치의학박사 학위논문

Effects of 4-Hexylresorcinol on the Reactive Oxygen Species induced by Oxidative Stress in Dental Pulp Cells of Rats

흰 쥐의 치수세포에서 4-Hexylresorcinol 투여가 산화스트레스로 인한 활성산소에 미치는 영향

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치의학과 치과교정학 전공

장 준 호

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ABSTRACT

Effects of 4-Hexylresorcinol on the Reactive Oxygen Species induced by Oxidative Stress in Dental Pulp Cells of Rats

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Objective: It is crucial for dental pulp to manage the stress avoiding wound healing delay and consequent inflammation. The application of anti-oxidant may be beneficial preventing the such complications. In this study, we aimed to evaluate the therapeutic effects of 4- Hexylresorcinol (4HR), one of the well-known antioxidants, on dental pulp cells.

Methods: In cellular experiments, dental pulp cells from rat mandibular incisor were cultured and treated with 4HR (1–100 μM). Changes in total antioxidant capacity (TAC) and glutathione peroxidase (GPx) activity in response to 4HR treatment were evaluated following hydrogen peroxide treatments. Primary

antibodies against tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were used in evaluation assays. The positive control was H_2O_2 treatment without 4HR pretreatment, whereas the negative control was no treatment group.
In animal experiment, twenty rats were subjected to the experiment. Two rats were used to measure the baseline activity of TAC and GPx after pulp tissue extraction. Eighteen rats were devided into two groups. The experimental group (n = 9) was treated with 2% 4HR ointment and the control group (n = 9) treated with the ointment base (lanolin) only. Three animals (n = 3 / 9) were used for protein extraction and Western blot analysis 3 days after the application. And six animals (n = 6 / 9) were used for histological and immunohistochemical analyses (n = 3) and for TAC and GPx analyses (n = 3) 5 days after the application.

Results: TAC and GPx activities were significantly increased ($p < 0.05$), while the expression levels of TNF-α and IL-1β were decreased following the exposure to 4HR pretreatment in an *in vitro* model ($p < 0.05$). Additionally, the application of 4HR ointment in an exposed dental pulp model significantly reduced the expression of TNF-α and IL-1β ($p < 0.001$).

Conclusions: 4HR exerted protective effects against oxidative stress in dental pulp tissues through downregulating TNF-α and IL-1β.

Keywords: 4-Hexylresorcinol; reactive oxygen species; dental pulp; oxidative stress

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I. INTRODUCTION

Dental treatments, such as removing hard tissue, conditioning tooth surfaces for the restoration, and even the restoration procedure itself, cause a variety of stresses to the teeth.[1] Like other dental treatments, orthodontic tooth movement induces oxidative stress, increasing the expression of proinflammatory factors, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β).[2] Oxidative stress may result in tissue regeneration or necrosis, depending on its duration and intensity.[3] Dental pulp tissue acts as a frontline marker of dental stress. Therefore, attempts have been made to reduce excessive oxidative stress through the administration of antioxidants, which may help in the healing of dental pulp tissue.

As part of the inflammatory response, reactive oxygen species (ROS) are generated; in turn, cells produce antioxidants to neutralize their harmful effects.[4] Failure to regulate oxidative stress may lead to multiple pathophysiological conditions.[5] As for the orthodontic treatment, it has been reported that pulpal side effects and root resorption can occur secondary to orthodontic tooth movement.[6] Therefore, the application of antioxidant may be beneficial in preventing such complications. At the cellular level, in dental pulp, ROS stress may damage a variety of organic molecules, including proteins and DNA.[7] The protective reaction against the damage occurs in the endoplasmic reticulum and mitochondria, producing several enzymes, including glutathione peroxidase (GPx), to reduce ROS production.[8]

4-Hexylresorcinol (4HR) is a widely used ingredient in cosmetics and antiseptics.[9] It inhibits ROS-induced damage in lymphocytes by activating GPx[10] and demonstrates a similar antioxidant profile to that of resveratrol in human umbilical vein endothelial cells (HUVECs).[11] In dentistry, 4HR has been shown to increase the rate of alveolar bone turnover and improves orthodontic tooth movement.[12] Additionally, 4HR injection increases the speed of mandibular incisor eruption in rat models,[13] and decreases root resorption resulting from excessive orthodontic force, and increases the expression of osteoprotegerin, a receptor activator of nuclear factor kappa-B ligand, alkaline phosphatase and runt-related transcription factor 2.[6] However, the effects of 4HR treatment on dental pulp under oxidative stress have yet to be studied.

Oxidative stress often induces the expression of TNF-α and IL-1β.[14] Many models have been developed to simulate the condition of oxidative stress, one of which uses hydrogen peroxide in cellular experiments.[15] Dental therapeutics that influence pulp-cell homeostasis can provide *in vivo* models. The application of heavy orthodontic force or removal of hard dental tissues is often used in *in vivo* models to induce dental-pulp stress. In this study, we aimed to evaluate the antioxidant effect of 4HR on ROS induced by oxidative stress in dental pulp cells. First, primary cultured pulp cells were subjected to oxidative stress by the application of hydrogen peroxide, and then the protective effect of 4HR was evaluated. Thereafter, the removal of hard dental tissue was used as an *in vivo* model to evaluate the antioxidant effect of 4HR on pulp tissues. The total antioxidant capacity (TAC) and GPx activity were measured. The expression levels of TNF-α and IL-1β were also measured

II. REVIEW OF LITERATURE

1. Reactive oxygen species and oxidative damage

Both oxygen-free radicals and non-radical oxygen derivatives are involved in the formation of reactive oxygen species (ROS), which cause oxidative damage. The main ROS are hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl (•OH) radicals. The resulting ROS are the most significant contributors to oxidative damage to cells and tissues, because they affect the peroxidation of double-chain fatty acids, proteins, and DNA, while also increasing oxidative stress.[16-18]

Periodontal inflammation is among the most prominent ROS generators in the oral cavity.[19] Xenobiotics (alcohol, smoke, pharmaceuticals), food (acrolein, high-protein high-fat diets), dental therapy (laser light, ultraviolet light, ozone, ultrasound, non-thermal plasma), and dental materials (fixed orthodontic appliances, dental composites, implants) are other ROS sources.

ROS have been shown to have beneficial biological effects at physiological concentrations.[20] However, because of strong environmental stressors, ROS concentrations can soar, resulting in oxidative stress. Another instance of oxidative stress can be observed in the scenario in which abnormally high levels of reactive oxygen species (ROS) impair cellular metabolism and cause cellular damage.[21] Because of their high reactivity, greater concentrations of ROS can be a mediator of harm to all biological macromolecules.[22] Oxidative modification

products have been shown to accumulate in many tissues and organs, causing morphological and functional alterations that impair normal function. Furthermore, positive feedback from oxidative modification products has the potential to drive further ROS production (for instance, by increasing NADPH oxidase activity), thereby amplifying redox disturbances.[22,23]

It is widely acknowledged that oxidative stress plays a role in the onset and development of most oral diseases,[24] particularly periodontal disorders. This mechanism is important for the disease progression of chronic inflammation, breakdown of the extracellular matrix of the periodontium, and bone remodeling. Oral inflammation is caused by oxidative stress.[25] The quantity and functional status of neutrophils engaged in phagocytosis have been shown to influence the rate of ROS generation in the oral cavity. Overproduction of ROS by neutrophils has been shown to cause collagen degradation, proteoglycan disruption, and hyaluronic acid depolymerization, decreasing periodontal tissue integrity and biochemical performance.[26]

One preventive method for avoiding oral and systemic illnesses is to recognize external sources of ROS and restrict exposure to them. Thus, antioxidant supplements may be beneficial for patients whose oral cavities produce excessive ROS.[27]

Resveratrol (3,5,4′-trihydroxystibene) is a natural phenol and phytoalexin found in grapes and peanuts. It inhibits hydroperoxidase and cyclooxygenase activity, and mediates anti-inflammatory effects. Resveratrol has been shown to inhibit cellular events associated with cancer initiation, promotion, and progression.[28] The inhibition of oxidative damage in human lymphocytes induced

by hydrogen peroxide could be caused by increased levels of GSH and their modulation of antioxidant enzymes (GPx, GR, and GST).[10]

2. Oxidative stress in orthodontic treatment

Reactions to the mechanical stresses that occur during orthodontic treatment involve a significant variety of inflammatory mediators.[1] Oxidative stress, which is linked to increased production of pro-inflammatory markers, is one of the physiological reactions to orthodontic treatment that is commonly observed and the resulting inflammation in the oral cavity.[2,29]

Oxidative stress is described as the inability of the body to prevent or mitigate the detrimental effects of free radicals by neutralizing them with antioxidants.[30] Several pathophysiological disorders, including inflammatory illnesses, cancer, and mutations, as well as multiple oral cavity diseases, including periodontitis and squamous cell carcinoma, are linked to oxidative stress. Therefore, assessment of the oxidative state of the oral cavity has been recommended as a powerful method for diagnosing and monitoring the course of multiple diseases.

A small number of articles have been reported that suggest orthodontic therapy may play a role in salivary oxidative stress.[31,32] Ozcan et al.[32] utilized gingival crevicular fluid and saliva to measure selected oxidative stress markers to assess oxidative damage occurring during orthodontic treatment; they proposed that orthodontic materials and orthodontic tooth movement do not alter markers beyond their physiological limits, which is suggestive of oxidative damage in both GCF and saliva. The oxidative–antioxidative equilibrium in the saliva of clinically

healthy individuals is altered by orthodontic treatment.[27] Changes in the oxidative state of saliva appear to be caused by elevated nickel concentrations, emitted from orthodontic appliances.

Dental composite resins are used to bond brackets to teeth. The effective polymerization of their monomers determines the mechanical, physical, and chemical characteristics of composites, as well as their biocompatibility. Unfortunately, monomer polymerization is never complete, always leaving residual monomers. These monomers have been shown to decrease intracellular glutathione stocks.[33] A decrease in glutathione levels leads to a decrease in glutathione peroxidase expression and increase in hydrogen peroxide levels. This increase in turn acts as a powerful inducer of catalase activity, feeding back to inhibit SOD activity. Furthermore, ROS can be generated not only as a result of glutathione shortage, but also as a consequence of GSH-monomer adduct formation.[35]

The blue light used for resin polymerization causes the formation of hydrogen peroxide and other ROS such as singlet oxygen, hydroxyl radicals, lipid peroxide radicals, and superoxide anions in dental pulp vascular cells.[36] Small quantities of metal ions, particularly nickel and cobalt, are leached from dental materials and react with hydrogen peroxide to produce hydroxyl radicals via the Fenton reaction. Other metal ions, such as chromium and cobalt, exhibit redox cycling and directly produce ROS.[37] Metal ion concentrations are significantly increased by fixed orthodontic appliances. Buczko et al. discovered a link between Ni concentration and ROS levels in these patients' saliva.[27]

3. Pulpal reaction to orthodontic force

Orthodontic tooth movement inevitably evokes significant reactions in the PDL and the dental pulp. Although several researchers have studied the reaction of the PDL to orthodontic force, few have focused on pulpal alterations. These include inflammation, cell damage, and wound healing, all of which can be harmful to dental pulp. Histologically, the response of pulp to orthodontic forces ranges from circulatory vascular stasis to necrosis.[38] After orthodontic force application, tissue respiration and alkaline phosphatase activity are decreased; apoptosis, vacuolization of odontoblasts, and tissue damage are observed.[39] In humans, angiogenic alterations in the dental pulp have been observed, as well as an increase in the number of microvessels, suggesting an increase in angiogenic growth factors in the dental pulp.[40] Others have reported that the angiogenic response of the pulp is influenced by epithelial growth factor (EGF) generated after orthodontic force application.[40-43] The inflammatory process appears to be influenced by neuropeptide stimulation and the generation of inflammatory cytokines, including IL-1, IL-3, and TNF-α.

The clinical significance of pulpal alterations produced by force application is whether they compromise the long-term viability of teeth. There is a paucity of data on the prevalence of long-term dental pulp damage caused by orthodontic treatment. Estimates for root canal obliteration (2%–17%) and pulpal necrosis (1%–14%) in orthodontically treated teenagers are wide.[44] There have been some cases of tooth vitality loss due to poorly regulated jiggling forces.[45] Pulp necrosis

has been reported after various orthodontic tooth movements.[46,47] Despite demonstrating pathologic alterations in the pulp tissue as a result of force application in general, no clear understanding was obtained of the association between orthodontic force magnitude and pulp tissue reaction. It is commonly assumed that stronger orthodontic pressures cause more severe pulpal alterations and their consequences,[48] but there is no solid scientific evidence to support this hypothesis.

Oxidative stress has the potential to harm a wide range of biological substances involved in the activities of the dental pulp, including nucleic acids, proteins, and lipids.[6] Generally, enzymes, including glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), are the first line of defense against oxidative stress.[49] Hydrogen peroxide, hydroperoxides, and superoxide radicals are all converted to harmless molecules by these enzymes.[50]

Heat stress increases the generation of reactive oxygen species (ROS) and the activation of antioxidant defense systems in human dental pulp cells.[51]

4. Biological properties and applications of 4HR

The substituted phenolic lipid 4HR, commonly known as 4-hexyl-1,3-dihydroxybenzene or 4-hexyl-1,3-benzenediol, has the chemical formula $CH_3(CH_2)_5C_6H_3$-1,3-$(OH)_2$.[52]

4.1. Biological properties of 4HR

4HR is employed in topical antiseptics and throat lozenges because of its antibacterial and antiparasitic effects.[53] When coupled with cisplatin, it inhibits squamous-carcinoma cell proliferation and has an anti-carcinogenic effect.[54] It has been utilized as a component in topical antiseptics for the treatment of sore throats in recent years.[55]

Assays have shown that 4HR suppresses bacterial, fungal, and parasitic growth and exhibits antioxidant and antigenotoxic effects.[56] It also has bactericidal properties against oropharyngeal bacteria that cause acute sore throats.[53] Because 4HR is a phenolic lipid derivative, its antimicrobial effect is mediated by the biological activity of phenolic lipids. Phenolic lipids react with biological membranes and cellular metabolism to exert cytotoxic, antibacterial, and antiparasitic effects. They disrupt the phospholipid cell membrane, altering its structure and permeability. They also alter intercellular protein and DNA structure.[56]

TNF-α is a pro-inflammatory cytokine produced by lymphocytes, neutrophils, macrophages, and mast cells.[57] It stimulates osteoclast differentiation through the RANKL pathway. In RAW264.7 cells, it is suppressed by 4HR application.[58] TNF-α is produced during the acute inflammatory phase and inhibits wound healing if it remains present for a long time. In burn wounds, TNF-α is abundantly expressed, whereas 4HR treatment lowers TNF-α expression and accelerates wound healing.[58]

4.2. Application of 4HR

4HR has been utilized as a component in burn ointments.[58] A 4HR ointment was used to treat a burn on the rear skin of a rat. Compared with the single ointment control, it exhibited rapid epithelization and collagen regeneration. Fourteen days following treatment, the 4HR ointment group had a smaller denuded region and a thicker epidermis. Furthermore, immunohistochemistry revealed reduced expression of TNF-α. The silk cocoon protein sericin was used as a dressing material.[59] In a diabetic burn wound model, sericin and 4HR combination ointment hastened wound healing and promoted epithelial regeneration.[60] Wound healing was better with both sericin and 4HR than with either alone. Mechanistically, 4HR ointment promotes epithelial and collagen regeneration in burn wounds by reducing TNF-α expression.[58]

A variety of biomaterials include 4HR, including silk sutures, vascular patches, membranes, and bone substitutes.[61-63] The medium was prepared with a 3 percent 4HR solution, although 4HR concentrations can be modified and altered.[64,65] A high concentration of 4HR may cause cell death and rapid biomaterial breakdown. A lower 4HR concentration is required for the inclusion of xenografted bone. Depending on dosage, 4HR can cause apoptosis.[66] To identify the appropriate concentration of 4HR for each type of biomaterial, more research is needed.

III. MATERIALS AND METHODS

Part 1. Cellular experiments

1.1. Cell culture and 4HR treatment

The dental pulp tissue was collected from the mandibular incisors of rats as described in a previous study.[13] The collected pulp tissue was washed with phosphate-buffered saline (PBS; CAT#17–602E; Lonza, Walkersville, MD, USA), then sliced with sterilized scissors and placed in a culture dish. Type I collagenase (CAT#17100017;Gibco™, Carlsbad, CA, USA) was added to the dish and incubated for two hours. Cells were cultured in 10 mL of minimal essential medium Eagle, alpha modification (α-MEM; CAT#SH30265, HyClone Laboratories, Logan, UT, USA) supplemented with 10% FBS (CAT#12483020, Gibco™), 50 U/mL penicillin G, 50 μg/mL streptomycin sulfate, 2 g/L sodium carbonate and 0.11 g/L of sodium pyruvate. This cell suspension was pelleted at 15,000 rpm for 10 min. Resuspended cells were cultured in 60-mm dishes. Resveratrol and 4HR (Sigma-Aldrich, St. Louis, MO, USA) stock solutions were prepared by dissolving in dimethyl sulfoxide (DMSO). Both were diluted in culture medium prior to application. The final DMSO concentration was 0.1%.

1.2. Assessing GPx activity and TAC

Changes in TAC in response to 4HR treatment were evaluated using commercial kits. The culture conditions for the primary cultured dental pulp cells and H_2O_2 treatment were the same as those described in section 1.1 above. Resveratrol was used as a positive control. Resveratrol or 4HR were added to dental pulp cells at concentrations of 1, 10, or 100 μM for 45 min. Cells were then treated with 0.1 mM H_2O_2 for 30 min. TAC was evaluated 24 h after resveratrol or 4HR treatment using a total antioxidant capacity assay kit (CAT#ab65329, Abcam, Cambridge, UK) according to the manufacturer's instructions. Optical density was measured at 570 nm after 90 min of incubation at room temperature. The GPx assay was performed using a commercial kit (CAT#ab102530, Abcam), according to the manufacturer's instructions. After adding cumen hydroperoxide, optical density was measured at 340 nm in kinetic mode.

1.3. Evaluating TNF-α and IL-1β Expression in Dental Pulp Cells after 4HR treatment

Primary antibodies against TNF-α and IL-1β (Santa Cruz Biotech, Santa Cruz, CA, USA) were used in all evaluations. Cell culture and H_2O_2 treatment conditions were as described in section 1.2 above. Primary cultured pulp cells were treated with 1, 10, or 100 μM 4HR. The positive control was H_2O_2 without 4HR pretreatment, whereas the negative control was no treatment. Cells were harvested 8 or 24 h after 4HR treatment and lysed using radioimmunoprecipitation assay (RIPA) buffer. Lysates were sonicated for 10 s. Protein concentrations were

measured using an RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were subjected to electrophoresis. After separation, proteins were transferred to nitrocellulose membranes. Blocking and antibody incubation steps were performed as described previously.[13] The dilution of primary antibody was 1:500. Blots were imaged using a ChemiDoc XRS system (Bio-Rad Laboratories). β-Actin was used as an internal control for normalization.

Part 2. Animal experiments

2.1. Animals and experimental design

Rats used in this study were purchased from Orient Bio Inc. (Sungnam, Korea). Twenty male rats (8-week-old Crl; CD-specific pathogen-free/viral antibody-free) were used. Caging and breeding conditions were as described previously.[13] This animal study was approved by the Gangneung-Wonju National University Committee for Animal Research (GWNU-2021-01).

Eighteen rats were subjected to incisor cutting. Pulp tissue was extracted from two rats to determine the baseline activity of TAC and GPx. The right incisor was cut at the free gingival margin; pulp exposure was identified as pinpoint bleeding. The experimental group was treated with a 2% 4HR ointment, and the control group was treated with the lanolin ointment base only. Three animals from each group were euthanized three days after application; teeth were harvested for protein extraction and western blotting. Another six animals from each group were euthanized 5 days after ointment application. Among these, half were used for histological and immunohistochemical analyses. Pulp tissue from the incisors was extracted from the other three rats in each group. They were then processed and homogenized for TAC and GPx analysis.

2.2. Histological, Immunohistochemical, Western blot, GPx activity and TAC analysis

Routine hematoxylin and eosin (H&E) staining was performed for histology. To measure the expression of TNF-α and IL-1β in pulp tissues, immunohistochemical staining was performed using anti-TNF-α and anti-IL-1β antibodies (Santa Cruz Biotech). Briefly, deparaffinized slides were treated with 1 mg of porcine trypsin (Sigma-Aldrich) for 10 min for antigen retrieval. Endogenous peroxidase activity was blocked by treatment with 30% hydrogen peroxide (H_2O_2) for 7 min. Slides were then washed and subjected to protein blocking for 1 h. Then, primary antibodies (dilution ratio 1:50) were applied and the slides were covered with parafilm. Slides were incubated in a humid chamber at 4 ˚C for 8 h. After washing, a universal secondary antibody (Dako REAL™ EnVision™/HRP, Rabbit/Mouse; Dako North America Inc., Carpinteria, CA, USA) was added. Unreacted secondary antibodies were removed by washing with PBS and slides were stained with a chromogen substrate (Dako REAL™ DAB+Chromogen and Dako REAL™ Substrate Buffer; Dako North America Inc.).

Fresh mandibular incisors on the affected side were separated from the mandible, homogenized in a buffer containing a protease inhibitor cocktail, and evaluated using western blotting as described above (Section 1.3). Pulp tissue from the incisor was processed and homogenized for TAC and GPx analysis. Subsequent procedures were performed as described above (Section 1.2). The relative activities of TAC and GPx in the no-injury group were assessed for comparison with 4HR and ointment base-only (control) groups.

Part 3. Statistical analysis

For both cell and animal experiments, the levels of TAC and GPx and the relative expression of TNF-α and IL-1β were compared between groups using the independent sample *t*-test and Wilcoxon rank sum test for measurements satisfying the parametric assumption and for those that did not, respectively. All statistical analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). The level of significance was set at $p < 0.05$.

For western blotting, relative protein expression levels between groups were compared using one-way analysis of variance (ANOVA) and a post-hoc test using the Bonferroni method. The level of significance was set at $p < 0.05$.

IV. RESULTS

Part 1. Cellular experiments

1.1. Application of 4HR Increases GPx Activity and TAC in Dental Pulp Cells

The control group showed a TAC of 0.223 ± 0.003 mM (Figure 1a). The resveratrol group had values of 0.231 ± 0.002 mM, 0.236 ± 0.004 mM, and 0.245 ± 0.005 mM for 1, 10, and 100 μM, respectively. The 10 μM and 100 μM resveratrol groups showed significantly increased TAC compared to controls (p = 0.002 and 0.001, respectively). Treatment 4HR produced a similar, dose-dependent increase in TAC: 0.228 ± 0.003 mM, 0.235 ± 0.007 mM, and 0.247 ± 0.002 mM for 1 μM, 10 μM, and 100 μM, respectively. Treatment with 10 μM and 100 μM 4HR significantly increased TAC levels over those of controls (p = 0.013 and p <0.001, respectively), while there were no significant differences between the 4HR and resveratrol groups (p > 0.05). GPx activity in the H_2O_2 treated control was 1.089 ± 0.022 mU/mL (Figure 1b), while for resveratrol treatment, it was 1.244 ± 0.001 mU/mL, 1.246 ± 0.002 mU/mL, and 1.559 ± 0.008 mU/mL for 1 μM, 10 μM, and 100 μM, respectively. Treatment with 10 μM and 100 μM resveratrol significantly increased GPx activity compared to the controls (p = 0.009 and < 0.001, respectively). GPx activity in the 4HR group was 1.247 ± 0.021 mU/mL, 1.309 ± 0.015 mU/mL, and 1.411 ± 0.017 mU/mL for 1 μM, 10

μM, and 100 μM, respectively. Treatment with 10 μM and 100 μM 4HR significantly increased GPx activity compared to controls (p = 0.005 and 0.001, respectively); however, no significant differences were observed between 4HR and resveratrol ($P > 0.05$).

1. 2. 4HR Decreases the TNF-α and IL-1β Expression Induced by Hydrogen Peroxidase

Hydrogen peroxide treatment increased TNF-α and IL-1β expression, which was then decreased by pretreatment with 4HR (Figure 2). The relative expression of TNF-α in H_2O_2-treated controls was 0.852 ± 0.054 (Figure 3). The relative expression of TNF-α at 8 h after 4HR treatment was 0.741 ± 0.058, 0.642 ± 0.043, and 0.655 ± 0.049 for 1, 10, and 100 μM, respectively. Treatment with 10 μM and 100 μM 4HR significantly decreased TNF-α expression compared to that of the controls (p = 0.006 and 0.010, respectively). The relative expression of TNF-α 24 h after 4HR treatment was 0.684 ± 0.045, 0.510 ± 0.045, and 0.350 ± 0.023 for 1 μM, 10 μM, and 100 μM, respectively. Treatment with 1 μM, 10 μM, and 100 μM 4HR significantly decreased TNF-α expression compared to that of the controls (p = 0.014, 0.001, and < 0.001, respectively). The relative expression of IL-1β in the H_2O_2 treated control was 1.108 ± 0.067. The relative expression of IL-1β at 8 h after 4HR treatment was 0.859 ± 0.056, 0.739 ± 0.053, and 0.598 ± 0.043 for 1, 10, and 100 μM, respectively. Treatment with 1, 10, and 100 μM

4HR significantly decreased TNF-α expression compared to that of the controls (p = 0.008, 0.001, and < 0.001, respectively). The relative expression of IL-1β 24 h after 4HR treatment was 0.688 ± 0.047, 0.491 ± 0.031, and 0.120 ± 0.012 for 1 μM, 10 μM, and 100 μM, respectively. Treatment with 1, 10, and 100 μM 4HR significantly decreased IL-1β expression compared to that in the controls (p = 0.001, < 0.001, and < 0.001, respectively). Decreased TNF-α expression after administration of 4HR and resveratrol after treatment with H_2O_2 was verified by confocal microscopy using fluorescein isothiocyanate (Figure 4).

Part 2. Animal experiments

2.1. 4HR Decreased Inflammatory Reaction of Pulp Tissue Induced by Physical Stress

Immunohistochemistry demonstrated that 4HR treatment of exposed pulp tissue decreased the expression of TNF-α and IL-1β compared to controls. H&E staining showed that the ointment base-only controls exhibited vascular dilatation and massive hemorrhage in response to stress (Figure 5a). The expression levels of TNF-α (Figure 5b) and IL-1β (Figure 5c) were also higher in ointment base-only controls. However, 4HR treatment reduced hemorrhage areas and active remodeling and regeneration (Figure 5d). The expression levels of TNF-α (Figure 5e) and IL-1β (Figure 5f) were reduced by 4HR treatment.

Relative expression was measured by staining intensity (0, no staining; 255, highest intensity). The densitometric measurement of relative expression of TNF-α was 74.87 ± 9.39 and 53.78 ± 11.95 in the control and 4HR groups, respectively (Figure 6) and the difference between these groups was statistically significant ($p < 0.001$). The relative expression level of IL-1β was 64.57 ± 6.72 and 49.26 ± 4.18 in the control and 4HR groups, respectively; the difference between these groups was also statistically significant ($p < 0.001$).

Western blot data were in accordance with the results from immunohistochemistry. Administration of 4HR to exposed incisor pulp tissues reduced the expression of both TNF-α and IL-1β (Figure 7a).

The relative expression of TNF-α was 1.36 ± 0.32 and 0.59 ± 0.49 in the control and 4HR groups, respectively (Figure 7b) and was statistically significant (p = 0.049). The relative expression level of IL-1β was 0.57 ± 0.08 and 0.15 ± 0.11 in the control and 4HR groups, respectively (Figure 7b) and was also statistically significant (p = 0.005).

Application of 4HR ointment reduced oxidative stress in the dental pulp compared to the ointment base-only control group. The relative level of TAC was 1.37 ± 0.02 and 1.20 ± 0.05 in the control and 4HR groups, respectively (Figure 8) and the difference between these groups was statistically significant (p = 0.009). GPx activities were 1.51 ± 0.05 and 1.21 ± 0.03 in the control and 4HR groups, respectively (Figure 8); the difference between groups was also statistically significant (p = 0.001).

V. DISCUSSION

Excessive stress is known to increase the production of reactive oxygen species (ROS), one of the major causes of inflammation.[10,11] Dental pulp tissue healing requires transient activation of the inflammatory response, which suggests that proper repair requires that ROS inducers for this response also should be expressed transiently. Therefore, the application of antioxidants may improve tissue regeneration under ROS stress.[67]

Resveratrol is a natural polyphenol and antioxidant activity.[68] Sirtuins (SIRTs) are class III histone deacetylase, and their activity is related to relieving cellular stress and extending the lifespan.[69] Resveratrol was found to activate SIRT1 in chondrocytes and blocked NFκB activation and suppressing IL-1β induced expression of iNOS in human chondrocytes.[70] SIRT activity is also increased by 4HR.[69] The administration of 4HR to microorganisms induces a dormancy-like state, in which they are much more resistant to the environment and can extend their lifespan.[70] Based on these observations, we hypothesize that 4HR administration might increase SIRT activity. Similar to resveratrol, we found that 4HR suppressed IL-1β expression. Thus, 4HR-associated IL-1β suppression may be similar to that of resveratrol. These include antioxidant activities and SIRT activation.

Dental pulp responds to the stress applied to the surrounding tooth.[71,72] Pathological tooth stress includes caries, periodontitis, and fracture. Orthodontic tooth movement and cavity preparation are examples of iatrogenic stresses on

teeth. Dental pulp generates reactive oxygen species (ROS) that induce an inflammatory response to these stresses[68] by altering blood flow in the dental pulp.[73]

Antioxidants have been candidates to reverse inflammation in injured dental pulp to help smoothen the transition to healing. Since tooth fracture is a frequent dental injury in which pulp is exposed and consequently exhibits increased inflammation and edema,[74,75] there have been several studies that tested antioxidants to reduce the oxidative damage in tooth-fracture models.

In this study, we simulated inflammatory oxidative conditions by applying hydrogen peroxide, which increases ROS production, to the dental pulp tissue and incisors of rats for cellular and animal experiments, respectively. The four rat HR applications were in the form of an ointment.

Antioxidant screening, using a hydrogen peroxide-induced ROS production model, was used in our study. Catalase (CAT), GPx, and superoxide dismutase (SOD) are important enzymes that protect cells from oxidative injury[76] and could be rulers for measuring the level of antioxidative reactions.

In this study, GPx activity and TAC were significantly upregulated by 4HR (10–100 μM) compared to the untreated controls ($p < 0.05$, Figure 1), indicating that pretreatment with 4HR or resveratrol protected the pulp cells from oxidative damage. This was consistent with a previous study showing that resveratrol and 4HR increased glutathione levels and GPx activity in human lymphocytes following hydrogen peroxide stimulation.[10] Similar results were reported in studies using HUVECs.[11,77]

It is clear that reducing oxidative stress alleviates inflammatory cytokine production, thereby reducing the availability of various signaling factors, including TNF-α and IL-1β.[78] Therefore, we performed immunohistochemistry to measure the expression of TNF-α and IL-1β. Administration of 4HR decreased the expression of both, after both were elevated in response to hydrogen peroxide (Figure 2). As DAPI primarily binds to AT-rich DNA domains, its increased lifetime in the 4HR group suggests that 4HR also is bound to AT-rich domains.[79] Interestingly, the *TNF A* promoter has an AT-rich domain and is a transcription factor-binding domain. Thus, accumulation of 4HR might result in decreased transcription and decreased TNF-α levels.

The activities of antioxidant enzymes varies between models. The levels of TAC and GPx activity were significantly reduced by 4HR treatment compared to those in the ointment base-only controls ($p < 0.05$, Figure 8). In chronic inflammatory conditions, ROS levels may be persistently elevated and the elevation of antioxidant enzyme activity follows.[80, 81] Both the 4HR-treated and the ointment base-only (control) groups exhibited elevated GPx activity and TAC (relative activity >1, Figure 8) compared to the non-injured pulp control. CAT activity is increased in inflamed dental pulp.[80] The application of antioxidants neutralizes ROS and decreases GPx activity and TAC in the dental pulp.[81] Antioxidant application to inflamed tissues should help in the early resolution of inflammation. The injured dental pulp showed chronic inflammation (Figure 5). The ointment base-only group showed massive hemorrhage and blood clots in the dental pulp (Figure 5a), and cellular regeneration in the hemorrhagic area was rare when the teeth were treated with the ointment base only. However, 4HR treatment

reduced the number of blood clots and markedly improved cellular regeneration (Figure 5d). An increase in ROS production stimulates the production of inflammatory cytokines such as TNF-α and IL-1β. In this study, the expression levels of TNF-α and IL-1β in the rat model were decreased by 4HR treatment (Figure 7). These results are consistent with the *in vitro* data (Figure 2).

The application of antioxidants in dental practice has been shown to exert beneficial effects. Titanium-based dental implants or plates induce a redox imbalance and oxidative damage to the periosteum.[82, 83] Lactoferrin (LF) is another well-known antioxidant.[84] Incorporation of LF in titanium discs improved the antibacterial and osteoinductive ability of dental implants.[85] A similar approach has been used in the development of bone cement.[86] According to previous research,[87] 4HR-integrated dental implants improve new bone formation in the case of bacterial contamination.

The possible antioxidant activity of 4HR suggests that it can be useful in multiple orthodontic treatment conditions, including gingivitis[88] and root resorption[13]. However, the method of applying 4HR varies by dental practice. When 4HR is prescribed for oral intake, its absorption from the gastrointestinal tract is poor.[89] Because of this poor absorption rate, it has been used for killing intestinal pathogens. Russian scientists found that microorganisms surviving 4HR administration undergo dormancy and are more resistant to external environmental stress.[79] However, intraoral administration was judged to be inappropriate for 4HR in dental pulp because of poor absorption. Although 4HR has been demonstrated to be beneficial for orthodontic side effects, identifying an appropriate method of carrying 4HR to target tissue is necessary.

In this study, a single application of 4HR ointment to the exposed dental pulp resulted in a reduction in levels of ROS and the inflammatory cytokines TNF-α and IL-1β. Treatment with 4HR ointment decreased TNF-α expression and increased epithelialization in a deep burn model[58] and in a diabetic animal model and 4HR topical application has been shown to increase capillary regeneration.[91] Ointment application is an easy and simple method for dental interventions.

However, this study had some limitations. Although the rat mandibular incisor is a good candidate because of its relatively large size and ease of approach, it is not appropriate for studying long-term changes in dental pulp because it erupts continuously and rapidly.[12] This continuous eruption is also an important difference between rats and humans. Despite these limitations, the application of 4HR has several obvious advantages, including reduced oxidative stress and downregulation of the expression of inflammatory cytokines. These have the potential to improve therapeutic outcomes in several dental practices, including orthodontic treatment. Altogether, these data suggest that further investigation in other models is warranted to better evaluate the effects of 4HR on stressed dental pulp tissue.

VI. CONCLUSIONS

The total antioxidant capacity and glutathione peroxidase activity were significantly increased following 4HR treatment *in vitro* model. 4HR pre-treatment alleviates hydrogen peroxide induced oxidative stress in dental pulp cells through decreasing the expression of TNF-α and IL-1β. Moreover, 4HR treatment also reduced the expression of TNF-α and IL-1β via antioxidant activity *in vivo*. In conclusion, 4HR exerts protective effects against oxidative stress in dental pulp tissues through the downregulation of TNF-α and IL-1β.

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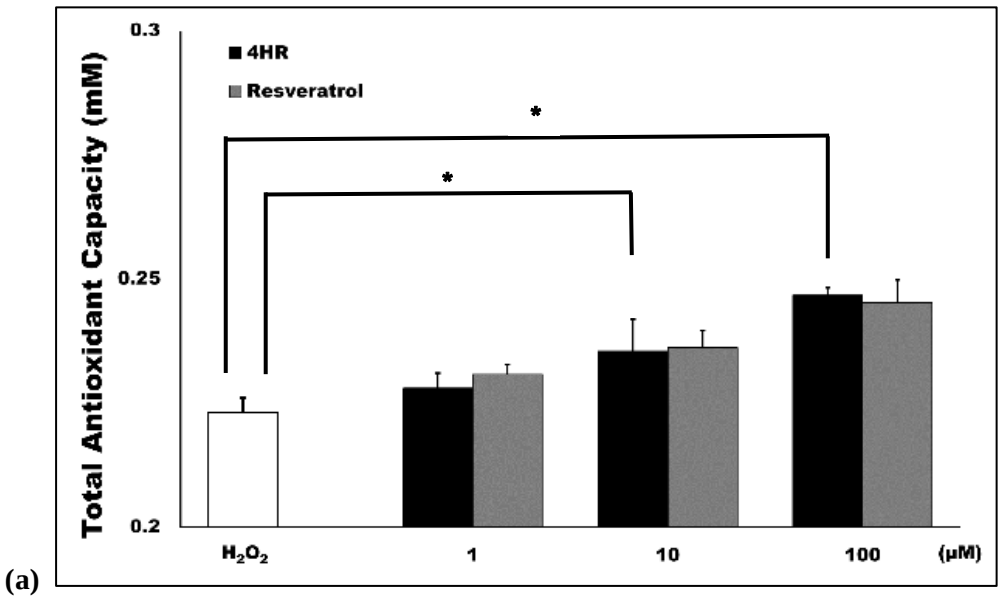
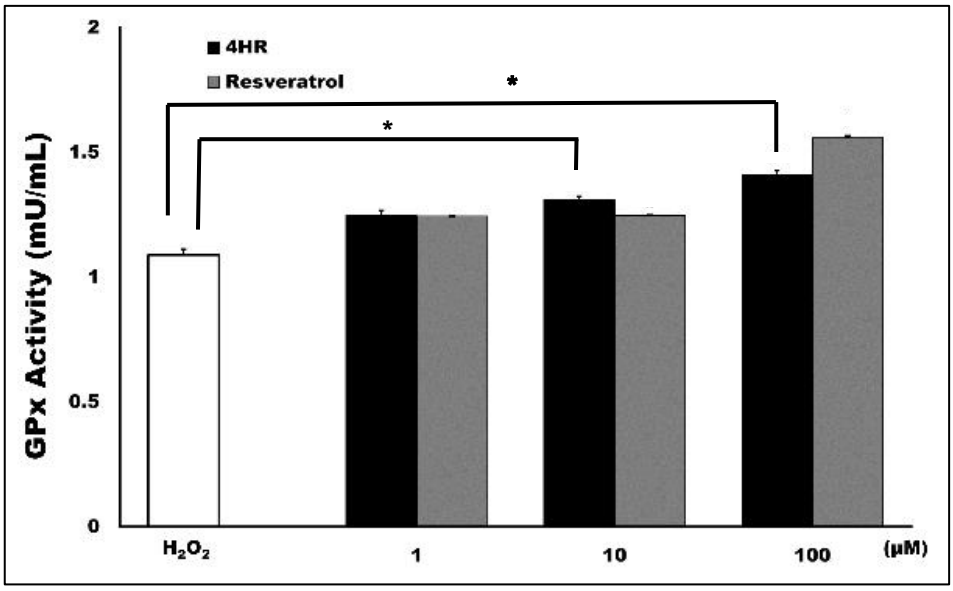


(a)



(b)

Figure 1. Antioxidant activity in dental pulp cells.

(**a**) Total antioxidant capacity (TAC). 4-Hexylresorcinol (4HR) (black) induced a similar level of TAC production as that of resveratrol (grey). TAC in the 4HR group increased in a dose-dependent manner. Treatment with 10 μM and 100 μM 4HR significantly increased TAC compared to controls (p = 0.013 and <0.001, respectively)

(**b**) Glutathione peroxidase (GPx) activity. 4HR (black) and resveratrol (grey) increased GPx activity over that of the H_2O_2-only control in a dose-dependent manner. 4HR (10 µM and 100 µM) significantly increased GPx activity over that of controls (p = 0.005 and 0.001, respectively).

* $p < 0.05$, compared with the hydrogen peroxide group. Data are shown as mean ± standard deviation.

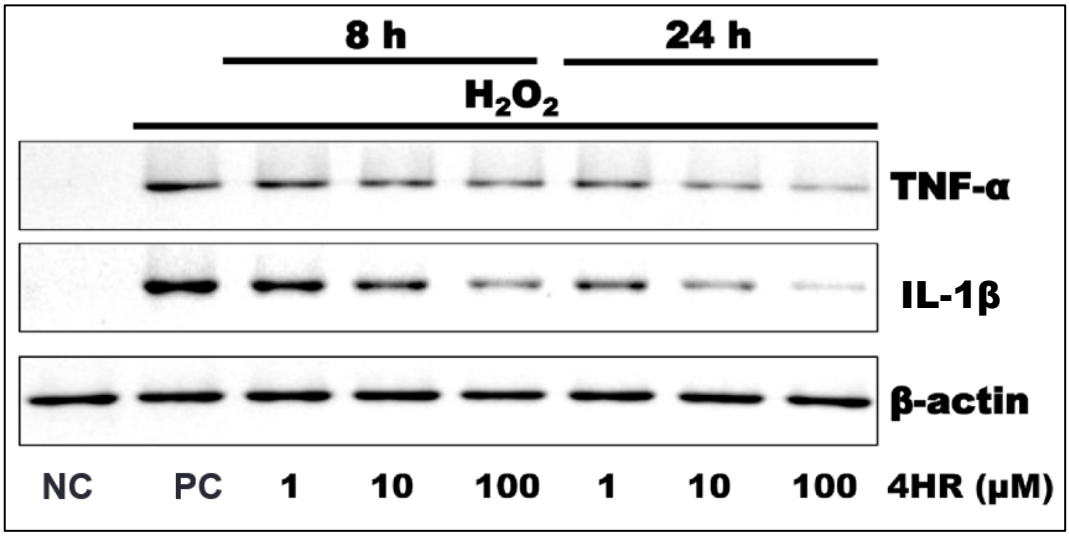


Figure 2. Western blot analysis in dental pulp cells after treatment with 0.1 mM H_2O_2. Hydrogen peroxide treatment increased the expression levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) expression, which was then downregulated in response to pre-treatment with 4-Hexylresorcinol (4HR) in a time- and dose-dependent manner. NC, negative control (no treatment group). PC, positive control (H_2O_2 group without 4HR pretreatment).

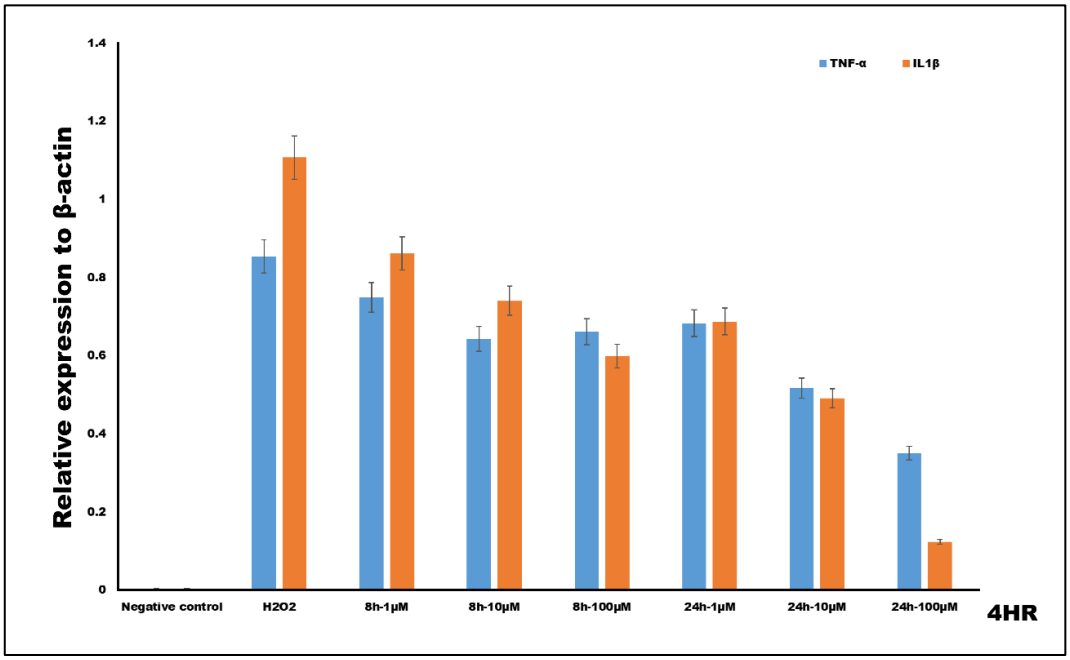


Figure 3. Relative TNF-α and IL-1β expression in dental pulp cells after 4HR administration. The administration of 4HR decreased the expression of TNF-α and IL1-β in a time- and dose-dependent manner after oxidative damage induced by H_2O_2 application. Data are shown as mean ± standard deviation.

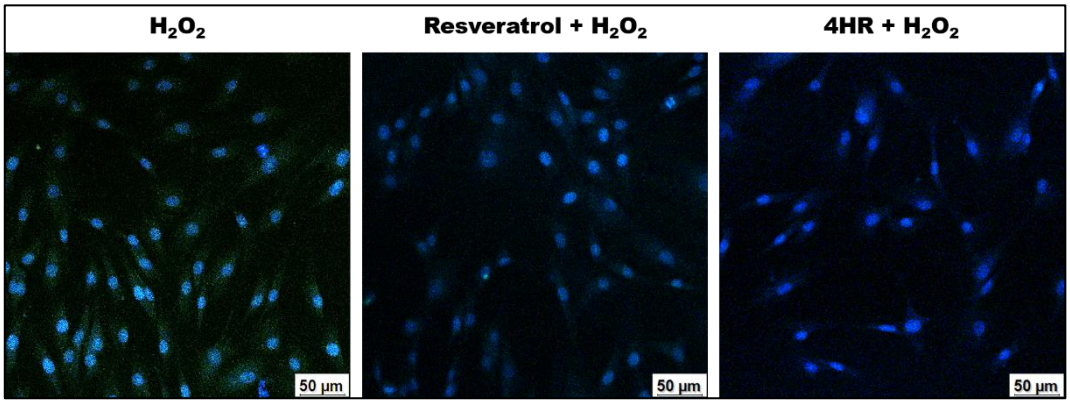


Figure 4. TNF-α expression decreased in dental pulp cells by administration of 4HR and resveratrol after treatment with H_2O_2 (Confocal microscopy of fluorescein isothiocyanate)

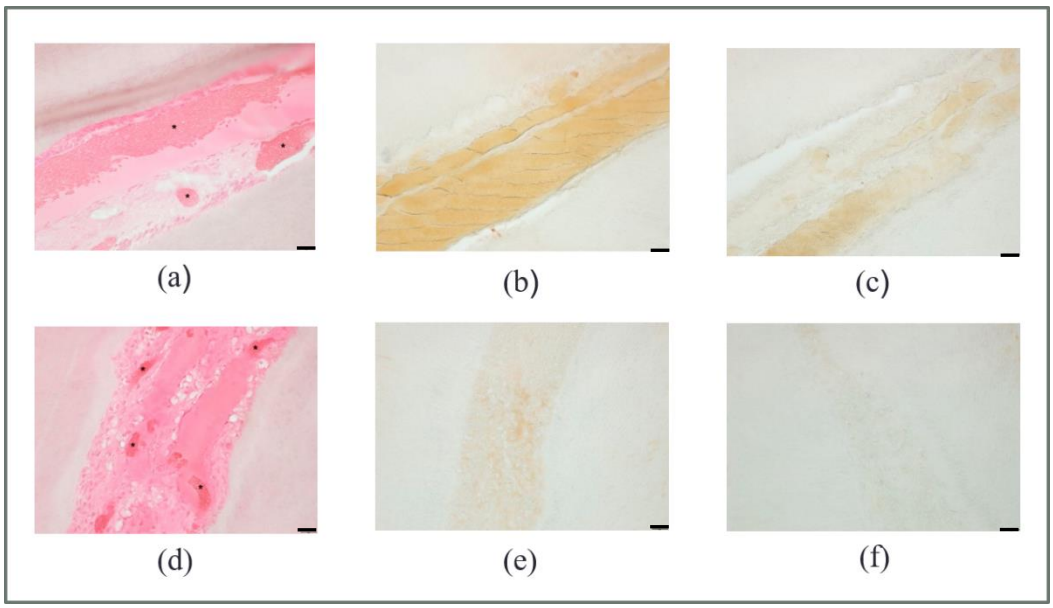


Figure 5. Histological and immunohistochemical analyses of the incisor damage model. (**a**). Hematoxylin and eosin (H&E) staining showing that the ointment base-only controls exhibited vascular dilatation and massive hemorrhage (*) in response to the stress, Expression levels of both (**b**) TNF-α and (**c**) IL-1β were increased in the ointment base-only controls. However, 4HR treatment reduces hemorrhage areas (*) and active remodeling and regeneration (**d**). Expression levels of both (**e**) TNF-α and (**f**) IL-1β decreased by 4HR treatment (bar = 20 μm, original magnification ×200).

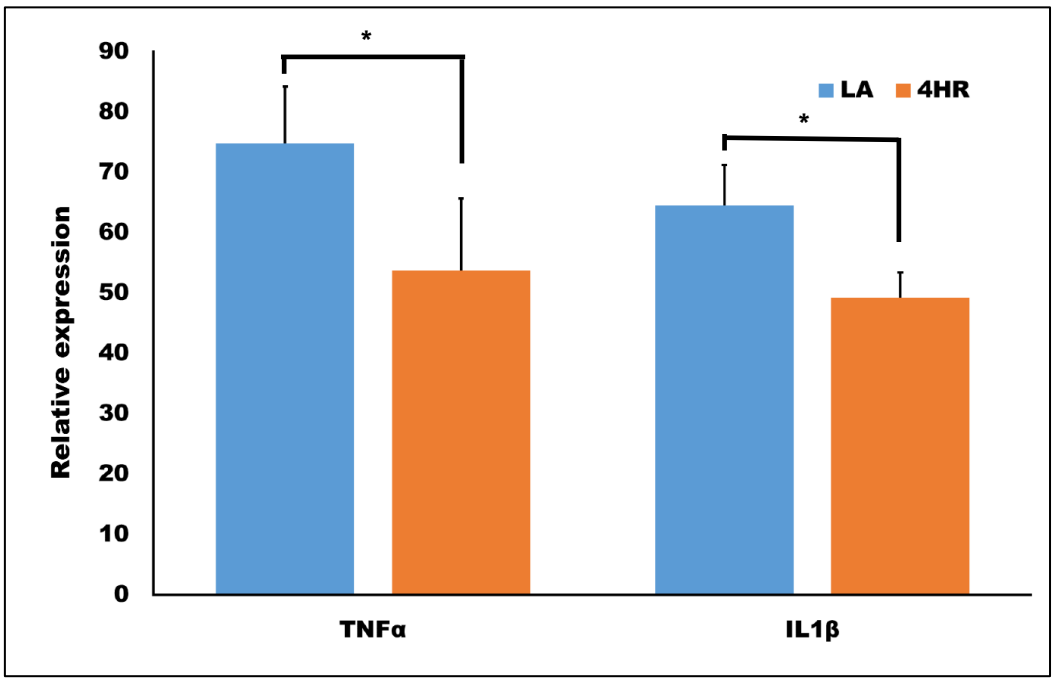
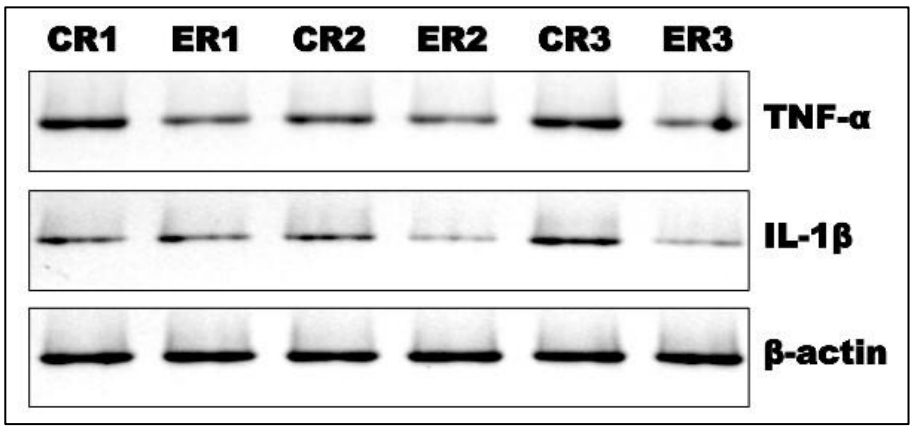


Figure 6. Relative expression of TNF-α and IL-1β. Administration of 4HR significantly decreased the expression of both in tissue sections after oxidative damage induced by cutting incisors ($p < 0.05$). Expression was measured by staining intensity (0, no staining; 255, highest intensity). * $p < 0.05$ compared to the ointment base-only control. Data are shown as mean ± standard deviation.

(a)



(b)

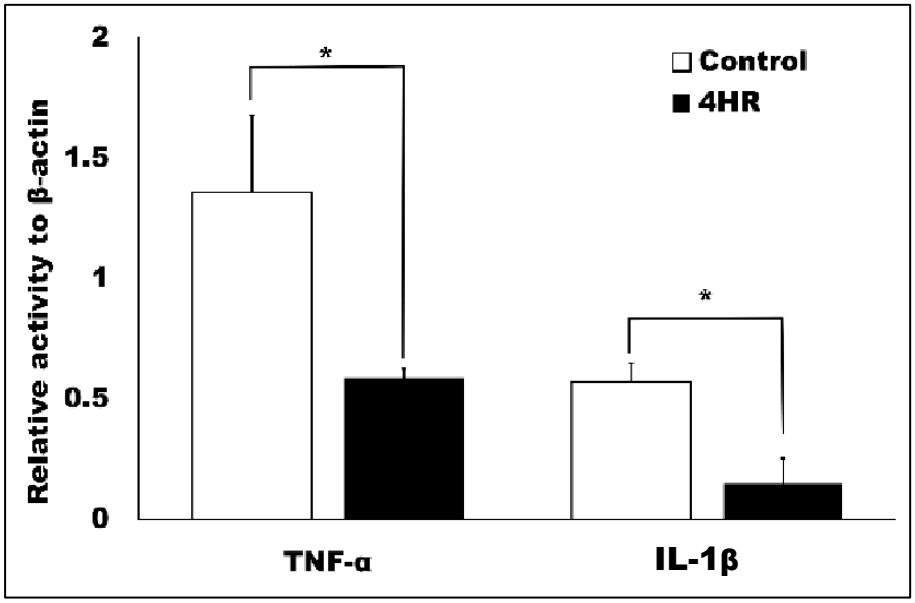


Figure 7. TNF-α and IL-1β expression in tissue samples is decreased by 4HR treatment, measured by western blotting. (**a**) Samples from ointment base-only controls are designated CR1, CR2 and CR3, while those taken after 4HR treatment designated ER1, ER2 and ER3. (**b**) Expression normalized against β-actin and presented as relative increase in TNF-α and IL-1β expression versus no-treatment controls (*$p < 0.05$). Data are presented as mean ± standard deviation.

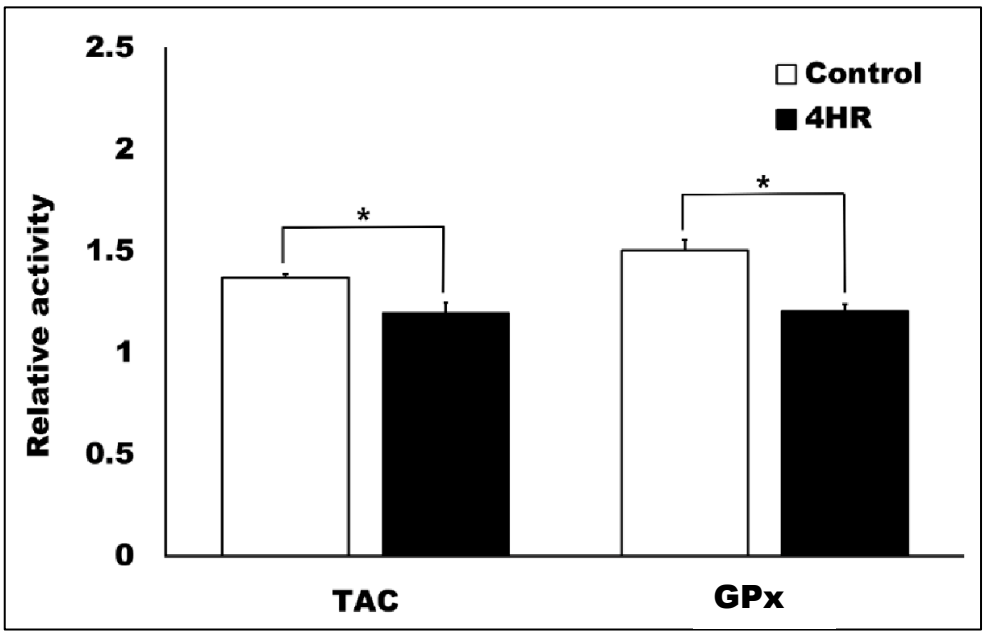


Figure 8. Total antioxidant capacity (TAC) and glutathione peroxidase (GPx) activity in dental pulp tissue. Controls received only the ointment base. The relative activity of the uninjured incisor pulp was set to 1. Pulp injury increased TAC and GPx activity, while the application of 4HR attenuated the oxidative stress significantly compared to the ointment base-only controls (*$p < 0.05$). Data are presented as mean ± standard deviation.

국문초록

흰 쥐의 치수세포에서 4-Hexylresorcinol 투여가 산화스트레스로 인한 활성산소에 미치는 영향

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1. 목 적

외상이나 교정력에 의해 치아는 스트레스를 받게 되며, 이에 대한 생물학적 반응으로 발생하는 산화 스트레스(oxidative stress)는 tumor necrosis factor-α (TNF-α)와 interleukin-1β (IL-1β) 같은 염증 촉진(pro-inflammatory) 요인들을 증가시켜 구강내 염증 반응을 일으킨다. 4-Hexylresorcinol (4HR)은 resveratrol 과 유사한 항산화제로 밝혀졌으며, 항산화제를 투여하여 과도한 산화 스트레스를 경감시키는 것은 치수조직의 치유에 도움을 줄 수 있다. 본 연구의 목적은 치수에서 산화스트레스에 대한 항산화제로서 4HR 의 치료 효과를 평가하는 것이다.

2. 방 법

생체 외(*in vitro*) 실험을 위하여 흰쥐의 하악 전치에서 치수 조직을 채취하여 배양한 dental pulp cell에 다양한 농도의 4HR (1,10,100 μM)을

전처리하고 H_2O_2를 처치한 후 total antioxidant capacity (TAC)와 glutathione peroxidase (GPx) 활성도를 측정하였다. TNF-α와 IL-1β의 항체에 대한 western blot을 시행하여 단백질 발현정도를 평가하였다.

생체 내(*in vivo*) 실험으로는 18마리(실험군, 대조군 각 9마리)의 흰 쥐에 하악 우측 절치를 절단하고 각각 2% 4HR ointment, ointment base (lanolin)를 도포하였다. 희생 후 TNF-α와 IL-1β에 대한 western blot을 시행하였으며 면역조직화학분석(immunohistochemical analysis) 및 TAC 와 GPx 활성도를 측정하였다. 하악 절치를 절단하지 않은 2마리는 TAC 와 GPx의 baseline activity를 측정하는데 이용하였다.

3. 결 과

1) 생체 외(*in vitro*) 실험: 4HR 투여 시 치수배양세포에서 TAC 와 GPx 활성도가 증가하였으며 ($p < 0.05$), TNF-α 와 IL-1β 발현 수치는 감소하였다 ($p < 0.05$).

2) 생체 내*(in vivo*) 실험: 외상에 의한 스트레스를 받은 치수조직에 4HR ointment 적용시 산화 스트레스를 완화시키며, TNF-α 와 IL-1β 발현 수치 역시 감소하였다 ($p < 0.001$).

4. 결 론

이상의 결과를 바탕으로, 4HR 투여가 TNF-α 와 IL-1β 를 감소시켜 산화스트레스로부터 치수 조직을 보호하는 효과가 있다고 판단된다.

주요어: 4-Hexylresorcinol, 활성산소, 치수조직. 산화스트레스

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