

# Antioxidant Effect of Eugenol in Human Periodontal Ligament Fibroblasts

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**Abstract** : Periodontal disease (PD) is the most common osteolytic disease of alveolar bone, oral infection seen in humans worldwide. PD is a common, chronic immunoinflammatory disease initiated by a complex subgingival bacterial and results in the inflammatory destruction of periodontal tissues, including the alveolar bone periodontal ligament, and gingivae. The effects of eugenol on periodontal ligament fibroblasts (PDLF) cell under oxidative injury have not been fully studied. Despite many studies in regard to the antioxidant effect of eugenol, the protective effect of eugenol against oxidative damage to PDLF cell, as well as the relationship between eugenol and apoptosis, has not been investigated so far. The aim of this study was to assess the protective effect of eugenol against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in PDLF cell.

Cell lines were separately grown as monolayers at 5% CO<sub>2</sub> and 37°C humidified atmosphere using appropriate media supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 µg/mL penicillin-streptomycin. DMEM/F12 was used as the culture medium for periodontal ligament fibroblast cells.

The viability of the PDLF cells which induced by the different concentrations of H<sub>2</sub>O<sub>2</sub> (control, 50, 100, 200, 400 µM) for 24 h was detected by MTT assay. Cell viability was significantly reduced in a H<sub>2</sub>O<sub>2</sub>-concentration dose-dependent manner. The mitochondria-dependent pathway of apoptosis is regulated by Bcl-xl family, such as the anti-apoptotic protein Bcl-xl, pro-apoptotic protein Bak. With H<sub>2</sub>O<sub>2</sub> injury, the protein level of Bak was up-regulated while the protein level of Bcl-xl was down-regulated. In group treated H<sub>2</sub>O<sub>2</sub> and eugenol, the ratio was reduced and the expression of Bak decreased at the same time, indicating that eugenol can attenuate apoptosis through mitochondrial related pathway in PDLF cells.

Therefore, although the findings of this study are limited to an in vitro interpretation, we suggest that eugenol preconditioning may have a beneficial effect in the recovery of periodontal ligament from oxidative stress.

**Keywords** : Antioxidant, Eugenol, Oxidative stress, Periodontal ligament fibroblasts

## Introduction

Periodontal disease (PD) is the most common osteolytic

infection of alveolar bone seen in humans worldwide. PD is a common, chronic immunoinflammatory disease initiated by subgingival bacteria and results in the inflammatory destruction of periodontal tissues, including the alveolar bone periodontal ligament, and gingivae. It is characterized by gradual destruction of periodontal tissue, in the end leading to tooth loss. One major mechanism by which PD exerts systemic effects is through the generation of oxidative stress [1-4]. In recent years, evidence has emerged to

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implicate reactive oxygen species (ROS) oxidative stress and the pathogenesis of periodontal disease in humans. The presence of excess reactive oxidants is thought to provide a basis for the progression of various diseases [5]. Eugenol, 2-methoxyphenol, which is contained in cloves as well as in cinnamon and other aromatic spices is used as a supplement or a therapeutic ingredient in various medications and foods. Eugenol is used to treat digestive disorders and skin infections and is found in insect attractants and in UV absorbers [6]. It is a beneficial antioxidant when ingested in moderate amounts reducing the level of free radicals. However, there are some reports that excessive doses of undiluted eugenol oil can cause symptoms. According to some studies, eugenol in excessive doses can be considered poison [7,8]. Previous studies reported that oxidative stress can directly induce cell death or apoptosis in various cell types, including osteoblasts, intestinal endothelial cells, and hepatocytes [9-11]. Studies demonstrated that eugenol has an antiapoptotic effect on *in vivo* and *in vitro* [12-14]. In ancient times, natural products were the main source of health care products. In modern medicine, they are still major sources of new drug development [15,16]. The effects of eugenol on periodontal ligament fibroblasts (PDLFs) exposed to oxidative injury has been widely investigated its protective effect against oxidative damage of PDLFs, as well as its relationship with apoptosis, has not been studied. The aim of this study was to assess the protective effect of eugenol against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in PDLFs cell.

## Materials and Methods

### 1. Reagents

The Human periodontal Ligament Fibroblasts (PDLF) was purchased from Lonza (Basel, Switzerland). The following chemicals and reagents were obtained from the indicated companies: eugenol, Hoechst 33342 was purchased from Sigma. The following reagents were obtained commercially: 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide (MTT), apoptosis detection kit was obtained from Biovision (Milpitas, CA, USA). Antibodies used in the study were as follows: cleaved Caspase 3 (1 : 1,000), : Bcl-x1 (1 : 1,000), : Bax (1 : 1,000), Santa Cruz. Secondary antibodies against rabbit (1 : 3,000), and mouse (1 : 3,000),

immunoglobulins were purchased from Bio-Rad.

### 2. Cell culture

Cell lines were separately grown as monolayers at 5% CO<sub>2</sub> and 37°C humidified atmosphere using appropriate media supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 µg/mL penicillin-streptomycin. DMEM /F12 was used as the culture medium for periodontal ligament fibroblast cells. Cells were passaged 3 times a week by treating with trypsin-EDTA and used for experiments after 5 passages.

### 3. Treatment of eugenol

Eugenol which were made by dissolving them in DMSO were kept frozen at -20°C until use. The stock was diluted to their concentration with DMEM when needed. Prior to eugenol treatment cells were grown to about 80% confluence and then exposed to eugenol at different concentrations (0, 50, 100, 200, 400 µM) for 24 h. Cells grown in medium containing an equivalent amount of DMSO without eugenol served as control. The groups were randomly divided into the following groups: Control, eugenol, H<sub>2</sub>O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>+eugenol.

### 4. MTT assay

Cell viability assay was measured using a quantitative colorimetric assay MTT solution, showing the mitochondrial activity of living cells. PDLF cells ( $3 \times 10^4$ ) were seed in 96-well plates. After drug treatment as indicated, cells were incubated with 300 µL MTT (final concentration 0.5 mg/mL) for 1.5 h at 37°C. The reaction was terminated by addition of 200 µL DMSO. Cell viability was measured by an ELISA reader (Tecan, Männedorf, Switzerland) at 570 nm excitatory emission wavelength.

### 5. Flow cytometer analysis

Cells were seeded into a 6-well plate at  $1 \times 10^6$  cells/mL and incubated overnight. Cells treated with eugenol were incubated for various time points. In each time point, the harvested cells were washed with PBS containing 1% bovine serum albumin and centrifuged at 2,000 rpm for 10 min. The cells were resuspended ice-cold 95% ethanol with 0.5% Tween 20 to a final concentration of 70% ethanol.

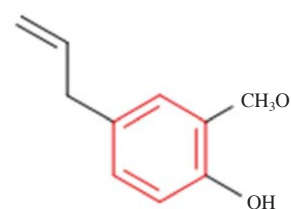
Fixed cells were pelleted, and washed in 1% BSA-PBS solution. Cells were resuspended in 1 mL PBS containing 20  $\mu\text{g}/\text{mL}$  RNase A, incubated at 4°C for 30 min, washed once with BSA-PBS, and resuspended in PI solution (10  $\mu\text{g}/\text{mL}$ ). After cells were incubated at 4°C for 5 min in the dark, DNA content were measured on a CYTOMICS FC500 flow cytometry system (Beckman Coulter, FL,CA,USA) and data was analyzed using the Multicycle software which allowed a simultaneous estimation of cell-cycle parameters and apoptosis.

### 6. Immunofluorescent staining to detect cytochrome c, AIF translocation

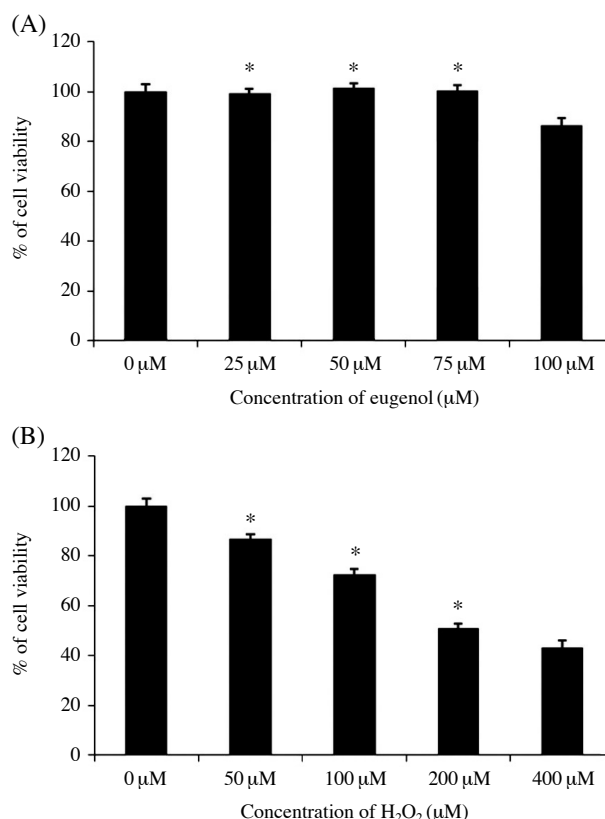
The cells were plated on coverslips and treated with eugenol. After 24 h, the cells were stained with 50 nM Mito-Tracker Red at 37°C for 30 min. After washing two times with PBS, the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min and washed three times with PBS. After permeabilization with Triton X-100 and blocking 1% BSA in PBS, the cells were incubated with primary antibodies in 1% BSA overnight at 4°C. After washing with PBS, cells were incubated with FITC-conjugated secondary antibodies in 1% BSA-PBS for 60 min and rinsed in PBS. Fluorescent images were observed and analyzed using a Zeiss LSM 750 laser-scanning confocal microscope (Göttingen, Germany).

### 7. Western blot analysis

Cells ( $2 \times 10^6$ ) were washed twice in ice-cold PBS, resuspended in 200  $\mu\text{L}$  ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100, 2 mM PMSF, 2  $\mu\text{L}/\text{mL}$  aprotinin and 2  $\mu\text{L}/\text{mL}$  leupeptin] and incubated at 4°C for 30 min. The lysates were centrifuged at 14,000 rpm for 15 min at 4°C. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA, USA) and 20  $\mu\text{g}$  of proteins were resolved by 10% SDS/PAGE. The gels were transferred to Polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and reacted with appropriate primary antibodies. Immunostaining with secondary antibodies was detected using SuperSignal West Femto (Pierce, Rockford, IL, USA) enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, Santa Clara, USA).



**Fig. 1.** Chemical structure of eugenol.



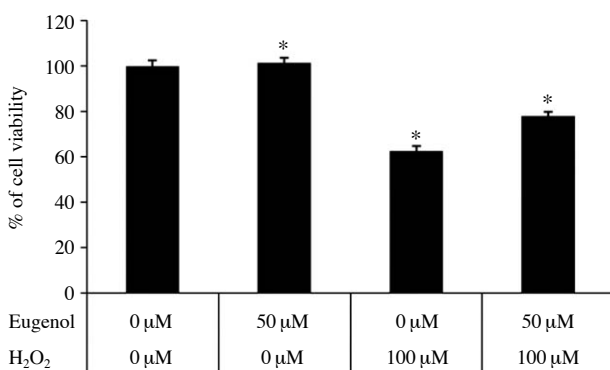
**Fig. 2.** Effect of eugenol preconditioning on cell viability. (A) The normal PDLF cells were treated with different concentrations (0, 25, 50, 75, 100  $\mu\text{M}$ ) of eugenol for 24 h. (B) PDLF cells were treated  $\text{H}_2\text{O}_2$  with various doses (0, 50, 100, 200, 400  $\mu\text{M}$ ). Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The values are denoted as mean SE of three independent experiments. \* $P < 0.05$  versus untreated samples.

## Result

### 1. Eugenol improved the cell viability of $\text{H}_2\text{O}_2$ -induced apoptosis in PDLFs

The effect of eugenol on PDLFs was investigated over a wide concentration range. Eugenol suppressed  $\text{H}_2\text{O}_2$ -de-

pendent programmed cell death, pointing to its potential as a potent antioxidant. The chemical structure of eugenol is shown Fig. 1. The PDLF cells with various doses of eugenol (below 100  $\mu\text{M}$ ) and exposed the cells to  $\text{H}_2\text{O}_2$  injury and then we measured cell viability by the MTT assay (Fig. 2A). Alteration of cell viability was not observed in euge-



**Fig. 3.** Protective Effect of eugenol on PDLF cell viability. Cells were pretreated with eugenol (50  $\mu\text{M}$ ) after 24 h treated with different concentrations of  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ) for 24 h, other group treated single  $\text{H}_2\text{O}_2$ . Cell viability was analyzed using the MTT assay.

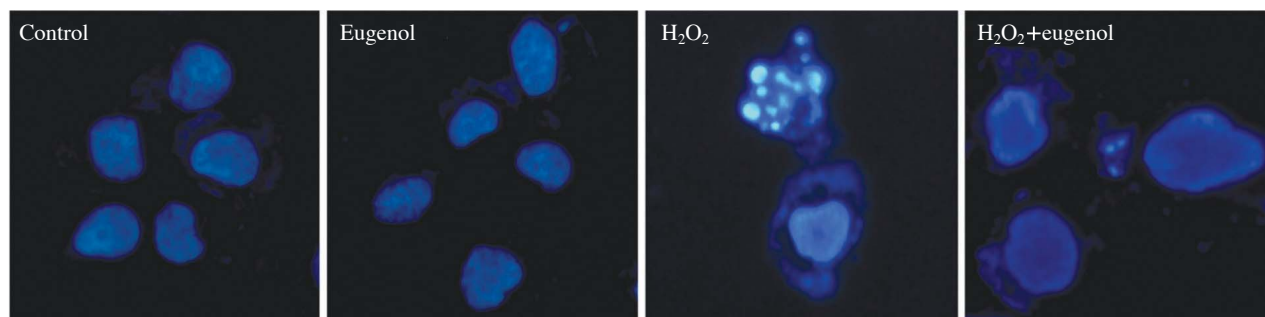
nol treatment group ( $p < 0.05$ ). The viability of the PDLF cells which induced by the different concentrations of eugenol (0, 25, 50, 75, 100  $\mu\text{M}$ ) for 24 h was detected by MTT assay. However, the cell viability was significantly reduced in a  $\text{H}_2\text{O}_2$ -treated in PDLFs dose-dependent manner (Fig. 2B).

## 2. Eugenol protected against $\text{H}_2\text{O}_2$ -induced apoptosis in PDLFs

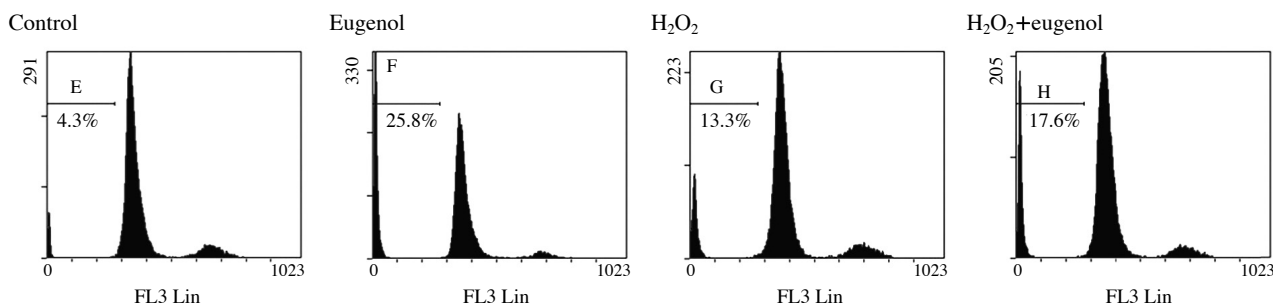
To investigate the protective effect of eugenol, the PDLFs cells were treated 50  $\mu\text{M}$  of eugenol for 24 h and exposed to  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ). After 24 h, the cells were assayed for cell viability. Treatment of the cells with 50  $\mu\text{M}$  of eugenol significantly increased the cell viability compared to that observed in the cells exposed exclusively to  $\text{H}_2\text{O}_2$  (Fig. 3).

## 3. Eugenol treatment led to a decrease in $\text{H}_2\text{O}_2$ -induced apoptosis in the PDLFs

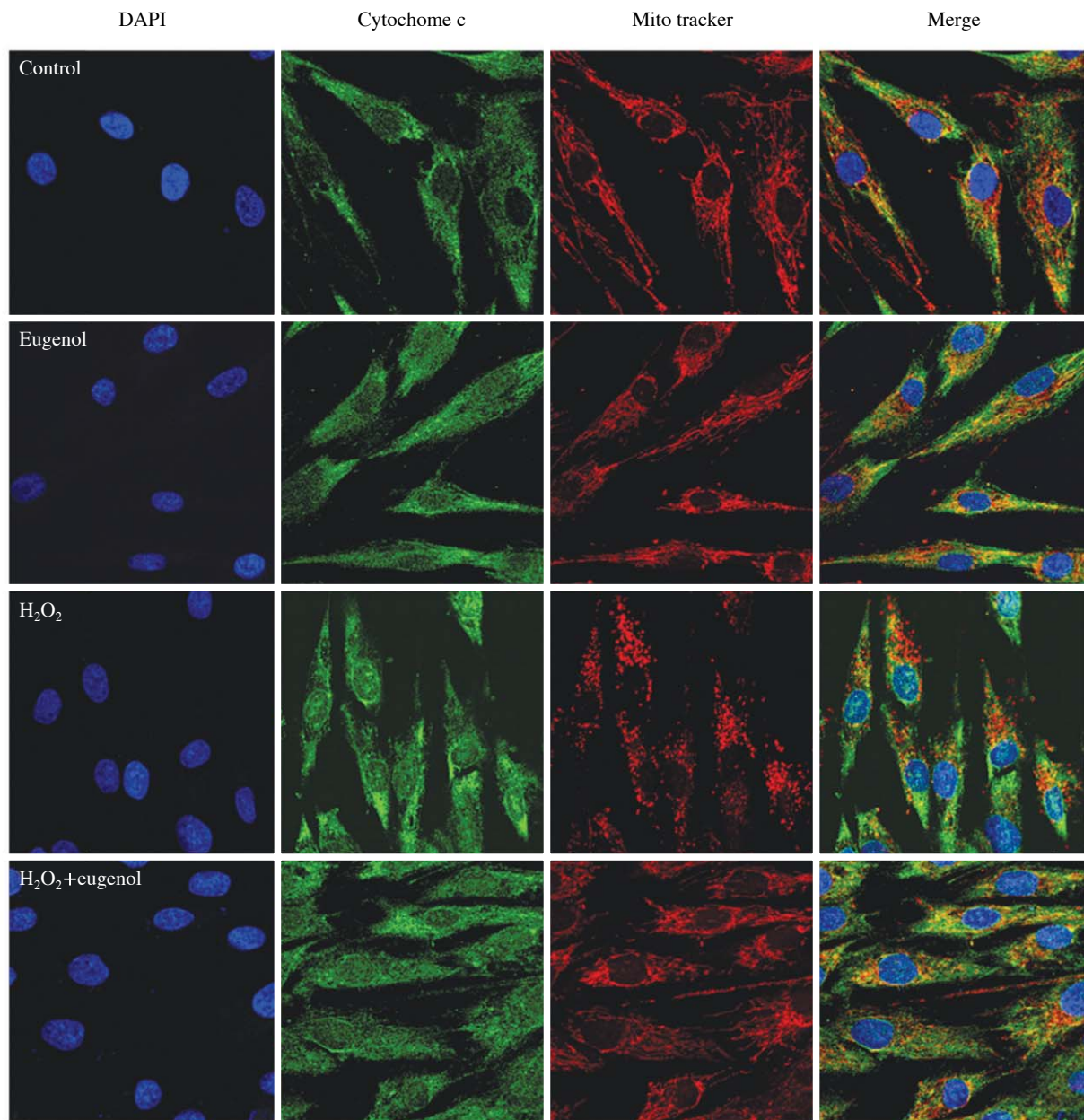
The changes in nuclear morphology were assessed by Hoechst 33342 staining after the  $\text{H}_2\text{O}_2$  treatment. The con-



**Fig. 4.** Nucleus condensation signally was observed on PDLF cells stimulated with  $\text{H}_2\text{O}_2$ . Apoptotic bodies in the  $\text{H}_2\text{O}_2$ +eugenol group were markedly reduced.



**Fig. 5.**  $\text{H}_2\text{O}_2$ -induced apoptosis in PDLF cells. Cells were treated with eugenol (50  $\mu\text{M}$ ) for 24 h, and ratio of apoptotic cells was determined by flow cytometry analysis.

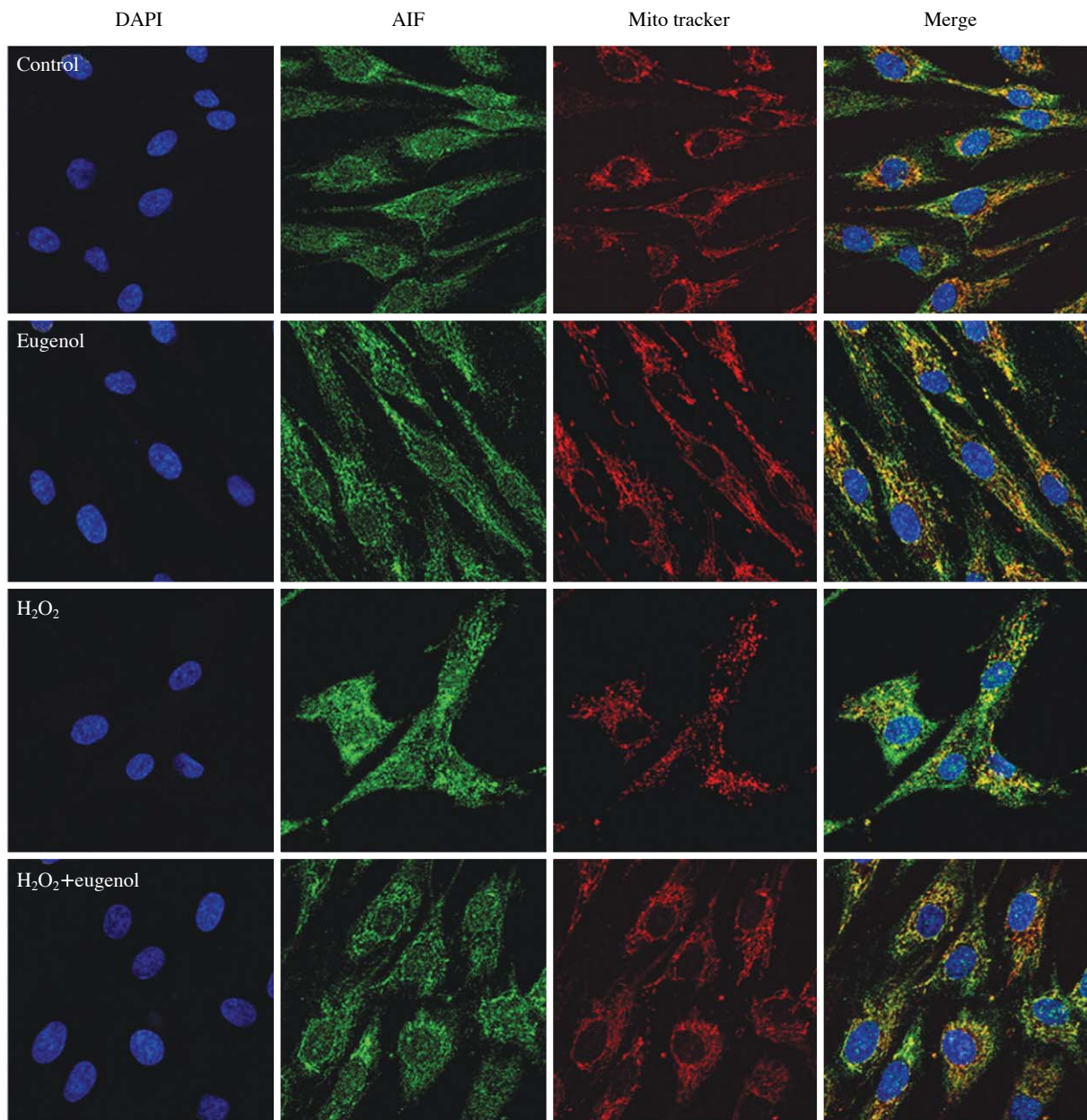


**Fig. 6.**  $H_2O_2$  induced translocation of cytochrome c from mitochondria into cytosolic fraction in PDLF cells. Cells were incubated with 50  $\mu M$  eugenol for 24 h and then stained with MitoTracker (red), cytochrome c (green), and DAPI (blue) to visualize mitochondria, cytochrome c, nuclei respectively.

control PDLF nuclei had a normal round shape. However, when the cells were exposed to  $H_2O_2$  for 24 h, nuclear condensation and fragmentation appeared. The eugenol treatment rescued the  $H_2O_2$ -induced nuclear morphological change (Fig. 4). Treatment of the cells with eugenol alleviated the cell damage. Based on these results eugenol significantly reduced apoptosis in the  $H_2O_2$  exposed PDLFs.

#### 4. $H_2O_2$ induced mitochondrial dysfunction and caspase-mediated apoptosis

Mitochondria play a key role in the intrinsic pathway of apoptosis and dissipation of the mitochondrial membrane potential ( $\Delta\psi_m$ ) is associated with mitochondrial dysfunction [17-19]. Thus, we verified the change of  $\Delta\psi_m$  in  $H_2O_2$ -



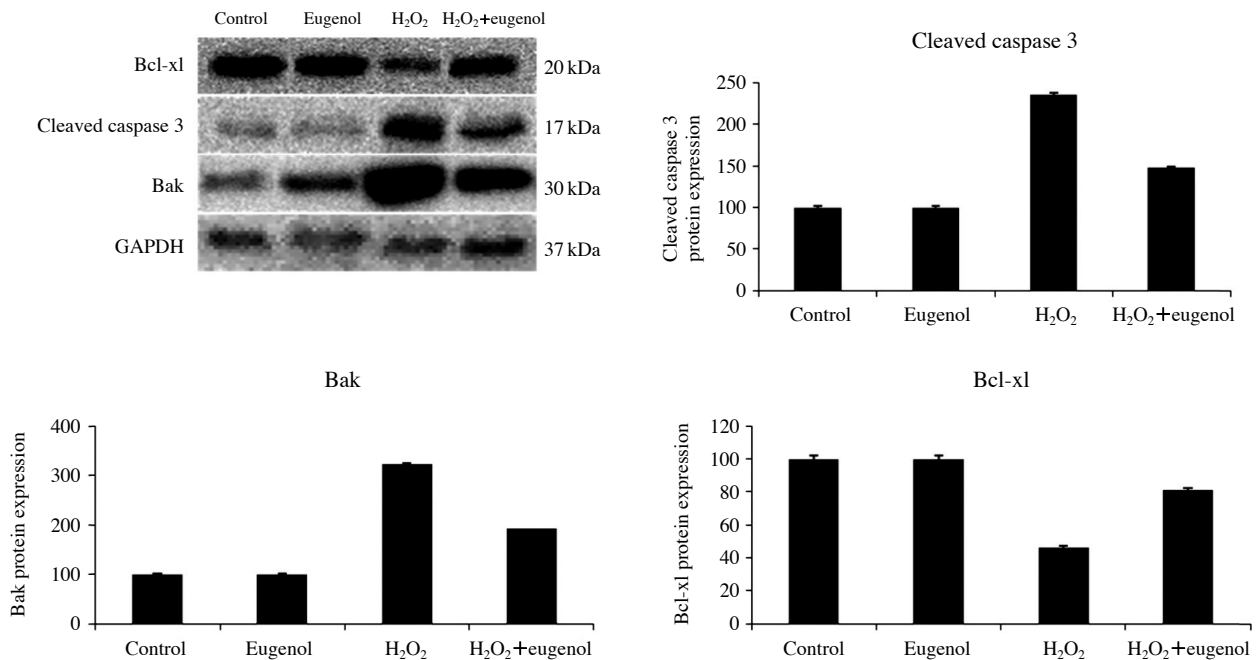
**Fig. 7.** Cells were incubated with 50  $\mu$ M eugenol for 24 h and then stained with MitoTracker (red), AIF (green), and DAPI (blue) to visualize mitochondria, cytochrome c, nuclei respectively. Images were observed by Confocal microscopy.

induced apoptosis using DiOC<sub>6</sub>. H<sub>2</sub>O<sub>2</sub> triggered the loss of  $\Delta\psi_m$  in the PDLFs, as compared to the controls. The loss of  $\Delta\psi_m$  resulted in the release of cytochrome c into the cytosolic fraction. We next investigated the translocation of cytochrome c using immunofluorescent staining. Eugenol treated group reduced compared H<sub>2</sub>O<sub>2</sub>-induced cause release cytochrome c and AIF (Figs. 6, 7).

### 5. Effect of eugenol treatment on apoptosis activation

The activation of cleaved caspase 3 is a key upstream event in the initiation and execution of apoptosis.

Cleaved caspase 3 was up-regulated in the H<sub>2</sub>O<sub>2</sub> group, and decreased in the eugenol and H<sub>2</sub>O<sub>2</sub>+eugenol groups. The mitochondria-dependent pathway of apoptosis is regulated by the Bcl-x1 family, such as the anti-apoptotic pro-



**Fig. 8.** Eugenol changed expressions of apoptosis-related proteins in PDLF cells. Cells were treated with eugenol for the indicated levels of Bcl-x1, cleaved caspase 3, Bak were analyzed by western blotting.

tein Bcl-x1 and the pro-apoptotic protein Bak. With H<sub>2</sub>O<sub>2</sub> injury, the protein level of Bak was up-regulated and the protein level of Bcl-x1 was down-regulated. In the H<sub>2</sub>O<sub>2</sub>+eugenol group, the level was reduced and the expression of Bak decreased at the same time, indicating that eugenol attenuated apoptosis through a mitochondrial related pathway in PDLFs (Fig. 8).

## Discussion

The objective of the current study was to test the hypothesis that eugenol, a natural plant constituent widely used in food products and dental materials, protects against oxidative stress and apoptosis caused by H<sub>2</sub>O<sub>2</sub> [20]. This study has three principal findings. First, the eugenol treatment conferred increased protection of PDLFs in oxidative injury (Fig. 2A). Second, the eugenol treatment protected human PDLFs against oxidative stress induced apoptosis. Using western blot analysis, we showed that the eugenol treatment decreased cleaved caspase 3 levels via a caspase-dependent pathway, and that it reduced the ratio of Bak and Bcl-x1 which are associated with a mitochondrial related pathway. The activation of upstream regulators of

cleaved caspase 3 is key to the initiation and induction of apoptosis [21,22]. It is known that the mitochondria-dependent apoptotic pathway is regulated by Bcl-x1 protein family, such as the anti-apoptotic protein Bcl-x1 and pro-apoptotic protein Bak, which are critical downstream regulators in caspase activation [14,23]. Third, we found that eugenol reduced apoptotic cell death in PDLF cells that ROS played a crucial role in this process [24]. Western blot analysis showed a cleaved caspase 3 and Bak, known pro-apoptotic proteins, and Bcl-x1 a known anti-apoptotic protein [25-27]. The results showed that the anti-apoptotic effect of eugenol was mediated by a mitochondria related pathway. In summary, the present study, eugenol treatment less than 100  $\mu$ M have not shown the cytotoxic effect in PDLFs and among of eugenol concentrations, 50  $\mu$ M treatment groups showed that to stimulate the expression of anti-apoptotic proteins under oxidative stress. This study was performed to investigate the effects of eugenol on the periodontal healing process. Although the findings of this study are limited to an in vitro interpretation, we suggest that eugenol preconditioning may have a beneficial effect on the recovery of periodontal ligament from oxidative stress.

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## 치주인대 섬유모세포에서 유지놀의 산화스트레스 억제 효과

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**간추림** : 치아주위조직 질환은 전세계적으로 구강감염 원인이 되는 질환 중 가장 흔히 관찰되며 골용해를 동반한다. 치주염의 발생은 치은, 치주인대, 치조골을 서서히 파괴하는 것을 특징으로 하며 결국에는 치아를 잃게 된다. 기존의 연구결과에서 치주질환의 기전은 염증성 세균에 의해 형성된 산화스트레스가 주된 원인으로 알려져 있다. 유지놀은 항산화 작용으로 산화스트레스를 억제하는 물질로 여러 차례 보고된바 있으며 본 연구의 목적은 치주인대 섬유모세포에서  $H_2O_2$ 에 의해 유도된 산화스트레스에 대한 유지놀의 보호 효과를 평가하고자 수행되었다.

유지놀의 항산화 효과를 평가하기 위해 세포생존율 분석, 세포주기분석, 면역형광염색법, western blot분석을 이용하여 실험하였다.

세포생존율은  $H_2O_2$  단독으로 처리한 그룹보다 유지놀 전처리한 그룹에서 세포생존율이 더욱 높게 나타났으며 세포자멸사와 관련된 단백질 cleaved caspase 3, Bak, Bcl-x1은  $H_2O_2$  단독 처리한 그룹과  $H_2O_2$ 처리 후 유지놀을 처리한 그룹을 비교하였을 때  $H_2O_2$  단독처리 한 그룹에서는 cleaved caspase 3와 Bak의 단백질 발현이 높게 나타났으며 Bcl-x1의 발현은 낮게 나타났다.

본 연구결과는 산화스트레스를 받은 치주인대세포의 세포자멸사를 유지놀이 억제하며 또한 산화스트레스에 의한 치주조직 손상에 대한 치료제 후보군에 유지놀의 높은 가능성이 있음을 제시한다.

**찾아보기 낱말** : 산화스트레스, 유지놀, 치주인대세포, 항산화