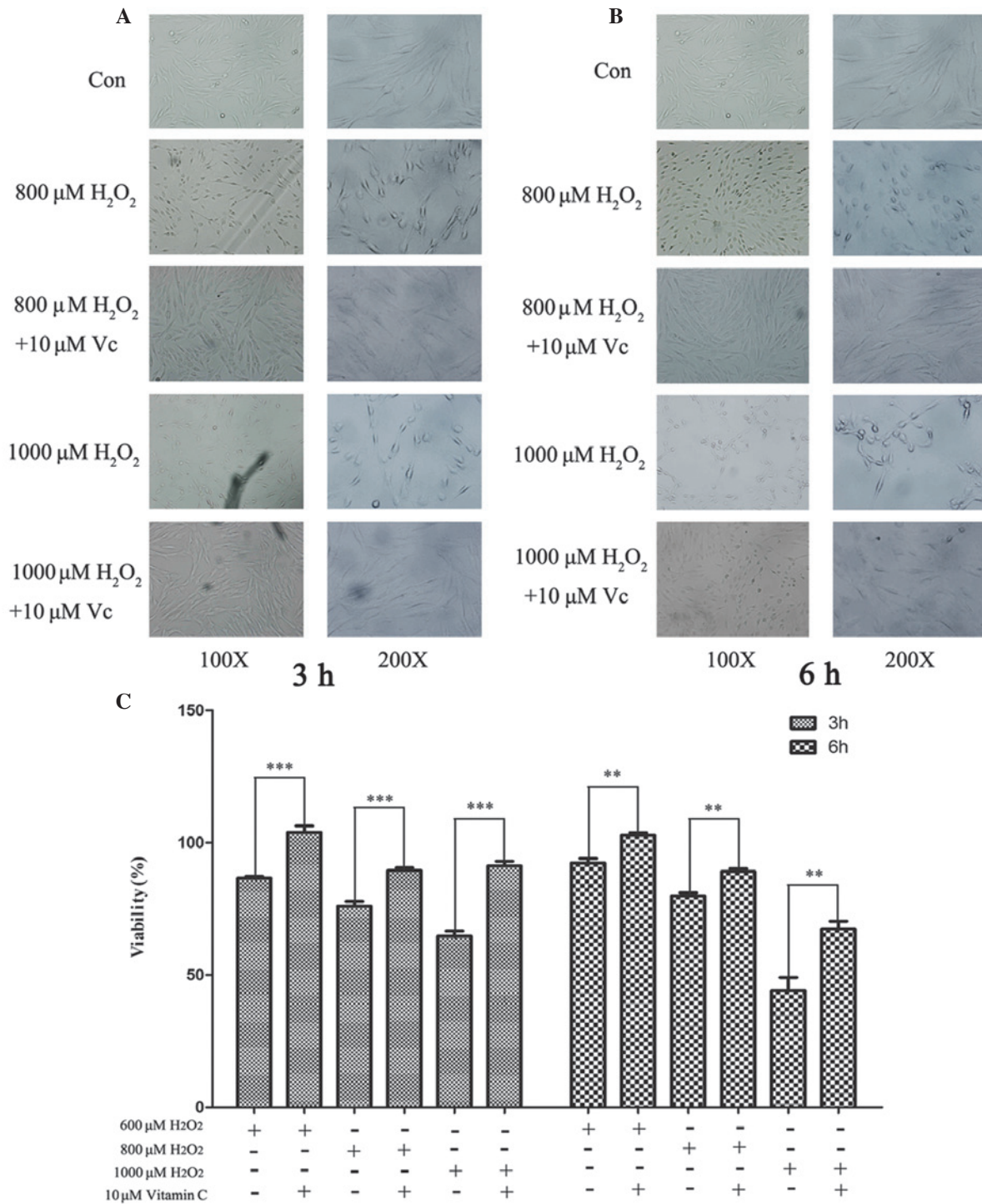


Figure 4. Vc partially rescues  $\text{H}_2\text{O}_2$ -treated PDLCs. Morphological alterations of  $\text{H}_2\text{O}_2$ -challenged PDLCs treated with  $10 \mu\text{M}$  Vc for (A) 3 and (B) 6 h. (C) MTT quantification of antioxidant effects of Vc at 3 and 6 h. PDLCs were treated with or without  $10 \mu\text{M}$  Vc under conditions of stress with 600, 800 and  $1000 \mu\text{M}$   $\text{H}_2\text{O}_2$ . \*\*P<0.01 and \*\*\*P<0.001 compared with the control group. PDLCs, periodontal ligament cells;  $\text{H}_2\text{O}_2$ , hydrogen peroxide; Vc, vitamin C; Con, control.

The viability of challenged PDLCs was increased at all  $\text{H}_2\text{O}_2$  concentrations 3 and 6 h post stimulation (Fig. 4C). These results suggested that optimal Vc administration increased the viability of PDLCs under oxidative stress and exerted a cytoprotective effect.

*Optimal Vc rescues PDLCs under oxidative stress by decreasing apoptosis.* The results of the present study demonstrated that

treatment of PDLCs with  $>10 \mu\text{M}$  Vc consistently exhibited a robust cytoprotective effect. Subsequent investigation was performed using flow cytometry to determine whether Vc had a potential anti-apoptotic function. The results demonstrated that  $1,000 \mu\text{M}$   $\text{H}_2\text{O}_2$  was capable of initiating apoptosis in the PDLCs, while Vc protected the PDLCs from  $\text{H}_2\text{O}_2$  exposure at 3 h post stimulation (Fig. 5A). In the cells treated with lower concentrations of  $\text{H}_2\text{O}_2$  (600 and  $800 \mu\text{M}$ ), apoptosis was not

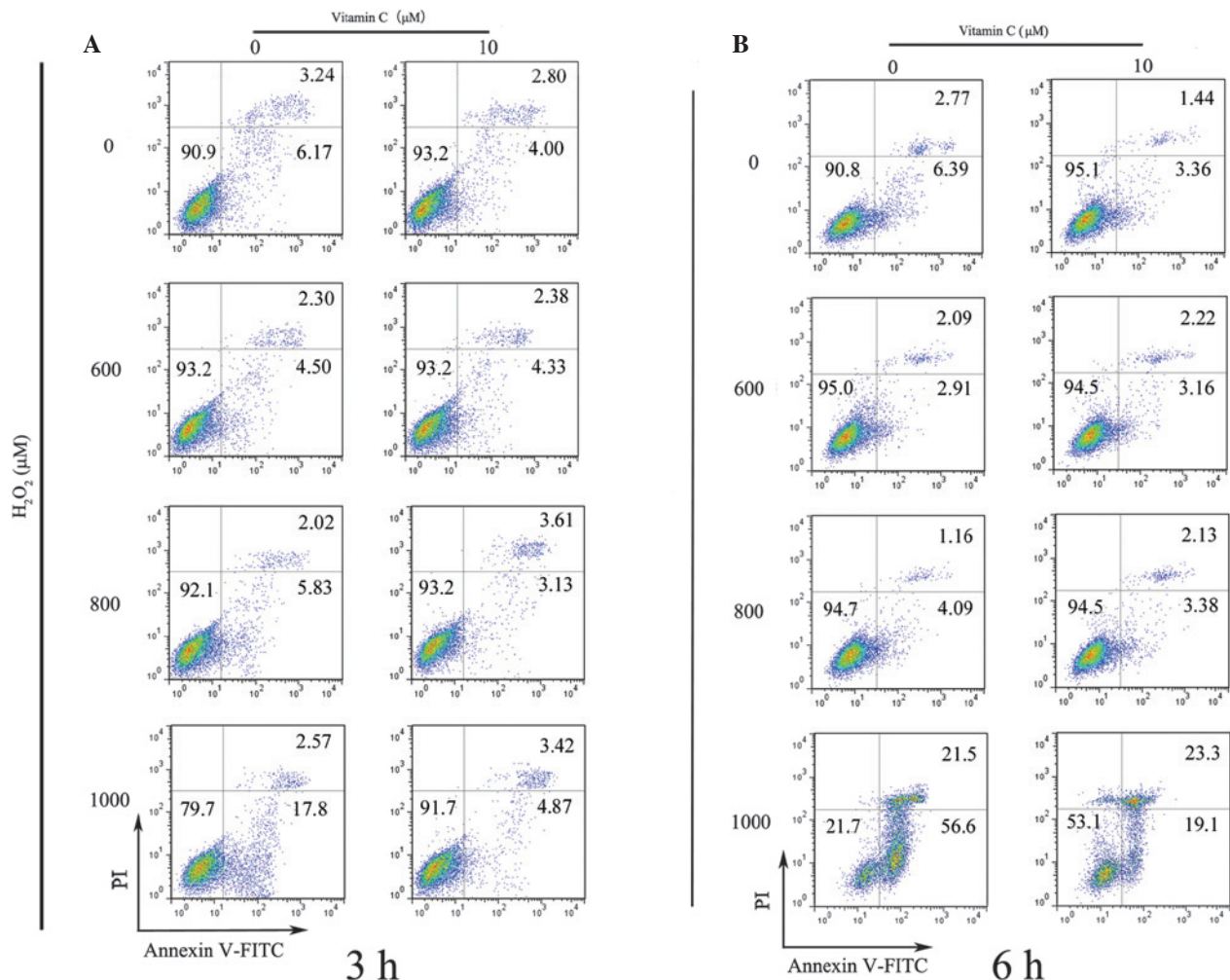


Figure 5. Vc decreases the apoptosis of H<sub>2</sub>O<sub>2</sub>-treated PDLCs. PDLCs were challenged with different concentrations of H<sub>2</sub>O<sub>2</sub> with or without 10 μM vitamin C for (A) 3 and (B) 6 h, respectively. Apoptosis was quantified using flow cytometric analysis. PDLCs, periodontal ligament cells; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; PI, propidium iodide; FITC, fluorescein isothiocyanate.

attenuated by the addition of Vc at 3 h, possibly due to the limited time scale (Fig. 5A). Similar results were observed when the apoptotic effects were evaluated at 6 h (Fig. 5B). The apoptosis observed following H<sub>2</sub>O<sub>2</sub> exposure at 800 and 1,000 μM was also verified by the occurrence of cleaved caspase-3, caspase-9 and PARP using western blot analysis (Fig. 6). However, following the addition of 10 μM Vc, the level of cleaved caspases-3, caspases-9 and PARP all decreased implying impairment of the apoptotic response (Fig. 6). These results suggested that optimal Vc administration antagonized the oxidative effect of H<sub>2</sub>O<sub>2</sub> and effectively improved the survival of PDLCs at high levels of H<sub>2</sub>O<sub>2</sub>, even at earlier time points.

## Discussion

Oxidative stress is central to periodontal tissue damage and periodontitis, either due to excess ROS production/activity, antioxidant deficiency or indirectly as a result of activating specific transcription factors that lead to a pro-inflammatory state. A significant association between abnormal oxidative status and periodontal diseases has been demonstrated implying that oxidative stress may be a determinant in the progression of periodontitis (13). Therefore, detailed investiga-

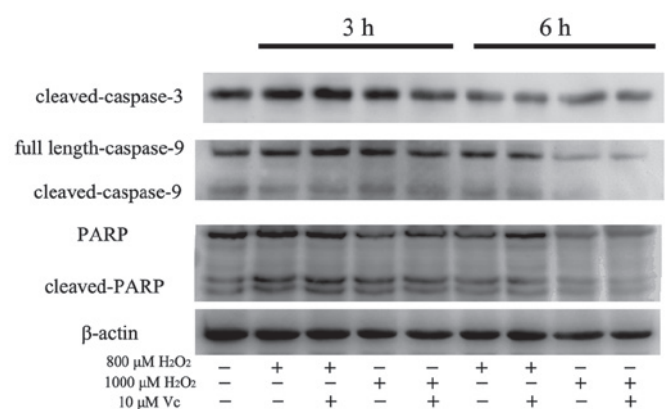


Figure 6. Vc protects PDLCs from H<sub>2</sub>O<sub>2</sub>-induced apoptosis. PDLCs were treated with the indicated doses of H<sub>2</sub>O<sub>2</sub> with or without 10 μM Vc and cellular apoptosis was evaluated at 3 and 6 h. PDLCs, periodontal ligament cells; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; Vc, vitamin C; PARP, poly (ADP-ribose) polymerase.

tion of the oxidative status in periodontal tissues may provide crucial insights into the pathological and pharmaceutical intervention of periodontitis. In the present study, the role of H<sub>2</sub>O<sub>2</sub>

was examined and  $H_2O_2$  was found to induce the apoptosis of PDLs in a dose- and time-dependent manner (Figs. 2 and 3). Furthermore, Vc supplementation partially reversed the adverse effect of  $H_2O_2$  (Figs. 4, 5 and 6), however, the addition of Vc did not always improve survival of the PDLs, supporting the existence of an optimal level of Vc to decrease PDL death (Fig. 3).

Several studies have associated oxidative stress with the pathogenesis of periodontitis (15-17). For example, high levels of ROS in the gingivae and saliva are associated with the progression of periodontal diseases (14). In addition, excess ROS and a marked reduction in antioxidant levels in gingival crevicular fluid are responsible for chronic periodontitis (14,16). The local assembly of pro-inflammatory lymphocytes and the release of ROS are major triggers of host defense for those suffering from periodontitis (18). Evidence from animal studies has also demonstrated a significant association between higher levels of ROS and periodontitis (14). Despite these findings, to the best of our knowledge, no studies have established a role of ROS levels in PDLs or investigated the mechanisms by which ROS may affect the viability of PDLs. The present study demonstrated that  $H_2O_2$  may decrease the viability of PDLs by inducing programmed cell death in a dose- and time-dependent manner. This apoptosis was verified using flow cytometric analysis and the occurrence of cleaved caspase-3, caspase-9 and PARP (Fig. 6). These observations suggest that at least the mitochondrial apoptotic pathway may be involved in eliminating PDLs exposed to  $H_2O_2$ . This mechanistic investigation provides insights into how increased ROS levels may lead to periodontal tissue destruction and supports the hypothesis that ROS excess further exacerbates periodontal tissue damage by inducing the apoptosis of PDLs. These findings also demonstrated the importance of ROS control in periodontitis. Therefore, administration of antioxidants may be favorable in the treatment of periodontitis, which prompted the present study to investigate the pharmacological effects of Vc exposure.

Few studies, to the best of our knowledge, have examined the role of the antioxidative effect of Vc in PDLs. Therefore, the current study investigated how oxidative status affected the viability of PDLs and whether stressed cells can be recovered by administration of the antioxidant Vc. Notably, Vc was found to rescue  $H_2O_2$ -challenged PDLs in a dose-dependent manner. However, the viability of  $H_2O_2$ -challenged PDLs did not monotonically increase with increasing Vc concentration. These results suggested that there may be an optimal Vc concentration for the maximal survival of PDLs in conditions of  $H_2O_2$  stress. Vc is able to either promote or inhibit apoptosis in a dose and cell type-specific manner (19-24). For example, Vc exacerbated rather than inhibited  $H_2O_2$ -induced apoptosis in epithelial and mesenchymal cells (19). Additionally, Vc alone has also been observed to exhibit cell type-specific effects in the regulation of apoptosis (19) and has a cytotoxic effect on the human gastric cancer cell line AGS, possibly by downregulating 14-3-3 $\sigma$  proteins (20). In human breast cancer cells, Vc acts through apoptosis inducing factor to initiate apoptosis (24). Therefore, the interpretation of Vc-induced cytotoxic effects may be complex and may depend on cell milieu, dose used and the availability of other cofactors. A dose-dependent

effect of Vc addition has been demonstrated in L6 muscle cells, where micromolar quantities of Vc increased apoptosis but millimolar doses were notably protective (25). Therefore, the clinical use of antioxidants, particularly Vc, requires cautious administration, as the addition of Vc may be cytotoxic under certain conditions. In the present study, it was clear that optimal use of Vc lowered the risk of cellular apoptosis at as early as 3 h post stimulation (Figs. 4 and 5) and this observation provides a deeper understanding of clinical intervention for the treatment of periodontitis.

Several experimental limitations in the present study must be noted. Although the mitochondrial apoptotic pathway was found to be involved in inducing the apoptosis of PDLs, the importance of other apoptotic pathways requires further investigation. In addition, whether PDLs are subject to other programs of cell death may be examined in future studies. The mechanism underlying the decreased survival of PDLs by excess Vc also requires investigation. Although the present study did not enable the inference of a causal association between  $H_2O_2$  exposure and the development of periodontitis, the importance of optimal Vc administration was evident, even without prior knowledge of the exact association.

In conclusion, the present study demonstrated that  $H_2O_2$  disrupted the morphology and decreased the viability of PDLs via apoptotic pathways. In addition, the optimal dose of Vc in treating periodontitis was revealed to be important, as excess Vc usage may otherwise induce cell death and exacerbate the progression of periodontitis. These results also clarified evidence that ROS exert toxic effects on PDLs. These findings may broaden current knowledge of the pathogenesis of periodontitis and may have significant implications for therapeutic intervention.

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