# Effects of Deferoxamine on Inflammation and Osteoclastogenesis: *in vitro* and *in vivo* model

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# Effects of Deferoxamine on Inflammation and Osteoclastogenesis: *in vitro* and *in vivo* model

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**Ko Eun Lee**

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**This certifies that the dissertation of Ko Eun Lee is approved.**

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# 감사의 글

먼저 연세대학교 치의학대학원에서 탁월한 실력과 훌륭한 인품을 지닌 교수님들의 지도 하에 소아치과학을 공부할 수 있도록 인도하신 하나님께 감사드립니다. 제가 박사논문을 완성할 수 있도록 애정과 지도해주신 지도교수님이신 최형준 교수님, 실험 및 논문 작성에 큰 도움을 주신 송제선 교수님, 경희대 이효설 교수님, 강정민 교수님, 전미정 박사님께 감사드립니다. 소아치과 의사로 올바르게 성장할 수 있도록 가르침을 주신 이제호 교수님, 김성오 교수님, 경희대 최성철 교수님, 지금은 은퇴하신 소아치과의 두 기둥이신 손흥규 교수님, 최병재 교수님께도 깊은 감사를 드립니다.

한결같이 곁을 지켜주신 부모님과 남동생, 조언을 해준 남편 김경남과 삶의 원동력인 딸 김하연에게도 고마움을 전합니다. 오랜 시간 동고동락 했던 소아치과 동기들과 실험에 도움을 준 선후배들에게도 감사의 마음을 전합니다. 이 모든 분들의 도움으로 박사과정을 마무리 할 수 있었습니다.

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# Abstract

## Effects of Deferoxamine on Inflammation and Osteoclastogenesis: *in vitro* and *in vivo* model

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Replacement and inflammatory resorption are serious complications associated with delayed replantation of avulsed teeth. This study aimed to assess whether deferoxamine (DFO) can suppress inflammation and osteoclastogenesis *in vitro* and attenuate inflammation and bone resorption in replanted rat tooth model. Cell viability and lipopolysaccharide (LPS)-induced inflammation were evaluated in RAW264.7 cells. Receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis was confirmed using tartrate-resistant acid phosphatase staining, measurement of the levels of reactive oxygen species (ROS) and quantitative reverse transcriptase-polymerase chain

reaction. RANKL-treated cells were exposed to different concentrations of DFO and hypoxia-related gene expression was confirmed by quantitative real-time polymerase chain reaction. The maxillary left molars of 31 six-week-old male Sprague Dawley rats were extracted and stored in saline (n = 10) or DFO solution (n = 21) before replantation. Micro-computed tomography (micro-CT) imaging and histological analysis were performed to evaluate inflammation and root and alveolar bone resorption. Results showed that DFO treatment led to a significant attenuation of the release of nitric oxide ($p < 0.01$) and expression of pro-inflammatory ($p < 0.05$) and osteoclastogenic genes ($p < 0.01$). DFO also reduced ROS production, mitochondrial biogenesis related gene encoding CAMP responsive element binding protein 1 / gene encoding PPARG coactivator 1 beta mechanism and stimulated nuclear factor erythroid 2-related factor 2 / heme oxygenase 1 signaling pathway. In addition, the expression of hypoxia-related genes increased. Furthermore, results from micro-CT and histological analysis provided evidence regarding decrease in inflammation and hard tissue resorption in the DFO group. Overall, these results suggest that DFO reduces inflammation and osteoclastogenesis in a tooth replantation model, and thus needs to be further investigated as a root surface treatment option for an avulsed tooth.

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**Keywords:** Deferoxamine, Tooth replantation, Inflammation, Osteoclastogensis, Reactive oxygen species

# Effects of Deferoxamine on Inflammation and Osteoclastogenesis: *in vitro* and *in vivo* model

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## I. Introduction

Replacement and inflammatory resorption are serious complications that can occur with the replantation of avulsed teeth (Day et al., 2012). It is associated with the damage of the periodontal ligament (PDL), viability of the PDL cells on the root surface, and presence of bacterial contamination (Poi et al., 2013). Short extra-alveolar time and use of a suitable temporary storage medium are critical for good prognosis of replanted teeth (Sigalas et al., 2004; Trope, 2002).

Depending on the site of the trauma and other related conditions, replantation cannot be conducted immediately and/or an appropriate storage medium cannot be used. Therefore, in cases where the replantation is delayed and/or an appropriate storage medium is unavailable, there have been several attempts to increase the viability of the tooth by applying root surface treatments with chemical materials (Panzarini et al., 2008), such as acidulated fluoride (Mahajan and Sidhu, 1981), alendronate (Lustosa-Pereira et al., 2006), and tooth enamel protein (Emdogain® ; Biora AB, Malmö, Sweden) (Poi et al., 2007).

Deferoxamine (DFO) is a Food and Drug Administration-approved iron chelator used to reduce acute and chronic iron overload and aluminum toxicity in patients with chronic kidney disease (Velasquez and Wray, 2020) and patients with thalassemia receiving a large amount of blood transfusion (Ballas et al., 2017). Recently, the use of DFO has been expanded to regenerative medicine (Fujisawa et al., 2018) as it can increase angiogenesis in wound healing and bone regeneration and has anti-inflammatory properties (Holden and Nair, 2019).

Recently in dentistry, DFO was known to induce osteoblastic and osteogenic differentiation in human periodontal ligament cells (Chung et al., 2014; Mu et al., 2017). As a representative hypoxia mimicking agent, the angiogenic capacity was investigated by confirming the increase of vascular endothelial growth factor and the related downstream angiogenic pathways. Not only in periodontal but also in dental pulp cells, DFO was found to promote odontoblast differentiation and because of its high healing potential, DFO is also applied to repair dental pulp stem cells (Jiang et al., 2014; Wang et al., 2017).

Reactive oxygen species (ROS) is an essential transmission cell signal with various physiological functions (Foyer, 2018). It is known that iron chelators, like DFO are closely connected with ROS signaling (Park and Chung 2019). However, excessive ROS can interfere with the balance between the oxidant and antioxidant systems (Tao et al., 2020). As shown in various studies on inflammation (Shen et al., 2021), rheumatoid arthritis (Phull et al., 2018), and osteoporosis, (Schröder, 2015), increased ROS is closely associated with increased bone destruction and inflammation. However, the effects of DFO on ROS production are still controversial.

Given its safety and potential efficacy, DFO needs to be considered as a candidate for root surface treatments before replanting the avulsed tooth. However, to the best of our knowledge, there has been no study to investigate the effects of DFO on inflammation and osteoclastogenesis in a tooth replantation model. Therefore, this study aimed to assess the effects of DFO on inflammation and osteoclastogenesis *in vitro* and in replanted rat molar tooth model.

# II. Materials and Methods

## 1. Cell culture

RAW264.7 cells were obtained from the Korean Cell Line Bank and cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA), containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 0.2% amphotericin B (Invitrogen), at 37 °C in 5% $CO_2$.

## 2. Cell viability assay

Measurement of cell viability was performed using Cell Counting Kit - 8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). RAW264.7 cells were plated onto 24-well plate (25,000 cells/well) and incubated for 24 hours, followed by treatment with different doses of DFO (Sigma, St. Lois, MO, USA) (0 μM, 10 μM, 20 μM, 50 μM, 100 μM) and incubation for another 24 h. Subsequently, the quantity of water-soluble colored formazan from the CCK-8 assay, formed by the activity of dehydrogenases in living cells, was measured using a spectrophotometer (Benchmark Plus microplate spectrophotometer, Bio-Rad Laboratories Inc., Hercules, CA, USA) at 450 nm. All experimental data were obtained from three independent experiments, with each sample run in triplicate.

### 3. Nitric oxide assay

RAW264.7 cells were treated with DFO (0 μM, 10 μM, 20 μM, 50 μM, 100 μM) for 2 h and lipopolysaccharide (LPS) (0.5 μg/mL; Sigma) was added. Cells were incubated for 20 h at 37 °C in $CO_2$. To determine the nitrite release in the culture media, presumed to reflect the nitric oxide (NO) levels, Griess reaction was used, wherein 100 μL cell culture medium was mixed with 100 μL of Griess reagent (Invitrogen) and incubated at room temperature for 30 min. The NO concentration was determined at 540 nm using a spectrophotometer (Bio-Rad Laboratories Inc.).

### 4. Tartrate-resistant acid phosphatase staining

RAW264.7 cells were plated in 24-well plates ($1\times10^4$ cells/well) and cultured in DMEM treated with DFO (0 μM, 10 μM, 20 μM, 50 μM, 100 μM) for 2 h. Next, DMEM was replaced with Minimum Essential Medium alpha (Invitrogen), containing 10% FBS and 50 ng/mL of receptor activator of nuclear factor-κB ligand (RANKL) (PeproTech Inc., Rocky Hill, NJ, USA), and the cells were incubated for 3 days at 37 °C in a $CO_2$ incubator. Finally, osteoclast formation was confirmed using tartrate-resistant acid phosphatase (TRAP)/alkaline phosphatase staining kit (WAKO, Osaka, Japan).

### 5. Reactive oxygen species assessment

ROS levels were assessed by incubating cells with $H_2$DCFDA (20 μM; Abcam, Cambridge, MA, USA) for 30 min at 37 °C. After incubation with $H_2$DCFDA, cells were washed with PBS and assessed for fluorescence intensity by employing a BD LSR II flow cytometer (BD Biosciences San Jose, CA, USA). Data were analyzed using FCS express Flow Cytometry Software (De Novo, Glendale, CA, USA).

## 6. Gene expression analysis by quantitative real time polymerase chain reaction

The integrity and concentration of the total RNA, isolated using RNeasy Mini kit (Qiagen, Valencia, CA, USA), was evaluated using a spectrophotometer (NanoDrop ND-2000, Thermo Scientific, Waltham, MA, USA). Next, 500 ng RNA aliquots were reverse transcribed to cDNA using a Maxime RT premix kit [oligo $d(T)_{15}$ primer; Intron Biotechnology, Seongnam, Gyeonggi, Korea]. A quantitative real-time polymerase chain reaction (qPCR) assay was performed with SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) and a real time PCR system (ABI 7300, Applied Biosystems, Carlsbad, CA, USA), as per manufacturer's instructions. The qPCR conditions were 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, with a final 5-min extension at 72 °C. The annealing procedures for all primers were performed at 60 ºC. Expression of each gene was normalized to that of *beta-actin*, and the relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The oligo-nucleotide primers are listed in Table 1.

**Table 1. Primers used for quantitative real time polymerase chain reaction analysis**

| Gene | Forward Primer sequence (5'–3') | Reverse Primer sequence (5'–3') |
|---|---|---|
| *CTSK* | GGGATGTTGGCGATGCA | CCAGCTACTTGAGGTCCATCTTC |
| *CREB1* | TGTACCACCGGTATCCATGC | TGGATAACGCCATGGACCTG |
| *GAPDH* | CTGGCACAGGGTATACAGGGTTAG | ACTGGTGCCGTTTATGCCTTG |
| *HIF1a* | AGCTTGCTCATCAGTTGCCA | CCAGAAGTTTCCTCACACGC |
| *HO-1* | GAAATCATCCCTTGCACGCC | CCTGAGAGGTCACCCAGGTA |
| *IL 1β* | GTTCCCCAACTGGTACATCA | CCATACTTTAGGAAGACACGG |
| *IL 6* | GTTGCCTTCTTGGGACTGATG | ATCAGAATTGCCATTGCACAA |
| *NFATc1* | CACTGGCGCTGCAACAAGA | CATTCCGGAGCTCAGCAGAATAA |
| *Nrf-2* | TGAAGCTCAGCTCGCATTGA | TGCTCCAGCTCGACAATGTT |
| *PGC-1β* | CTCTGACACGCAGGGTGG | AGTCAAAGTCACTGGCGTCC |
| *RelA* | CTCCTGAACCAGGGTGTGTC | GAGAGACCATTGGGAAGCCC |
| *TRAF6* | CTACCCGCTTTGACATGGGT | CACCTCTCCCACTGCTTGTT |
| *TRAP* | CAAAGGTGCAGCCTTTGTGTC | TCACAGTCCGGATTGAGCTCA |
| *VEGF* | TCCGAAACCATGAACTTTCTGC | AGCTTCGCTGGTAGACATCC |
| *ACTB* | TCACCATGGATGATGATATCGC | GGAATCCTTCTGACCCATGC |

Abbreviations: *CTSK*, gene encoding cathepsin K; *CREB1*, gene encoding CAMP responsive element binding protein 1; *GAPDH*, gene encoding glyceraldehyde-3-phosphate dehydrogenase; *HIF1a,* hypoxia-inducible factor 1 alpha; *HO-1*, heme oxygenase-1; *IL 1β*, interleukine 1 beta; *IL 6*, interleukine 6; *NFATc1*, gene encoding nuclear factor of activated T-cells, cytoplasmic 1; *Nrf-2*, nuclear factor erythroid 2–related factor 2; *PGC-1β* , gene encoding PPARG coactivator 1 beta; *RelA*, gene encoding REL-

associated protein; *TRAF6*, TNF receptor associated factor 6; *TRAP*, gene encoding tartrate-resistant acid phosphatase; *VEGF*, vascular endothelial growth factor; and *ACTB*, glyceraldehyde-e-phosphate dehydrogenase.

### 7. Extraction and replantation of rat maxillary first molar

The maxillary first molar of 31 six-week-old male Sprague Dawley rats (200-250 g) (Orient Bio, Seoul, Korea) was used for tooth replantation, in accordance with the ethical guidelines and regulations approved by the Institutional Animal Care and Use Committee of Yonsei University (#2018-0214). DFO, diluted to 500 μM in saline, was used for the *in vivo* study. To facilitate the extraction, 0.4% β-aminoproprionitrile (Sigma, St. Lois, MO, USA) mixed in distilled water was administered to the rats along with the feed 3 days prior to extraction. The maxillary left right molar was atraumatically extracted under anesthesia with ketamine (0.1 mL/100 g, Yuhan, Dongjak, Seoul, Korea) and xylazine (0.05 mL/100 g, Bayer Korea, Dongjak, Seoul, Korea). After performing peritomy using explorer, the teeth were extracted with minimal trauma from the alveolar socket using tissue forceps. To determine the drug effect on PDL healing, the extracted teeth were randomly divided into two groups based on the treatment. In the control group (n = 10), the extracted teeth were stored in 50 mL of 0.9% physiological saline (pH 6.0) at 4 ℃ for 5 min and cotton soaked with 0.9% physiological saline was applied to the alveolar socket to induce thrombus removal and hemostasis. In the experimental group (n = 21), the extracted teeth were stored in 500 μM DFO solution (pH 7.22) at 4 ℃ for 5 min, and cotton soaked with 500 μM DFO solution was applied to the alveolar socket. Immediately after the root surface treatment,

the teeth were replanted in the original sockets. After replantation, all animals received a single intramuscular dose of 20,000 UI penicillin G benzathine (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan). After four weeks of replantation, the animals were euthanized under ketamine anesthesia. The maxilla was acquired and fixed in 10% formalin after washing with saline. For each animal, four different investigators were involved as follows: The investigator 1 and 2 took care of animals and was responsible for the anesthesia. The investigator 3, the only person aware of the treatment group, administered the treatment materials assigned by a random number table. The investigator 4 replanted the extracted teeth and gave antibiotics. Finally, three reviewers (investigator 1,2,4) assessed micro-CT images and histological analysis scores with blinding of which treatment is receiving.

### 8. Micro-computed tomography image analysis

The extracted maxilla was scanned at 10 μm intervals using a micro-computed tomography (micro-CT) system (Quantum FX micro-CT, Parkin Elmer, Norwalk, CT, USA), and the tooth images were reconstructed using a software (TRI-3D, Ratoc System Engineering Co., Ltd., Tokyo, Japan). Mesiobuccal (MB) root was chosen as it is the largest and allows clear observation of changes. A longitudinal image of the replanted tooth was obtained through the long axis of the MB root in the mesiodistal direction. The scoring standards are listed in Table 2. The extension of bone surface resorption area in contact with the root was measured in micrometers. Then, the ratio of the total surface of the bone in contact with the root was calculated.

**Table 2**. **Scores for quantitative analysis in micro-computed tomography image**

| Characterization | Score |
|---|---|
| A. Bone resorption | |
| No resorption present | 0 |
| Resorption occurred in <1/3 of the bone surface | 1 |
| Resorption occurred in >1/3 and <2/3 of the bone surface | 2 |
| Resorption occurred in >2/3 of the bone surface | 3 |
| B. Root resorption | |
| No resorption present | 0 |
| Resorption occurred in <1/3 of the bone surface | 1 |
| Resorption occurred in >1/3 and <2/3 of the bone surface | 2 |
| Resorption occurred in >2/3 of the bone surface | 3 |
| C. Ankylosis (direct fusion of bone to root) | |
| Absence | 0 |
| Presence | 1 |
| D. Canal calcification | |
| Absence | 0 |
| Presence | 1 |

## 9. Histological analysis

After the micro-CT scan, the maxilla was decalcified with 10% ethylenediaminetetraacetic acid (pH 7.4; Fisher Scientific Co., Houston, TX, USA) for three weeks at room temperature. The degree of decalcification was confirmed by radiographical analysis. After cryotomy, 20 - 25-μm thick section of maxillary first molar was cut from the sagittal plane to the apical axis, and tissues that were well connected with the apical and apical roots were selected, stained with hematoxylin and eosin, and observed using an optical microscope (Axio Lab, Zeiss,

Germany). The degree of inflammatory resorption of the roots, substitutional substitution, PDL status, and degree of inflammation were observed for each group using an optical microscope.

For quantitative analysis, similar to the micro-CT image analysis, only MB root was observed. In the longitudinal section, the characters listed in Table 3 were investigated. The criteria defined in the study for scoring in micro-CT image analysis and histological examination were in accordance with those described by Poi et al. (Poi et al., 2018).

**Table 3. Scores for quantitative analysis in histological examination.**

| Characterization | Score |
|---|---|
| A. Bone resorption | |
| No resorption present | 0 |
| Resorption occurred in <1/3 of the bone surface | 1 |
| Resorption occurred in >1/3 and <2/3 of the bone surface | 2 |
| Resorption occurred in >2/3 of the bone surface | 3 |
| B. Root resorption | |
| No resorption present | 0 |
| Resorption occurred in <1/3 of the bone surface | 1 |
| Resorption occurred in >1/3 and <2/3 of the bone surface | 2 |
| Resorption occurred in >2/3 of the bone surface | 3 |
| C. Inflammation at the epithelial insertion | |
| Absence or occasional presence of inflammatory cells | 0 |
| Inflammatory process restricted to the lamina propria of the internal part of the epithelium | 1 |

| | |
|---|---|
| Inflammatory process extending apically to the small portion of the connective tissue underlying the lamina propria of the internal portion of the gingival epithelium | 2 |
| Inflammatory process reaching the proximity of the alveolar bone crest | 3 |
| D. Inflammation in the PDL | |
| Absence or occasional presence of inflammatory cells | 0 |
| Inflammatory process presents only in the apical, coronal or small lateral area of the PDL | 1 |
| Inflammatory process reaching more than half of the lateral PDL of the root | 2 |
| Inflammatory process in the whole PDL | 3 |

## 10. Statistical Analyses

All *in vitro* experiments were performed, at least, in triplicate. All statistical analyses were performed with SPSS (version 25.0, SPSS Inc., Chicago, IL, USA). The normality of the *in vitro* and *in vivo* data was evaluated using the Shapiro–Wilk test ($p < 0.05$). Mann–Whitney U test ($p < 0.05$) was used to compare the data of control and experimental, LPS treatment, and experimental, RANKL treatment and experimental, and saline and DFO groups.

# III. Results

## 1. Cell viability and anti-inflammatory effect of deferoxamine in RAW264.7 cells

The cytotoxic properties, measured with CCK-8 shows, that the cell viability has no significant difference between control and dose-dependent DFO treatment groups (Figure 1A). NO release significantly decreased at 20, 50, 100μM of DFO when compared with that in the LPS treatment group ($p < 0.01$, Figure 1B). The expression of pro-inflammatory cytokines, interleukin 1β (*IL 1β*) and interleukin 6 (*IL 6*) were also significantly decreased in DFO- treated group compared with that in the LPS-treated group ($p < 0.05$) (Figure 1C, D).

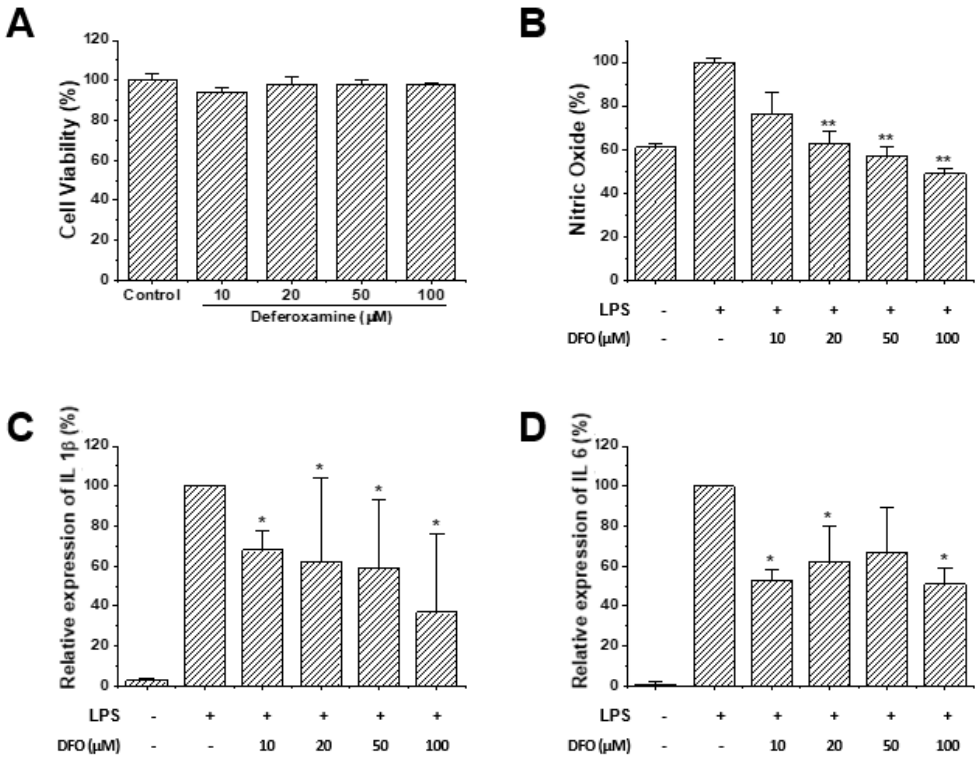


**Figure 1. Cell viability and anti-inflammatory effect of deferoxamine in RAW264.7 cells.** (A) Cell viability of DFO was assessed using CCK-8 assay kit in RAW264.7 cells. (B) NO release of DFO in RAW264.7 cells. Cells were incubated in the presence of different concentrations of DFO and 0.1 μg/mL LPS for 20 h. Then, the culture supernatant was analyzed for NO (C,D) *IL-1β* and *IL-6* expression on different concentration DFO-treated RAW 264.7 cells analyzed with qPCR (A-D) Cells were treated with different concentrations of DFO. The data are expressed as mean ± standard deviation. Data show

mean ± standard deviation values of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate significant differences compared to the control value (100%).

## 2. Deferoxamine affects osteoclast differentiation in RAW264.7 cells

To further investigate the role of DFO in osteogenesis, TRAP/ALP staining of RANKL-treated RAW264.7 cells was conducted, resulting in red-stained osteoclasts. Cells in the DFO treatment group showed lighter TRAP staining that those in the RANKL treatment group. Moreover, higher the DFO concentration, lower was the TRAP staining intensity (Figure 2). The expression of several genes related to osteoclast differentiation was confirmed by qPCR. TNF receptor associated factor 6 (*TRAF6*), nuclear factor of activated T-cell, cytoplasmic 1 (*NFATc1*), TRAP, and Cathepsin K (*CTSK*) expression was lower in the DFO treatment group and differed significantly compared to that in the RANKL treatment group (100% of RANKL treatment group, Figure 3B-E). These results suggest that DFO is involved in inhibiting the production of osteoclasts.

Since DFO attenuates osteoclast differentiation, we investigated several well-known factors associated with osteoclastogenesis to determine the signaling pathway. As a result, it was confirmed that treatment with DFO decreased the expression of ROS in RANKL-induced osteoclast differentiation (Figure 3A), and of *RelA*, known as p65, a subunit of NF-κB (Nolan et al., 1991) (Figure 3F). In addition, as significant regulators of ROS metabolism (Kong et al., 2010), the expression of *CREB* and *PGC-1β* was also decreased (Figure 3G, H). On the other hand, a key signal pathway for antioxidant and anti-inflammation, the expression of *Nrf-2* and the related *HO-1*, increased (Figure 3I, J).

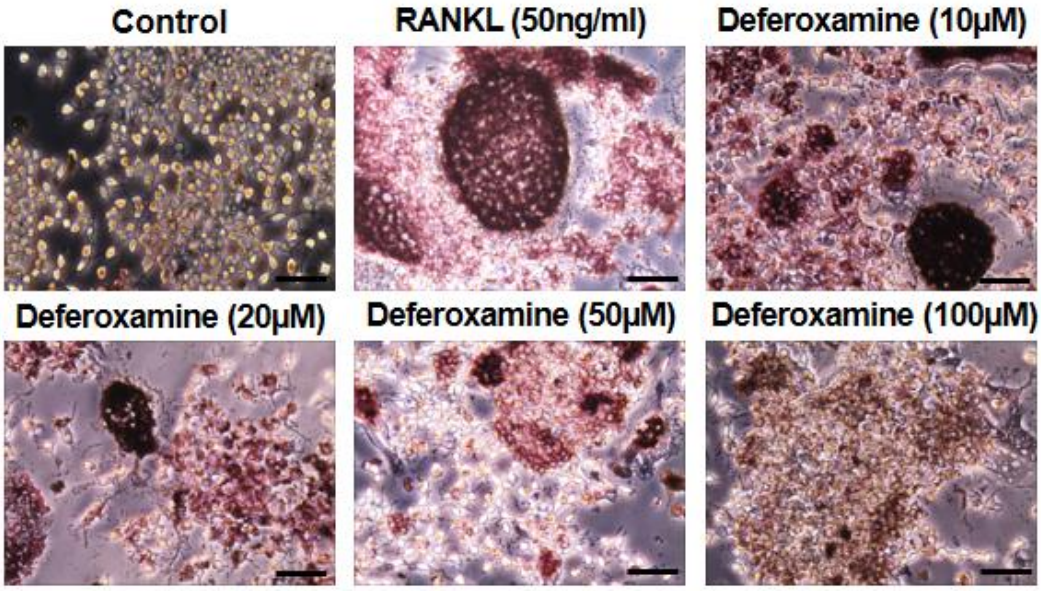


**Figure 2**. **Osteoclast differentiation of deferoxamine in RAW264.7 cells.** Tartrate-resistant acid phosphatase (TRAP) staining of DFO in RAW264.7 cells. Cells were applied with different concentrations of DFO for 2 h followed by treatment with 50 ng/mL receptor activator of nuclear factor-κB ligand (RANKL) for 3 days. Subsequently, RAW264.7 cells were fixed and stained to detect TRAP. The osteoclasts were stained red. Scale bars = 50 μm.

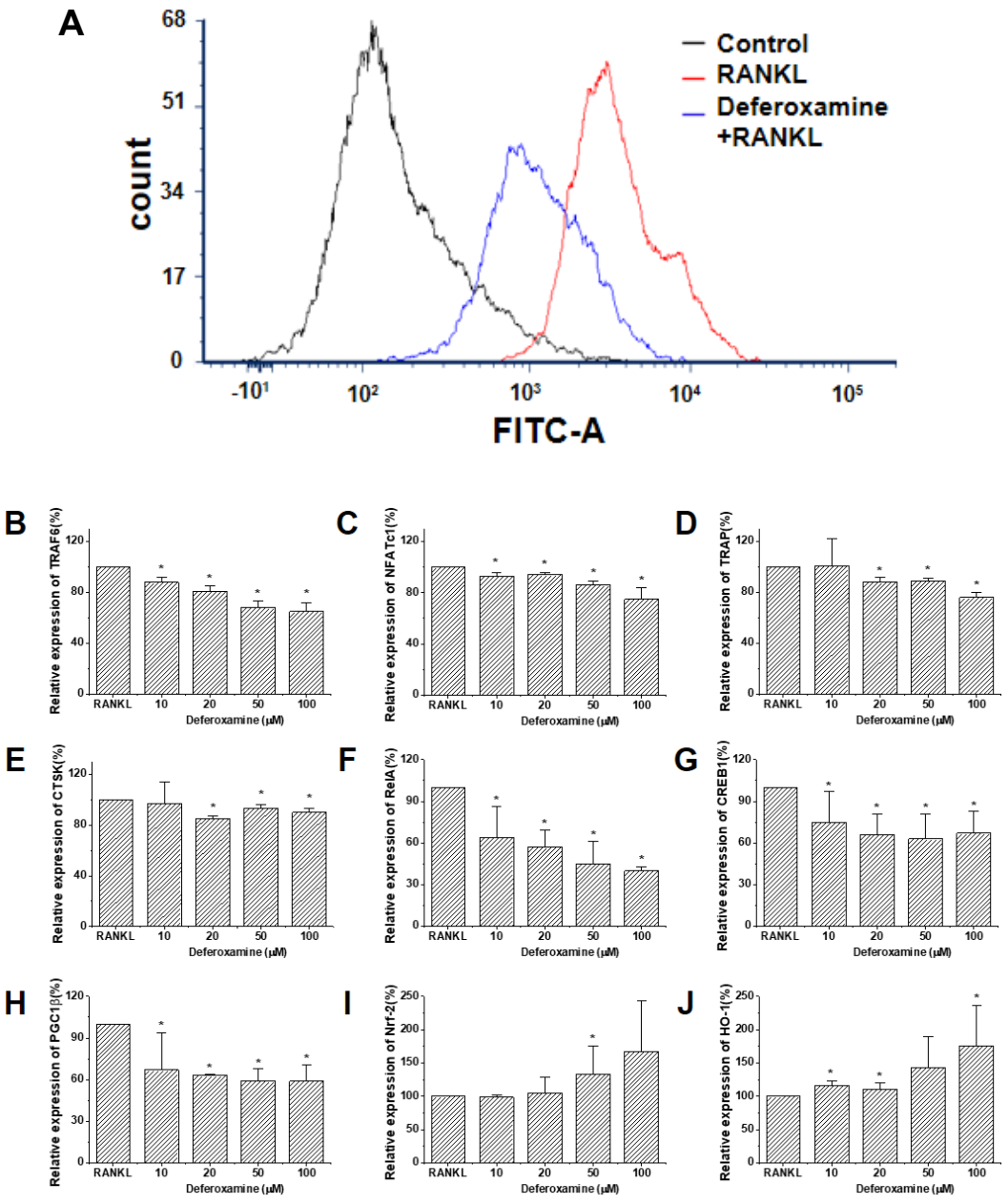


**Figure 3. Intracellular reactive oxygen species and related genes with osteoclast differentiation treated with deferoxamine in RAW264.7 cells.** (A) Intracellular ROS

levels determined using an $H_2$DCFDA ROS probe and analyzed with FCS express Flow Cytometry. (B-J) Changes in the expression of the osteoclast-related genes of DFO in RAW264.7 cells. Cells were pretreated with different concentrations of DFO for 2 h followed by treatment with 50 ng/mL RANKL for 3 days. RNA was isolated from RAW264.7 cells and cDNA was synthesized. Expression of tartrate-resistant acid phosphatase (*TRAP*), TNF receptor associated factor 6 (*TRAF6*), nuclear factor of activated T cell, cytoplasmic 1 (*NFATc1*) and CathepsinK (*CTSK*) were evaluated using quantitative RT-PCR relative to RANKL treatment group (normalized to 100%). Expression of v-rel avian reticuloendotheliosis viral oncogene homolog A (*RelA*), cyclic AMP-responsive element-binding protein 1 (*CREB1*), peroxisome proliferator-activated receptor gamma coactivator-1beta (*PGC-1β*), heme oxygenase-1 (*HO-1*) and nuclear factor erythroid 2–related factor 2 (*Nrf-2*) were evaluated using quantitative RT-PCR relative to RANKL treatment group (normalized to 100%). Data were obtained from five separate experiments, with all samples run in duplicate. The data are expressed as mean ± standard deviation values. The expression of the genes differed significantly; t-test and Mann–Whitney U test, * $p < 0.05$.

### 3. Deferoxamine as a factor promoting wound healing

The expression of several genes related to hypoxia was confirmed by qPCR. Hypoxia inducible factor 1 alpha (*HIF1α*), vascular endothelial cell growth factor (*VEGF*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were significantly increased in DFO-treated group compared with those in the RANKL-treated group (100% of RANKL treatment group, Figure 4).

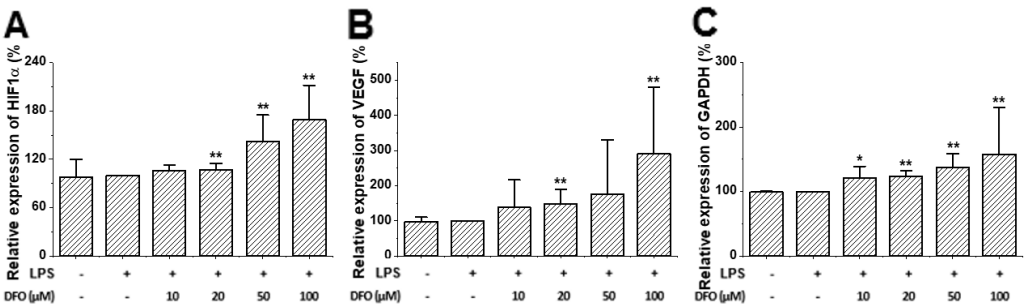


**Figure 4**. **Related genes with promoting wound healing with deferoxamine in RAW264.7 cells.** Changes in the expression of the hypoxia-related genes of DFO in RAW264.7 cells. Cells were pretreated with different concentrations of DFO for 2 h followed by treatment with 50 ng/mL RANKL for 3 days. RNA was isolated from RAW264.7 cells and cDNA were synthesized. Expression of gene encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxia-inducible factor 1 alpha (*HIF1a*), vascular endothelial growth factor *(VEGF)*.

### 4. Anti-inflammation and reduced bone loss in replanted rat first molar

In saline, the external root resorption happened heterogeneously and invaded the cementum and dentine irregularly. Extensive absorption was also observed in bones in contact with the replanted tooth (Figure 5B, E). Although inflammatory alveolar bone and root resorption were observed in the DFO group, the thickness and amount of absorbed bone and the amount of resorbed cementum and dentin of the root were small (Figure 5C, F). When the worst score was 3, wide range of inflammatory resorption in the alveolar bone (average score = 2.40) and extensive resorption of dentin and cementum of MB root (average score = 2.50) was observed in the saline group. However, in the DFO group, alveolar bone resorption showed a relatively smaller area, at an average score of 1.86. Root resorption was also reduced in most cases, with the average score of 1.95. However, difference in bone and root resorption between the groups was not statistically significant (Figure 5G, H). The difference in ankylosis was not statistically significant between the saline (average score = 0.30) and DFO (average score = 0.24) groups (Figure 5I). Furthermore, no statistical difference was observed in canal calcification between the saline (mean = 0.40) and DFO (average score = 0.33) groups (Figure 5J). Figure 6 shows the histological analysis of the interstitial space between replanted tooth and bone. The replanted root included fibrous tissue with inflammatory cells, such as lymphocytes, plasma cells, and neutral multinuclear cells. In addition, PDL-like structures were hardly observed in the saline group. An aggressive root replacement resorption was performed not only in dentine-cementum structure, but also in the inner dentine wall of the replanted root

(Figure 6B, E). In the DFO group, small area of depression was observed on the root surface, inflammation state was found in the apex 1/3 area, and the middle 1/3 showed relatively good adhesion of the fibers with less expanded PDL space (Figure 6C, F). The differences in the degree of root resorption and inflammation at the epithelial insertion were statistically different ($p < 0.05$) between the two groups (Figure 6H, J). There was no statistical difference in bone resorption and inflammation at the epithelial insertion between the saline (mean = 0.40) and DFO (average score = 0.30) groups (Figure 6G, I), which showed a reducing tendency.

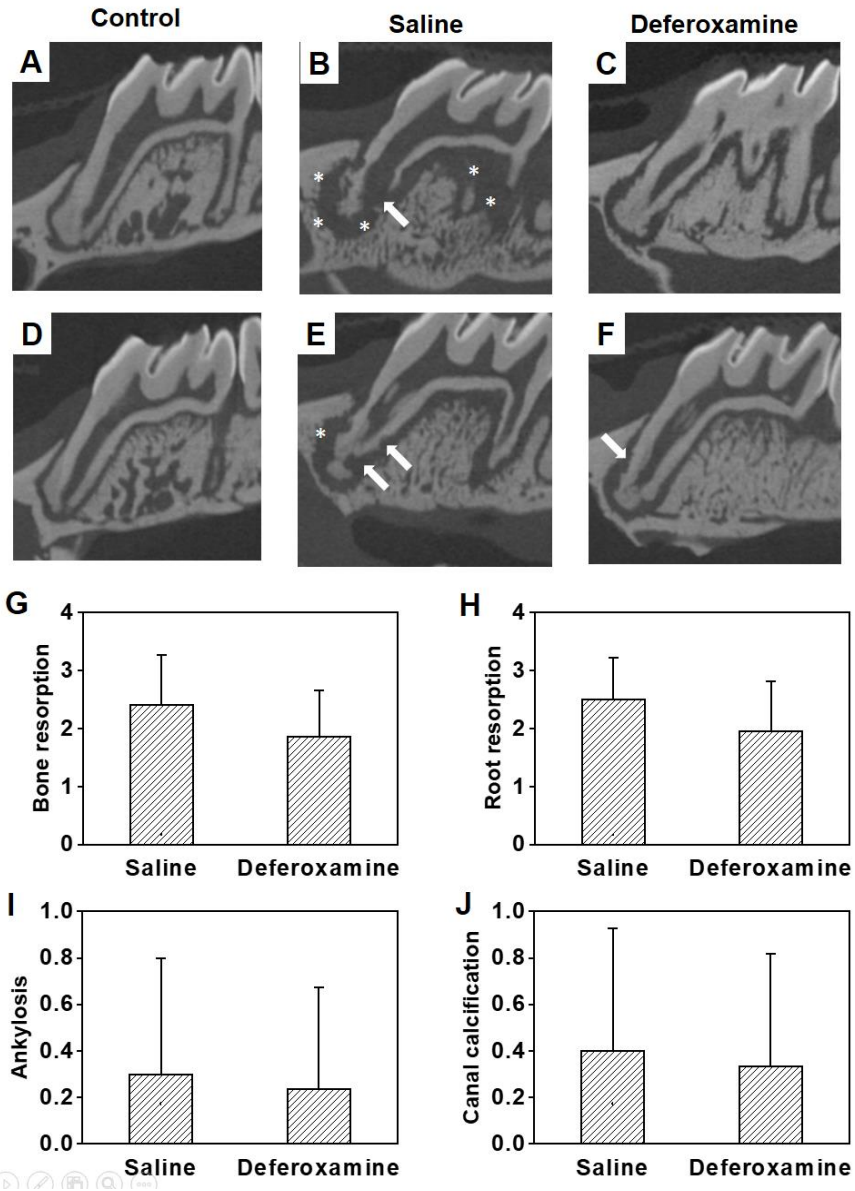


**Figure 5. Micro-computed tomography images of rat maxillary first molars 4 weeks after replantation.** (A, D) Control groups: The teeth without extraction and replantation.

Normal root surface and alveolar socket. (B, E) Saline groups: Before replantation, 0.9% physiological saline was applied to the extracted teeth surface and alveolar socket. Severe root resorption (arrows) and extensive alveolar bone resorption (asterisks) were observed. (C, F) DFO groups: 500 μM DFO solution was applied to the surface of the extracted teeth and alveolar socket. A relatively short range of bone resorption (asterisk) was observed compared to that in the saline groups. (G–J) Evaluated scores, represented as mean ± standard deviation, for (G) bone resorption, (H) root resorption, (I) ankylosis, and (J) canal calcification in the saline and DFO groups. However, there was no statistically significant difference between the saline and DFO groups.

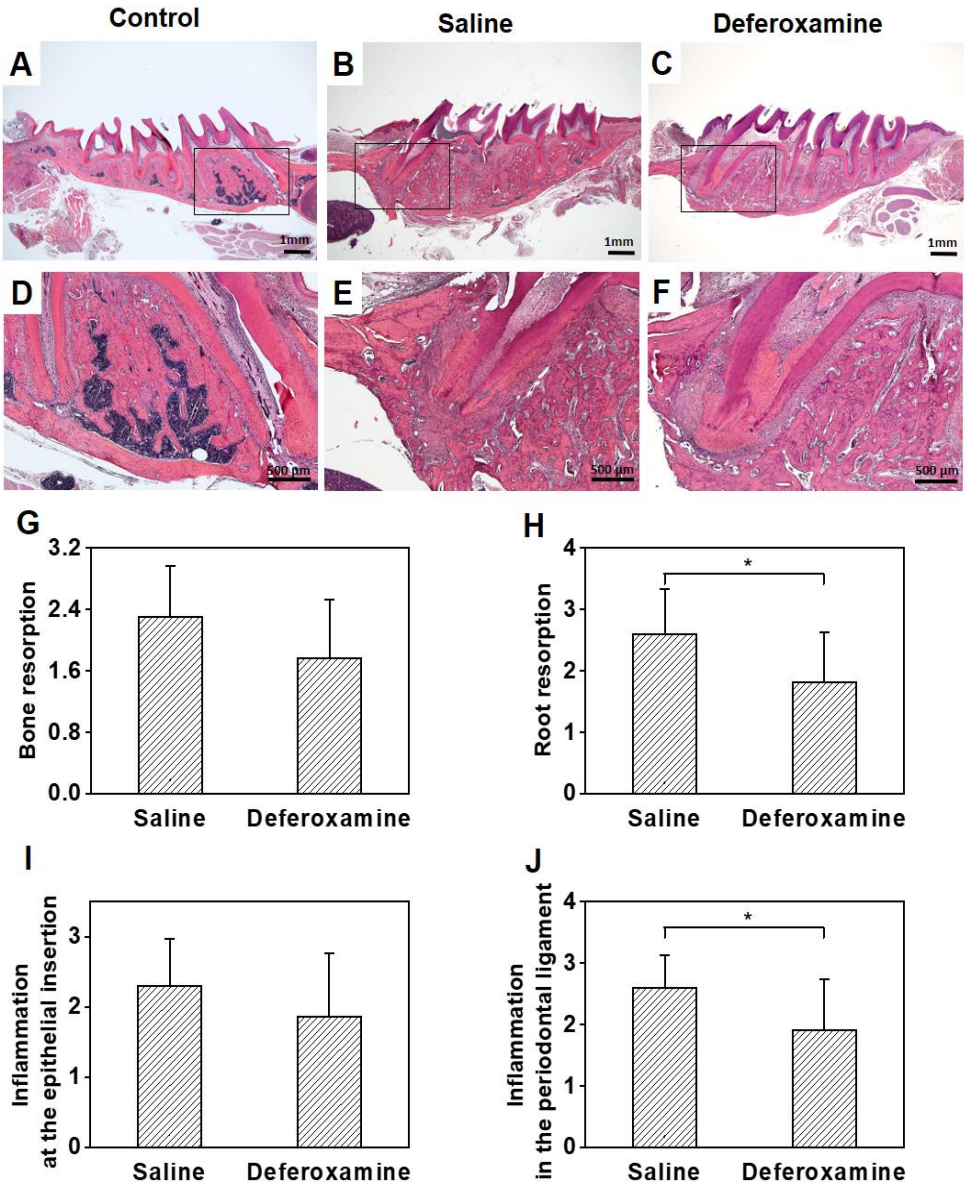


**Figure 6. Histological analysis of rat maxillary first molars 4 weeks after replantation.**

(A, D) Control groups: The teeth without extraction and replantation. Normal root surface

and alveolar socket. (B, E) Saline groups: Many cells related with inflammation were observed in the periodontal ligament space, severe inflammatory root resorption was found on all surface of root. (C, F) DFO groups: Localized root resorption in the apical third happened. (G–J) Evaluated scored, expressed as mean ± standard deviation, for (G) bone resorption, (H) root resorption, (I) inflammation at the epithelial insertion, and (J) inflammation in the periodontal ligament in the saline and DFO groups. Hematoxylin-eosin staining, Mann–Whitney U test, * $p < 0.05$ (applies to G–J). Scale bars: (A-C): 1mm, (D-F): 500μm

# IV. Discussion

Avulsed teeth followed by replantation are at risk for infection, inflammation, and root and bone resorption (Pohl et al., 2005). Although DFO is widely used in various medical conditions (Zhang et al., 2015), there have been no studies exploring the effects of DFO as a root surface treatment option in replanted avulsed teeth. In the present study, *in vitro* experiments such as expression of genes related to inflammation and osteoclastogenesis were significantly reduced. The stimulating Nrf2/HO-1 signaling pathway, which reduce the levels of nuclear factor-κB (NF-κB) and ROS, was suggested as one of the underlying mechanisms related to these findings (Figure 7). Topical application of DFO showed anti-inflammatory effects and prevented osteoclast differentiation in replanted rat molar teeth.

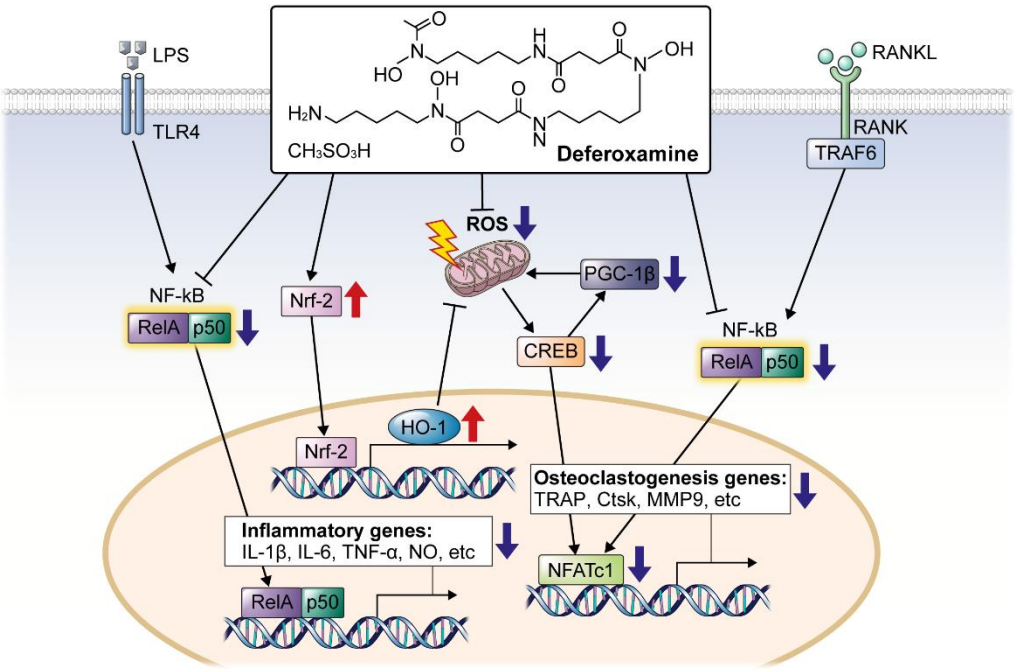


**Figure 7**. Scheme of a working model for inhibition of inflammation and osteoclast differentiation by deferoxamine in RAW264.7 cells.

DFO is an effective iron chelator, which binds to iron at a 1:1 ratio (Hershko et al., 2001). It is widely used in patients with iron overload, such as thalassemia, sickle cell anemia, and acute iron poisoning in small children. However, in recent years, DFO has gained attention due to its antioxidant and angiogenic properties. The antioxidant capability of DFO, an extension of iron-chelation characteristic, is involved in iron homeostasis and is closely related to the concentration of ROS, an important factor in cell signaling. The angiogenic property of DFO is closely connected to the upregulation of hypoxia-related genes, such

as HIF-1α (Holden and Nair, 2019).

We found that DFO application can reduce inflammation, consistent with previous studies (Zhang et al., 2010). Inflammation in the replanted tooth is related to rapid destruction of root surface and alveolar bone loss that can lead to loss of a tooth (Müller et al., 2020). Interleukin 1 (IL 1) stimulates bone resorption and interferes with bone production (Gowen et al., 1983). It stimulates the growth of osteoclast progenitor cells and promotes the differentiation and maturation of osteoclast progenitor cells (Pfeilschifter et al., 1989). The expression of IL 1 and IL 6 confirms that bone resorption can occur from inflammatory conditions (Gu et al., 2017). This reaction can rapidly degrade the cementum and dentin of the tooth (Levin et al., 2001).

*In vitro* studies revealed that DFO inhibited osteoclastogenic differentiation and showed that the activity of osteoclast-related factors (TRAF6, NFATc1, TRAP, and CTSK). The RANK/RANKL-dependent signaling pathway regulates osteoclastogenesis (Yamamoto et al., 2020). TRAF6 is a key protein that transmits the RANKL signals to the nucleus through pathways such as NF-κB and mitogen-activated protein kinase (MAPK). The expression of NFATc1 plays an important role in activation of osteoclast by RANK/RANKL-dependent signaling. NFATc1, in turn, increases the expression of TRAP and CTSK, which are necessary for releasing acid and producing degrading enzymes leading to bone resorption (Xu and Teitelbaum, 2013). DFO has an effect on inhibiting the expression of factors related to the differentiation of osteoclasts in bone (Zhang et al., 2019).

High intracellular labial iron pool can form ROS and damage capability (Hatcher et al., 2009). Moreover, ROS and free radicals are known to increase osteoclast differentiation and inflammation (Agidigbi and Kim, 2019). ROS can lead to oxidative stress and interrupt the cellular homeostasis, especially, under normal physiological conditions. ROS produced by osteoclasts, stimulates and facilitates resorption of bone tissue (Domazetovic et al., 2017). Our results confirmed that DFO can reduce ROS beyond the iron chelating function, and exert antioxidant effects (Holden and Nair, 2019). As ROS decreases, osteoclastogenesis through the NF-κB and MAPK pathways also decrease.

In addition, the activity of NFATc1 can be reduced due to biogenesis related genes. *PGC-1β* is member of a family of transcription coactivators which has a central role in the regulation of cellular energy metabolism (Liang and Ward, 2006). It simulates mitochondrial biogenesis and promotes the remodeling. In addition, CREB binds to cAMP response elements and DNA, and regulates the transcription of the genes (Wang et al., 2018). This pathway activates the osteoclastogenesis; however, if the ROS levels decrease after DFO treatment, its function is also degraded.

DFO not only reduces ROS, but is known to induce the activity of Nrf2, which was also confirmed in our study. Nrf2 is an essential transcription factor that regulates antioxidant defense gene expression, such as HO-1. Recently, antioxidant, anti-inflammatory, and immunomodulatory effects of HO-1 have been found in vascular cells, (Araujo et al., 2012), which has an enormous effect on osteoclastogenesis (Zwerina et al., 2005). Nrf2/HO-1

pathway reduces intracellular ROS and inhibits NF-κB signaling (Yang et al., 2013), which was also confirmed through our experiments.

VEGF, an inducer for vascular permeability and promoter of the growth of new blood vessels, is the key signal molecule (Bates and Jones, 2003). VEGF is activated by a hypoxia-related gene. DFO stabilizes HIF-1α even under normoxic conditions, and can facilitate wound healing (Holden and Nair, 2019). DFO improves vascular network and bone fracture healing (Donneys et al., 2013). A study on periodontal ligament on human teeth indicated that VEGF increases 1.8 fold in DFO treated group (Ratajczak et al., 2016). Taken together, DFO is an important candidate that can facilitate wound healing, positive bone remodeling, and healing of ischemic injuries.

Results from the *in vivo* study confirmed that DFO has an effect on reducing inflammation and osteoclastogenesis in 28 days compared with those from saline group. DFO-treated group showed reduced pulp damage, like obliteration and less ankylosis or root resorption with damaged PDL. PDL healing is an important factor for favorable result of replantation. Müller et al. investigated the complication of avulsed teeth and concluded that 33% teeth were extracted during the mean observation period of 3.5 years, especially because of inflammatory resorption. And reported that the unpredictable poor result could be reduced by regenerating PDL of the avulsed tooth (Müller et al., 2020).

To the best of our knowledge, this is the first *in vivo* study investigating DFO as a candidate for the root surface treatment in tooth replantation. Further studies conducted

among animals and, especially human, are needed to confirm the effectiveness of DFO in a tooth replantation and draw conclusive clinical implications.

Moreover, DFO is a widely used iron chelator of which the safety is confirmed by the Food and Drug Administration and has strengths as a treatment options for replantation of tooth. However, treatment dosage of DFO with optimal efficacy and sufficient safety margin is yet to be assessed for tooth replantation, which warrants further investigations.

There are some limitations of this study. First, bone remodeling, including bone resorption, is a result of the crosstalk between osteoblast and osteoclast, but we only focused on osteoclast. In addition, although representative NF-κB was examined in terms of the osteoclastogenesis mechanism, pathway through MAPK or Akt was not investigated despite of its importance in osteoclast differentiation. Secondly, as examined in the results, ankylosis and canal obliteration are one of the long-term side effects of tooth trauma and can be more accurately identified when performing a long-term follow.

# V. Conclusion

In conclusion, this study showed that DFO prevents inflammation and bone loss and may be potentially applied to the root surface and alveolar bone before replantation. This is the first *in vivo* study in which DFO was applied to teeth. Considering the wide underlying mechanism and therapeutic effect of DFO, more diverse and broader approaches for further research are needed.

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국문요약

# 데페록사민이 염증과 파골 세포 형성에 미치는 영향: *in vitro* 및 *in vivo* 모델

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탈구된 치아의 재식이 지연되는 경우 대치 및 염증성 흡수와 같은 심각한 합병증이 발병한다. 본 연구의 목적은 데페록사민이 *in vitro*에서 염증 및 파골세포의 형성을 억제하고, 쥐의 치아의 재식실험에서 염증과 골 흡수에 미치는 영향을 평가하기 위함이다. 본 연구는 *in vitro* 실험에서 세포 생존율과 지질다당류에 의한 염증 여부를 평가하였다. RANKL이 유도하는 파골 세포 형성은 tartrate-resistant acid phosphatase 염색법, 활성산소종의 농도 측정 및 실시간 중합효소 연쇄반응분석을 통해 분석하였다. *In vivo* 실험은 Sprague Dawley 수컷 31마리의 대구치를 발치하여 식염수 또는 데페록사민에 각각 5분 담근 후 재식하였다. 마이크로 컴퓨터 단층 촬영 영상과 조직학적 분석을 시행하여 염증과 치근 및 골흡수를 평가하였다. 데페록사민을 처리한 결과 *in*

*vitro*에서는 염증과 골분화가 현저하게 감소하는 것을 확인하였다. 또한 활성산소종의 발현 및 CAMP responsive element binding protein 1 / gene encoding PPARG coactivator 1 beta 신호 과정을 억제하였으며, nuclear factor erythroid 2−related factor 와 heme oxygenase 1 신호전달체계를 활성화시키는 것으로 나타났다. 그밖에 저산소증 관련 유전자의 발현이 증가하였다. *In vivo* 실험에 대한 방사선 및 조직학적 분석 결과 염증 및 경조직의 흡수가 감소하는 경향을 보였다. 이러한 결과는 데페록사민이 탈구된 치아 재식모델에서 염증과 파골 세포 형성을 감소시킴을 시사하며, 향후 탈구된 치아의 치근 표면 처리제로써 좋은 후보군이 될 수 있음을 나타낸다.