Role of RvD1 on Osteogenic Factors Involved in the Inflammatory and Resolution Processes in Periodontal Periapical Lesions

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ABSTRACT

Introduction: Inflammation is a protective body response against invading traumatic or microbial injury. However, persistent or unresolved acute inflammation can result in a chronic injury to local tissues that may lead to long term complications. Periodontal diseases (e.g. marginal and apical periodontitis) are examples of chronic inflammation affecting the periodontal tissues. Persistence of these conditions may lead to permanent tooth loss. Recent evidences suggest that Resolvins derived from ω-3 PUFAs play an important role in resolution of inflammation. Knowledge about the effects of Resolvin D1 on periodontitis is limited. Yet, it is postulated that RvD1 therapy demonstrates a great efficacy in reducing the inflammatory process without the side effects of chronic antibiotic usage.

Aims: to investigate the effect of different doses of RvD1 in the regulation of tissue destruction and resolution of inflammation in periodontal lesion models and to evaluate its potential in therapy of these conditions.

Methods: An in vitro study was performed in which periodontal ligament fibroblasts from three different donors were used. Cells were cultured in DMEM and further treated with different doses of RvD1 (1ng/ml, 10ng/ml and 100ng/ml) in the presence and absence of TNF-α (1ng/ml). Cell proliferation was observed using MTT cell proliferation assay in three different time points (1, 3 and 7 days). Cells were also cultured in osteoinductive medium (OM) and then treated with different doses of RvD1 (10ng/ml and 100ng/ml) in the presence and absence of TNF-α (1ng/ml). Expression of bone marker genes (ALP, Col-1 and OC) was assessed using RT-PCR. An in vivo pilot study then followed, in which nine mice were used. Pulp exposure (in the mandibular first molar) was performed for six mice and then they were divided into two groups (three mice each). One group received RvD1 injection and the other group received normal

saline injection. A third control group was also included (neither pulp exposure nor treatment). At the end of the experiment jaws were dissected and both TRAP staining and micro-CT were performed to assess the osteoclastic activity and the bone volume respectively.

Results: RvD1 induced the proliferation of PDL fibroblasts after three and seven days. Expression of ALP and Col-1 genes was increased when cells were treated with RvD1 (10ng/ml). RvD1 treated mice had lesser number of osteoclasts although no difference in the bone volume measurements observed between the groups.

Conclusion: Treatment of PDL fibroblasts with RvD1 leads to cellular proliferation which can improve the healing process. It can also lead to reduction of inflammation and stimulate bone formation. Further investigations on the effects of RvD1 on inflammation and wound healing are needed.

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ACRONYMS AND ABBREVIATIONS

µCT	**Micro-Computed Tomography**
ω3-PUFA	**Omega 3- Polyunsaturated Fatty Acid**
AA	**Arachidonic Acid**
ALP	**Alkaline Phosphatase**
ANOVA	**Analysis Of Variance**
AT-RvD	**Aspirin Triggered Resolvin**
BMPs	**Bone Morphogenic Proteins**
BSP	**Bone Sialoprotein**
Col-1	**Collagen-1**
COX	**Cyclooxygenase enzyme**
DHA	**Docosahexaenoic Acid**
EPA	**Eicosapentaenoic Acid**
FBS	**Fetal Bovine Serum**
FCS	**Fetal Calf Serum**
IFN-α	**Interferon Alpha**
IL	**Interleukin**
JE	**Junctional Epithelium**
LOX	**Lipooxygenase**
LPS	**Lipopolysaccheride**
LTs	**Leukotriens**
LXA4	**Lipoxin A4**
M-CSF	**Macrophage Colony Stimulating Factor**
MTT	**Methylthiazol Tetrazolium**

NF-κB	**Nuclear Factor Kappa B**
OC	**Osteocalcin**
OD	**Optical Density**
OM	**Osteoinductive Medium**
OPG	**Osteoprotegerin**
PBS	**Phosphate Buffered Saline**
PDL	**Periodontal Ligament**
PFA	**Paraformaldehyde**
PGE	**Prostaglandin E**
PMN	**Polymorphonuclear leukocytes**
RANKL	**Receptor Activator of Nuclear Factor Kappa B Ligand**
ROI	**Region of interest**
RT-PCR	**Real Time Polymerase Chain Reaction**
RvD	**Resolvin D series**
RvE	**Resolvin E series**
TNF-α	**Tumor Necrosis Factor Alpha**
TRAP	**Tartrate resistant Acid Phosphatase**
TV	**Tissue Volume**

INTRODUCTION

Acute inflammation (also known as innate immune response) is the first body response to injury. It is a protective body response against invading traumatic or microbial injury. The cardinal signs of inflammation include: hotness (*calor*), redness (*rubor*), swelling (*tumor*) and pain (dolor). These characteristics were described by Celsus more than 2000 years ago. In 1858 Virchow added loss of function (*functio laesa*) to the list [1, 2]. Immediately after microbial invasion, chemoattractants (e.g. endogenous chemical mediators) are released. Sources for these chemoattractants are various including; innate immune cells, platelets and microorganisms [3]. They are also released during complement system activation [2, 3]. These chemoattractants attract neutrophils to the injury site [4, 5]. Neutrophils immediately start the microbial killing process as well as wound decontamination and also attract macrophages and mast cells (granulocytes) to the injury site. If the inflammatory events last longer, T-lymphocytes appear at the injury site. They might aid in wound resolution and remodeling [3]. Macrophages engulf dead neutrophils as well as microbial debris before leaving the injury site [4-7]. They also produce growth factors important for fibrogenesis, epithelialization and angiogenesis. Outcomes of acute inflammation are either development into chronic inflammation or complete resolution of the events, which is the ultimate goal to be reached (fig. 1) [3].

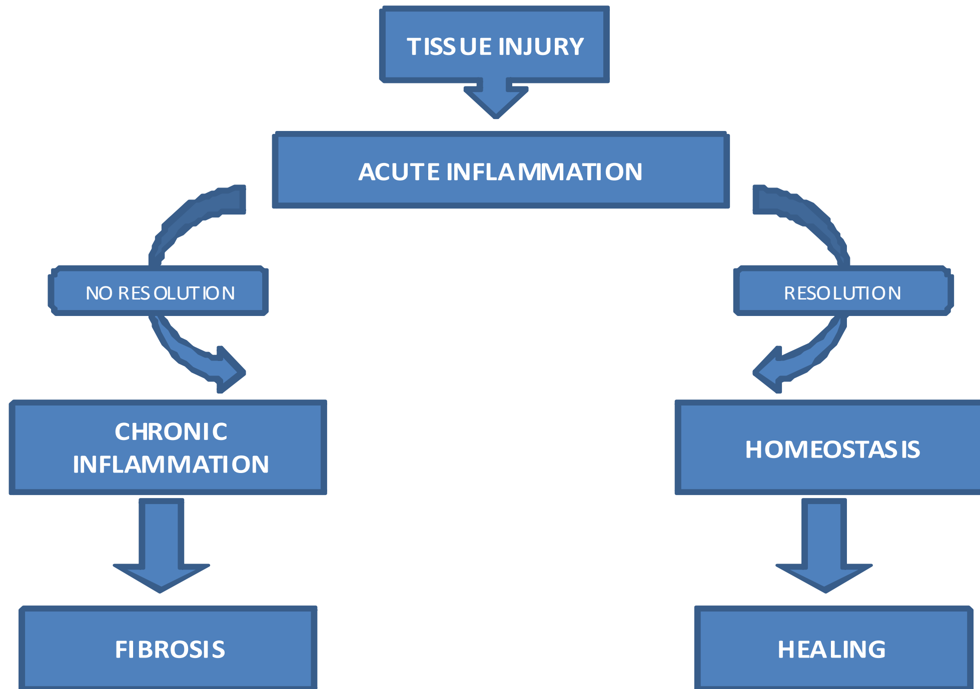


Fig. 1 Outcomes of inflammation. (Adapted from Serhan et al. 2005)

If neutrophils were completely eliminated and the number of macrophages and granulocytes returned to the normal pre-inflammatory state, it can be stated that the inflammation has subsided [7]. Inflammatory events, however, may not subside and necrosis of PMN occurs. This leads to cell membrane rupture and release of the intracellular contents into the outer environment [8] leading to various degrees of tissue destruction. This destruction can result from the release of certain components into the injury site [3]. These components might be released by bacteria (e.g.: Lipopolysaccharides "LPS": a component of the wall of gram negative bacteria) and help on the osteoclastic activity at the injury site[9]. Prostaglandin E2 (PGE2) and leukotrienes (LTs) are arachidonic acid products that can also aid in the tissue destruction process. Pro-inflammatory cytokines (e.g.: Interlukin-1 "IL-1" and tumor necrosis factor "TNF-α") also play a significant role in the inflammatory bone resorption and tissue destruction [9, 10]. This leads to tissue damage and a persistent condition develops instead (i.e. chronic inflammation) [7, 8, 11]

which contributes to a wide range of acute and chronic diseases (e.g.: Chronic periodontitis, aggressive periodontitis and cardiovascular diseases) with difficult therapeutic challenges [7, 11]. Chronic periodontitis, for example, is characterized by apical migration of the junctional epithelium (JE) and pocket formation, gingival ulcerations and alveolar bone resorption. These features combined together can result in pathological tooth mobility and eventually loss of the tooth [12]. Elimination of such conditions is challenging because the available treatments tend to block the active inflammatory process or to promote the healing process [11]. Another inflammatory condition that affects the oral cavity and may lead to tissue destruction is apical periodontitis.

Apical periodontitis:

Apical periodontitis (also called periapical periodontitis or periradicular periodontitis) is an infectious disease that is characterized by the destruction of the tooth supporting structures [13]. It occurs around the root apex as a defensive response of the body against the invading infection (when the body fails to defend against invading microbes). Chronic infection and pulp necrosis result in the colonization of the pulp space and root canals by micro-organisms [14-16]. They produce a wide range of irritating substances (e.g.: enzymes, acids and toxins). These substances may subsequently leak from the root canal into the periapical tissues leading to apical irritation. The body acts on walling off these invaders and cleaning the site by production of different inflammatory molecules. Beside the clearance of the invading pathogen, these molecules might lead to destruction of the surrounding tissues including alveolar bone [15, 17]. The inflammatory cells in the region try to confine the lesion into a specific area leading to granulation tissue

formation (apical granuloma). This tissue is made up by leukocytes (mononuclear and polymorphonuclear), together with fibroblasts and vascular tissue [18]. Once the microorganisms are trapped periapically, their elimination becomes difficult and needs intervention [15]. Persistent condition will eventually lead to localized apical abscess and suppuration that can develop into a chronic condition if not treated. This can further develop into a focal osteomyelitis [16]. Suppuration may spread through the cortical bone of the jaws into the superficial soft tissues forming a localized abscess. It can also break through the overlying oral mucosa and/or skin leading to sinus formation and further cellulitis [15, 16].

Periodontal ligament cells, characteristics and functions

Periodontal ligament is a soft (fibrous) connective tissue found between the tooth cementum and the inner wall of the alveolar bone socket. It provides protection and support for the tooth structure. It also aids in alveolar bone remodeling, physiological mobility during mastication and also in tooth eruption. It consists of various cell populations at each stage of its differentiation, including fibroblasts (predominant), endothelial cells, epithelial rests of Malassez, bone associated cells, sensory system associated cells and cementoblasts [19, 20]. These cells have an important role in the maintenance of periodontium as well as in the regeneration of destructed tissues following inflammation [21-23]. Periodontal ligament (PDL) fibroblasts contain stem cells that have a high proliferation, self-renewal and multi-lineage differentiation capacity. When isolated, they showed the capability to differentiate into adipocytes, collagen- forming cells and cementoblast-like cells that play an important role in the regeneration of the destructed periodontium [21, 24, 25]. Previous studies have showed their osteogenic potential when

stimulated with osteo-inductive medium [26, 27]. They also show a capability of mineralized tissue production by expression of alkaline phosphatase (ALP) during the mineralization process of both alveolar bone and cementum [20, 27]. Recent studies have demonstrated that PDL fibroblasts in close contact with bacteria produce cytokines and chemokines and amplify inflammation, suggesting the fibroblasts as a target for pharmacological intervention in periodontitis [18]. Multipotent stem cells, isolated from the periodontal ligament, have shown stem-like properties similar to bone marrow stem cells. They were later named periodontal ligament stem cells. They showed self-renewal properties and the ability to differentiate into different cell lineages, including adipocytes, chondrocytes, osteoblasts and neurocytes [28, 29]. This demonstrates their potential use in periodontal tissue regeneration.

Healing and resolution

The most critical, yet simple step in the elimination of the inflammatory response is the elimination of the injurious agent that initiated the process [1]. Resolution process serves as an agonist to the inflammation phase, as the pro-resolution molecules act to reduce the infiltration of neutrophils to the injury site. They also promote the clearance of apoptotic cells and microbes by macrophages. In addition, they stimulate the antimicrobial activities of the epithelial cells [1, 4]. If the inflammation resolved there will be minimal tissue destruction. However, in unresolved inflammatory conditions where elimination of the insulting molecules is not obtained, inflammation might continue. This will increase the production of the arachidonic acid derivative lipoxin A4 LXA4 (in response to pro-inflammatory mediators) that in turn function to minimize tissue damage and resolve the inflammation [30]. Anti-inflammatory cytokines (e.g.: interleukin-4 “IL-4”, interferon-alpha “IFN-α” and granulocyte-colony stimulating factor “G-CSF”) also get

released at the injury site. They try to counteract and regulate the effects of the pro-inflammatory cytokines and to drive tissues to the normal state [31]. The balance between the pro- and anti-inflammatory molecules determines the outcome of the inflammatory and healing processes. When the inflammatory process is resolved, tissue destruction is mild; consequently, regeneration occurs (replacement of the same type of dead cells). Persistent inflammation, however, leads to more tissue destruction as it is the case in marginal and apical periodontitis.

Bone remodeling

To keep a constant and normal mass of bone, there has to be a well maintained balance between bone formation and bone resorption. This active process is referred to as bone remodeling and it mainly depends on two types of cells, osteoblasts (bone formation) and osteoclasts (bone resorption). These cells have to work together in harmonious number, time and space to give the desired balance [32]. Bone remodeling process (Figure 2) occurs in several steps, starting by the activation phase, where some inflammatory cytokines (e.g.: interleukin-1, interleukin-6 and TNF-α) activate the bone lining cells. This activation in turn leads to expression of receptor activator of nuclear κB ligand (RANKL) in their surface. Expressed RANKL will interact with its receptor (RANK) expressed by pre-osteoclasts. This interaction leads to the differentiation of the pre-osteoclasts into multinucleated osteoclasts [32-36]. Then the resorption phase starts by migration of osteoclasts and their adhesion into bone surface. Osteoclasts then start dissolving the organic matrix of bone followed by its degradation. This releases several growth factors stored in the bone matrix. Immediately after that, osteoclasts undergo programmed cell death (apoptosis) [32]. Macrophage like cells reach the resorption site and remove all the debris

formed during the organic matrix degradation. This is called the reverse phase. Finally, growth factors released during the resorption phase (e.g.: fibroblast growth factor: FGF, and bone morphogenic proteins: BMPs) lead to recruitment of osteoblasts into the resorption site. These osteoblasts are differentiated through different stages and produce osteoid that later get mineralized into mature bone [32, 37].This is the final formation phase in bone remodeling as summarized in Fig. 2.

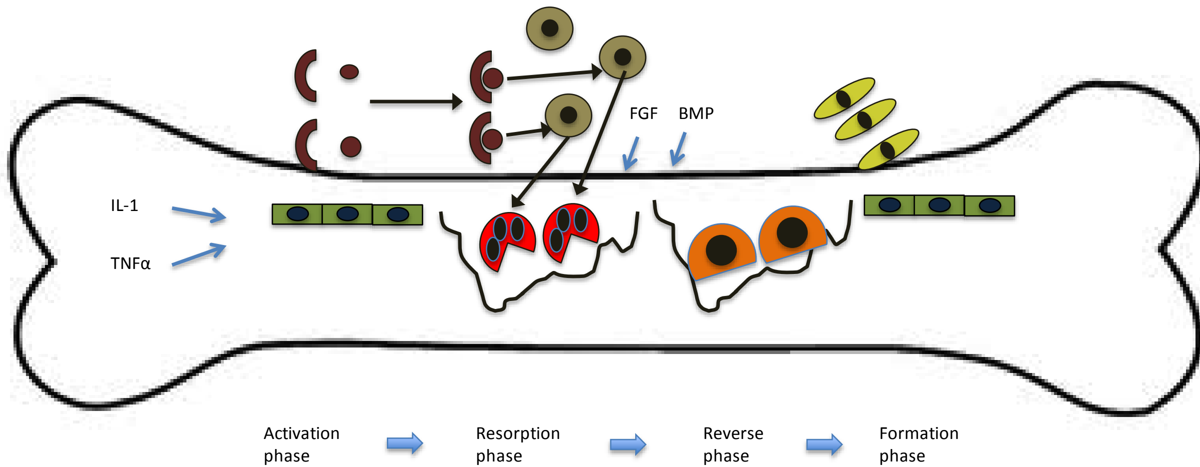
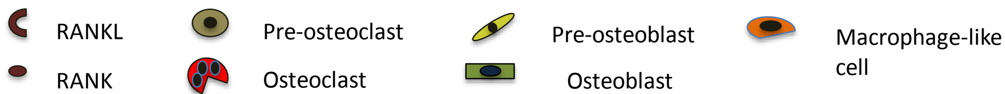


Fig. 2 Bone remodeling process.



IL-1: Interlukin-1, **TNFα**: Tumor Necrosis Factor, **FGF**: Fibroblast Growth Factor, **BMP**: Bone morphogenic Protein.

Collagen type 1 (Col-1) and alkaline phosphatase (ALP) are the markers found during the early stage of osteoblast differentiation. Late stage of osteoblast differentiation is marked by both

Osteocalcin (OC) expression and mineralization of extracellular matrix [32, 37]. Recent studies documented beneficial effects of Omega-3 polyunsaturated fatty acids and their derivatives in bone healing and new bone formation [11, 38].

Resolvin

Resolvins (resolution phase interaction products) are a new group of bioactive lipid mediators produced naturally in the human body from ω3-polyunsaturated fatty acids (ω3-PUFA). They have a significant role in the resolution of inflammation [7, 39-43]. They are produced by oxygenation of ω3-PUFA derivatives, docohexapentaenoic acid (DHA) and eicosapentaenoic acid (EPA). DHA is the precursor for D-series resolvins (RvD1, RvD2, RvD3 and RvD4) [7, 44, 45] while EPA is the precursor for E-series resolvins (RvE1, RvE2 and RvE3) [44, 46].

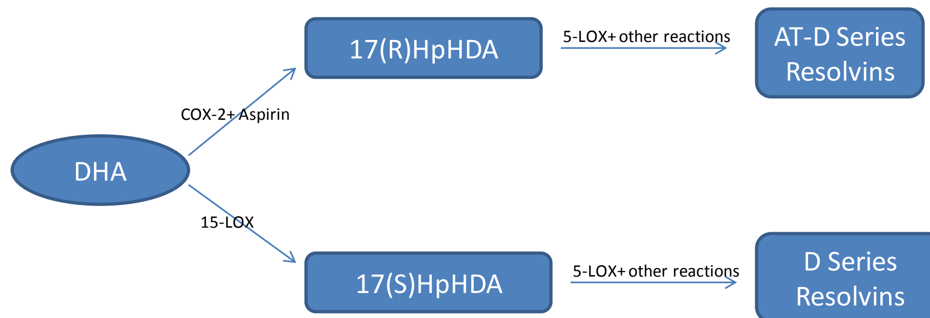


Fig. 3 Synthesis of RvD from DHA.
DHA: docosahexaenoic acid, COX: cyclooxygenase, LOX: lipooxygenase, HpHDA: hydroperoxydocosahexaenoic acid, AT: aspirin triggered. (Adapted from Weylandt 2012)

RvD1 is a DHA derivative (figure: 3) that is biosynthesized during the resolution phase of inflammation [43, 47]. 15-lipoxygenase enzyme (15-LOX) binds to the DHA substrate in the PUFA and forms 17S-hydroperoxy-DHA. This compound is an intermediate compound that can be converted into other bioactive compounds (e.g.: RvD1) [48]. It has potent anti-inflammatory and pro-resolving properties. DHA can also be converted into 17R-hydroxy-DHA by sequential oxygenation initiated by cyclooxygenase enzyme (COX-2) forming an aspirin triggered form or epimer (17R-RvD1). This epimer has the similar activity (both in-vitro and in-vivo) and uses the same receptors as RvD1. It was also found to be resistant to rapid inactivation by eicosanoid oxidoreductases and potentially has a longer duration of action in vivo [43, 47-49].

Role of Resolvins and their precursors in inflammation and resolution

Actions of Resolvins and their precursors have been under investigations and research for many years. They appeared to cause a clear and dramatic anti-inflammatory effect on many diseases. Arita et al. in 2005 [50] tested the anti-inflammatory effects of RvE1 in mouse peritonitis model. Their results revealed that systemic application of RvE1 counter-regulated both leukocyte-mediated tissue injury and pro-inflammatory gene expression. There was improvement in the survival rates in the animals used. Body weight was sustained and the leukocytic infiltration was decreased. In 2011, Bento et al. [51] studied the anti-inflammatory effects of Aspirin-Triggered RvD1 (AT-RvD1), its precursor (17(R)-HDHA) and RvD2 in induced colitis in mice. They concluded that their systemic application caused improvement in disease activity. Reduction in colonic damage and PMN infiltration were also observed. In 2008, Kasuga et al. [52] examined the effects of RvD1 and its precursor in peritonitis model. They spotted an organ protective role of Rvd1 in vivo by prevention of second organ injury. Tian and co-workers in 2009 [41] investigated the biosynthesis and anti-inflammatory actions of RvE1 and RvD1 in a co-culture of coroid retinal endothelial cells (CREC) and leukocytes. They observed an increase in the biosynthesis of both RvE1 and RvD1 under inflammatory conditions. They also observed a reduction in the inflammatory mediators release in the presence of Resolvin that led to reduced inflammatory condition. Liao et al. [53] in 2012 studied the effects of RvD1 on lipopolysaccheride induced lung injury in mice. Their experiments showed that RvD1 has markedly decreased the inflammation by suppression of NF-κB activation. In 2010, Yuan J. and co-workers studied the effects of PUFAs and their metabolites on osteoclastogenesis process in vitro [54]. They obtained some interesting results; one is that DHA has strongly inhibited the process, while DGLA (Dihomo-γ-Linolenic Acid), AA (Arachidonic Acid) and EPA

(Eicosapentaenoic Acid) has enhanced it. They also found that COX products of PUFAs, PGE1, PGE2 and PGE3, were increased during the process. Moreover they concluded that RvD1 had significantly inhibited the process. Resolvins also demonstrated beneficial impact in Alzheimer's disease, cardiovascular diseases, diabetes, eye diseases, lung diseases, pain, rheumatic diseases as well as effects on wound healing [41, 53, 55-62].

Role of Resolvins and their precursors in periodontal diseases

Recent evidences suggest that Resolvins derived from n-3 PUFAs play an important role in resolution of inflammation [7, 11, 63]. Studies on the effects of Resolvins on resolution of periodontal diseases are few. Yet, it is postulated that resolvin therapy demonstrates a great efficacy in reducing the inflammatory process without the side effects of chronic antibiotic usage [11].

Campan et al in 1997 [64] used a human model (pilot study) to assess the effects of omega-3 PUFAs on experimentally induced gingivitis. 37 healthy volunteers were involved in the study. They practiced intensive oral hygiene measures for 14 days and then stopped from brushing for 21 days. Volunteers were randomly divided into two groups, one received 30% PUFAs (treatment group) and the second received only 1% PUFAs (placebo group) 3 times a day for 8 days. PUFAs were administered systemically in both groups. The results revealed that omega-3 PUFAs reduced inflammation in the treatment group, but the significance was difficult to assess as this was a pilot study. In 2010, Elsharkawi et al [65] also used a human model to assess the effects of systemic application of omega-3 PUFAs in a chronic periodontitis model. They included 80 healthy subjects in the study. Subjects were divided into two groups (40 in each).

Members of one group were treated with scaling and root planing followed by a placebo drug. The other group members were treated with both scaling and root planing followed by a daily dose of omega-3 PUFAs (900mg EPA+ DHA) and Aspirin (81mg). The results revealed a significant reduction in the severity of chronic periodontitis (i.e.: attachment gain and decreased probing depth) in patients who used supplementation of omega-3 PUFAs in combination with Aspirin when compared to the placebo group. They also observed a significant reduction in salivary levels of RANKL in the treatment group which indicates lesser osteoclastic activity and hence lesser bone destruction. Hasturk and co-workers in 2005 [66] showed the effects of Resolvin E1 (RvE1) on bone destruction in periodontitis. They applied pathogenic organisms to a ligated tooth in order to develop periodontitis and bone destruction in a rabbit model. They assessed the effects of topically applied RvE1 (4micro gm/tooth 3 times/ week) both histologically and radiographically. Their results showed that topical application of RvE1 significantly inhibited bone and tissue damage. Herrera et al in 2008 [38] tried to assess whether RvE1 had direct effects on osteoclastic development and bone resorption in an in vitro periodontitis model. They used bone marrow cells flushed from freshly isolated femurs and tibia from mice. Cells were cultured for 7 days and osteoclastic differentiation was induced (using M-CSF and RANKL) in the presence of RvE1. Interestingly, they observed a marked decrease in osteoclastic growth and resorption pit formation in the presence of RvE1, a finding that well correlates with both resolution of inflammation and bone remodeling. Mustafa and co-workers in 2013 [62] obtained cells from healthy individuals and seeded them in 12-well plates until confluence then a linear scratch was created (3 mm wide). Cells were then incubated with either RvD1 (100ng/ml) or vehicle in DMEM for comparison. They observed an increase in PDL proliferation in the presence of RvD1. A recent study by Khaled [67] et al examined the effects

of RvD1 on human gingival fibroblasts and cytokine expression when these cells were exposed to P. gingivalis (one of the primary pathogenic bacteria in periodontitis). Their results showed that treatment with RvD1 has altered the cytotoxicity of P. gingivalis and its ability to produce inflammation. It also decreased the pro-inflammatory cytokine expression and increased the level of growth factors. The effects of topical application of RvE1 on infected pulps was investigated in a recent study by Dondoni and coworkers [68]. A rat pulpits model was used where the pulps of mandibular first molar teeth were exposed and left open into the oral environment. Teeth were treated 24 hr later with topical application of a corticosteroid/antibiotic blend, RvE1 or ethanol in a sterile cotton pellet. Cavities were then restored with amalgam fillings. Effects were histologically assessed 24 and 72 hour after teeth restoration. Results showed a protective role of RvE1 (marked reduction in both tissue cellularity and extent of inflammation) in comparison to the other two groups.

AIMS OF THE STUDY

Periodontal diseases such as marginal and apical periodontitis are among the most predominant causes of tooth loss. Knowledge about the effects of RvD1 on periodontitis is limited. However, there is evidence that the endogenous lipid mediator Resolvin D1 may have a therapeutic role in periodontitis. It was therefore the aim of this thesis to investigate the effect of RvD1 in the regulation of tissue destruction and resolution of inflammation in periodontal lesion models and to evaluate its potential in therapy of these conditions.

We investigated the role of different doses of RvD1 in inflammation resolution as well as in bone resorption. To achieve this, we performed an *in-vitro* study followed by an *in-vivo* study (pilot).

Specific aims

1. To assess the role of RvD1 on the proliferation of human PDL fibroblasts *in vitro.*

2. To study the effects of RvD1 on osteogenic factors' gene expression in human PDL cells *in vitro.*

3. To investigate the effect of systemic application of RvD1 on periapical lesion formation *in vivo* in a model of murine apical periodontitis.

MATERIALS AND METHODS

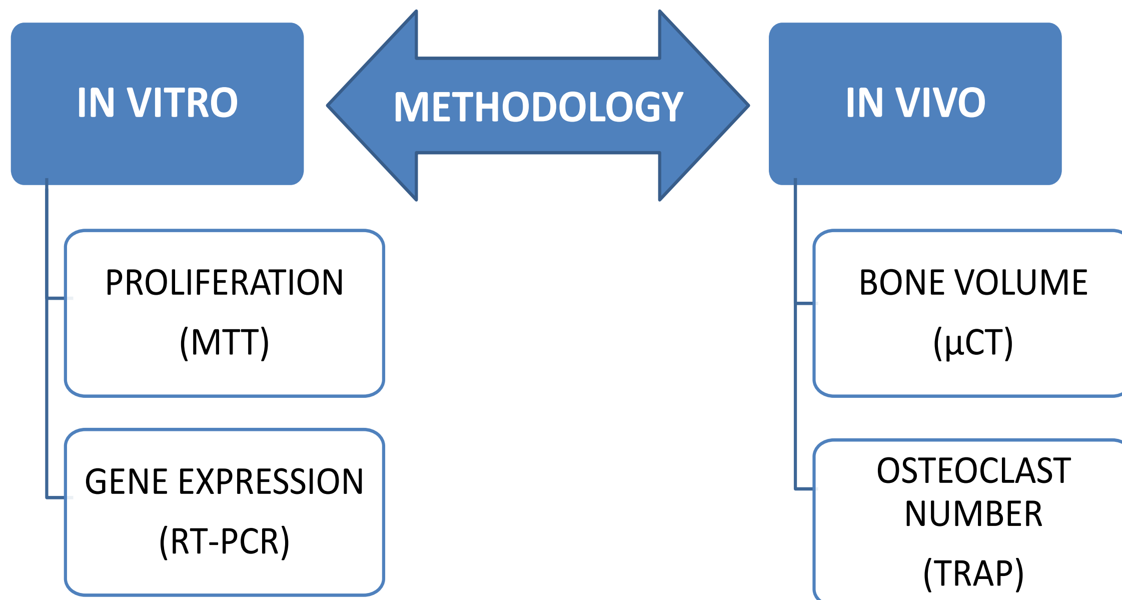


Fig.4 Parameters measured in the study.

Cell culture (in vitro model)

Our study was approved by the Ethics Committee at University of Bergen and Regional Committees For Medical And Health Research Ethics (REK). All the subjects participating in the study provided a signed informed consent. Primary periodontal ligament fibroblasts were obtained from extracted teeth (extracted for orthodontic reasons) from three different donors. Donors were healthy individuals with no clinical signs of periodontal disease. Pieces of periodontal tissues were scraped from the apical two thirds of the tooth root and then minced and

cultured according to previously published [23]. Cells were grown in 160 cm^2 Falcon culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with sodium-glutamine (4mmol), 5% fetal bovine serum (FBS) and 1% antibiotics (Penicillin and Streptomycin). Flasks were incubated in 37°C humidified incubator gassed with 5% CO_2. Medium was changed every other day and cells were routinely passaged using 0.25% trypsin containing 1mM EDTA. PDL fibroblasts used for the experiments proliferated in logarithmic phase between the 6th and 10th passages. Twenty four hours before starting each experiment, cells were counted by a Countess™ Automated Cell Counter (Invitrogen™, Carlsbad, California) and seeded into the plates with the wanted number.

Methylthiazol Tetrazolium assay (MTT)

Following the protocol published in [69], PDL fibroblasts from two donors (passages between 8 and 10) were seeded in three different 96-well cell culture plates (seeding density: 1×10^4 cells/well). Cells were 80% confluent after 24h where they were stimulated with different doses of Resolvin (1, 10 and 100 ng/ml) in the presence or absence of TNF-α (1ng/ml) in osteo-inductive culture medium (OM) (Minimum Essential Medium, alpha modification (α-MEM), supplemented with 10% fetal calf serum (FCS) 1% antibiotic (Penicillin/Streptomycin solution), 0.0001% Dexamethasone, 0.005% Ascorbic acid and 0.0035% beta-glycerophosphate (β-GP)). A control group was also included for both cases (incubated in OM in the presence (inflammation group) or absence (non-inflammation group) of TNF-α). Three different time points were set to the proliferation test (1, 3 and 7 days) where the viability test using MTT assay was performed. The culture medium was replaced by MTT solution and cells were incubated for 3 h. The viable cells (with functional mitochondrial dehydrogenase) were able to reduce the yellow MTT

solution into purple (formazan product). Medium was then discarded and the precipitate was dissolved in 100µl DMSO (dimethyl sulfoxide) containing 6.25% (v/v) 0.1M NaOH for 20 min with gentle shaking. Quantification was done for the end product using microplate spectrophotometer at 570 nm wave length. The quantity of purple color is proportional to the number of viable cells (dead cells do not have the ability to convert the MTT solution into formazan product) [70]. Results were presented as optical density units (OD) after subtraction of blank reading.

Real Time Polymerase Chain Reaction (RT-PCR)

Effects of different doses of Resolvin D1 on the expression of ALP, Col-1 and OC gene levels were studied using RT-PCR according to published protocols [71, 72]. PDL fibroblasts from three donors (passages between 6 and 8) were seeded in a 6-well plate (25×10^5 cells/well, in three replicates). Medium was changed every other day till cells were confluent. Plates were then stimulated with different doses of Resolvin (10, 100 ng/ml) in the presence or absence of TNFα (1ng/ml). After incubation for two weeks, plates were washed with PBS and stored at -80°C freezer for later use. For the total RNA extraction, the Maxwell© 16 LEV simplyRNA Cells Kit (Promega Corporation, Madison, MI, USA) and the Maxwell© 16 instrument (Cat. # AS2000) were used. 200µl of lysis buffer was added to each well and cells were scraped and moved to individual eppendorf tubes. They were vortexed vigorously for 15 seconds and then transferred to the first well in the plate. 5µl of DNAse I solution was added to the fourth well on the plate. The machine was then operated according to manufacturer's instructions. Quantity and quality of the RNA were then checked by spectrophotometry (NanoDrop Technologies, Inc., Wilmington, DE, USA) for cDNA synthesis.

The q-PCR was then performed using TaqMan Universal Fast PCR master mix. The PCR amplification of the bone markers was done in four replicates, each with 10μl reaction volume. PCR reaction mix containing 0.5μl of the respective TaqMan probes (ALP ”ALPL: Hs01029144_m1”, Col-1 “CoL1A2: Hs00164099_m1” and OC “BGLAP: Hs00609452_g1”), 3.5μl nuclease free water, 5μl of universal fast master mix and 1μl cDNA was prepared and run in 96-well microtiter plate using ABI StepOnePlus™Real-Time PCR system (Applied Biosystems) with thermocycling conditions set to 1 cycle of 95°C for 20s, 40 cycles of 95°C for 1s and a cycle of 60°C for 20s. Data were analyzed by the comparative C_T method for relative quantification. C_T (threshold cycles) were calculated automatically using StepOne v.2 software. The $2^{-\Delta\Delta Ct}$ method was used to quantify the gene expression level of the bone markers. GAPDH “GAPDH: Hs02758991_g1” was used as the endogenous control.

Murine apical periodontitis model (in-vivo pilot study)

Nine 7-10 weeks old male C3H mice were used. They were purchased from The Jackson Laboratory (Charles River, Germany). All experiments were performed with the approval of the Local Ethical Committee and in accordance with the Norwegian State Commission for Laboratory Animals.

As previously described [14, 73], mice were acclimatized for one week and then anaesthetized with a mixture of Ketamin (10 mg/ml) - Ketalar and Medetomidin (1 mg/ml)- Domitor with respective doses of 75 mg/kg and 1 mg/kg, intramuscularly (IM). Bilateral pulp exposures were performed under a stereomicroscope on the first mandibular molars. Round bur (number ½) was used. At the end of the procedure, atipamezole was given intramuscularly for reversal of the

anesthesia. Mice were divided into 3 groups (3 mice per group). The first group was the untreated control group (no intervention was done). In the second and third groups periapical lesion development was induced by exposing the pulp. The second group was injected with 100 µl normal saline (intramuscular injection), starting one day after pulp exposure. The same dose was repeated three times with one week interval. Mice in the third group were injected with 10 ng of Resolvin D1 dissolved in normal saline (intramuscular injection); in three separate injections of 100 µl, as described above. All animals exhibited normal feeding habits and gained weight during the period of the experiment.

Three weeks after pulpal exposure, mice were euthanized by an overdose of sodium pentobarbital intraperitoneally (Mebumal 50 mg/ml; Svaneapoteket, Bergen, Norway). Both upper and lower jaws were collected. One side jaws were stored in -80°C to later undergo PFA fixation and micro-CT scanning. The other side jaws were immediately fixed in paraformaldehyde (PFA) overnight at 4° C and afterwards were decalcified in 10% (w/v) EDTA. EDTA was changed three times every day and after approximately one week to 10 days the decalcification of the jaws was complete. Samples were washed in phosphate buffer and placed into PBS + 30% sucrose solution for overnight. Samples were then taken out and embedded in mounting compound (Tissue-Tek OCT; Sakura, Zoeterwoude, the Netherlands) and immediately stored in -20°C for later cryosectioning.

Tartrate-resistant Acid Phosphatase staining (TRAP)

Parasagittal sections of the mandibles were taken as previously described [14]. Briefly sections were cut in a freezing slide microtome (-20°C) with 10μm thickness. Tissue orientation was

taken into consideration. Tartrate-resistant acid phosphatase (TRAP) was used for detection of osteoclasts and pre-osteoclasts using Leukocyte Acid Phosphatase for TRAP kit (387A, Sigma-Aldrich, USA). Slides were fixed and stained according to the manufacturer's instructions. Briefly, TRAP staining solution was freshly prepared (Diazotized Fast Garnet GBC Solution+ Naphtol AS-BI Phosphate solution+ Acetate Solution+ Tartrate Solution all in pre-warmed Deionized water). Slides were immersed into the warm solution and incubated at 37°C for 1 hour. Slides were rinsed for 10 min, counterstained with hematoxylin solution and air-dried. Cells were observed under the light microscope and considered to be osteoclasts if they were TRAP-positive, multinucleated, and were located on the bone surface or residing in Howships's lacunae. Quantification of positive cells was done on TRAP-stained sections from each mouse under a photomicroscope (Nikon Eclipse E600; Nikon Instruments, Kanagawa, Japan) connected to a digital camera using Lucia imaging software and averaged for each mesial and distal root of the 1st mandibular molar of each animal. Results were expressed as number of TRAP-positive cells.

X-ray Micro-Computed Tomography (μCT)

Micro-computed tomography (micro-CT) scans were taken for quantitative evaluation of the bone structure on the mice periapical region at three weeks as described before in the literature, with some modifications [74, 75]. The SkyScan1172© microfocus X-ray system (SkyScan©, Kontich, Belgium) was used, with the CTAn 1.8© and NRECON RECONSTRUCTION© CT software (SkyScan©, Kontich, Belgium). A 0.5-mm aluminum filter was used for taking optimized images. Source voltage and current were set as 50 kV and 200 μA, respectively. Volumetric assessment of periapical bone was determined by drawing a region of interest (ROI)

around the root of mandibular first molar. The drawing was extended from the mesial extension of the cemento-enamel junction (CEJ), around the root apex and up to the distal extension of CEJ, and down again around the socket wall and up to the start point. The periapical bone was contoured carefully for every fifth slice and modified where necessary. The resultant region of interest was constructed into 3D ROI. Software was used to obtain tissue volume (TV: total volume of the selected region of interest (ROI)), bone volume (BV: hard tissue volume within the selected region of interest (ROI)) and bone volume fraction (BV/TV) out of the specified drawn ROI.

Statistical analysis:

All data was subjected to statistical analysis using R statistical package. For evaluation of differences between groups, one-way analysis of variance (ANOVA) followed by Tukey's HSD (honest significant difference) test were used. A p value < 0.05 is considered as statistically significant.

RESULTS

In-vitro

Since Resolvins were found to play a significant role in resolution of inflammation, their role in cellular proliferation and in gene expression on sites of inflammation is also of importance. In the current work, we studied the effect of different doses of RvD1 on PDL cell proliferation as well as their effects on the expression of certain osteogenic markers.

Effects of RvD1 on cell proliferation

MTT test was used to examine the effect of different doses of RvD1 (1, 10 and 100ng/ml) on cellular proliferation in the presence or absence of TNFα (1ng/ml). The test was done for two donors. One donor in three replicates (Donor A) and the second donor in five replicates (Donor B). Differences were evaluated using ANOVA test followed by Tukey's HSD test. Donors behaved differently in response to RvD1 application. Results are shown in figures 5 to 10.

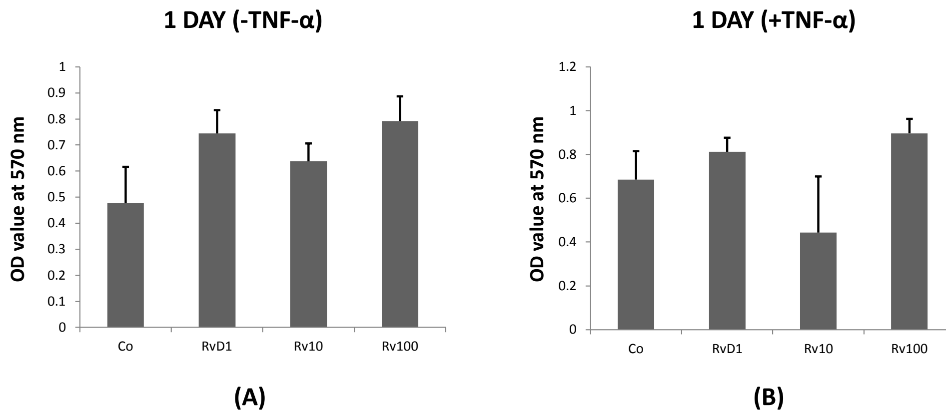


Fig. 5 Effect of different doses of Resolvin on proliferation of periodontal ligament fibroblasts (donor A) on one day time point in the absence **(A)** or presence **(B)** of TNF-α (I ng/ml). Optical density (OD) values are presented as median +/- StD.

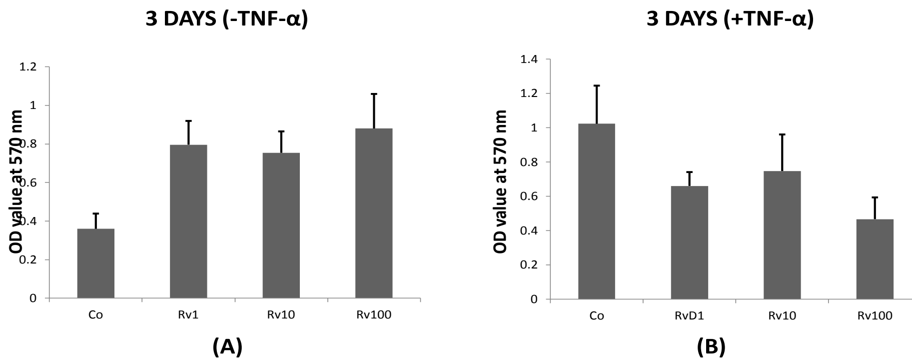


Fig. 6 Effect of different doses of Resolvin on proliferation of periodontal ligament fibroblasts (donor A) on three days time point in the absence **(A)** or presence **(B)** of TNF-α (I ng/ml). Optical density (OD) values are presented as median +/- StD.

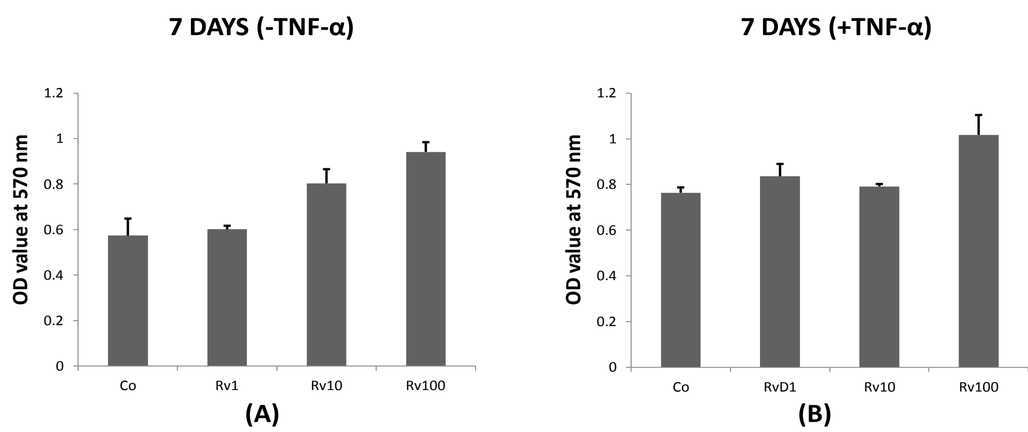


Fig. 7 Effect of different doses of Resolvin on proliferation of periodontal ligament fibroblasts (donor A) on seven days time point in the absence **(A)** or presence **(B)** of TNF-α (I ng/ml). Optical density (OD) values are presented as median +/- StD.

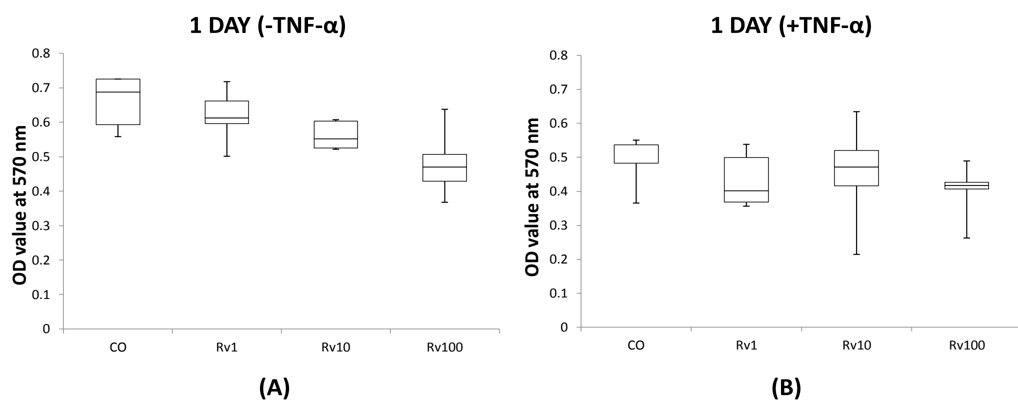


Fig. 8 Effect of different doses of Resolvin on proliferation of periodontal ligament fibroblasts (donor B) on one day time point in the absence **(A)** or presence **(B)** of TNF-α (I ng/ml). Optical density (OD) values of five replicates are presented as box plots (showing minimum, 25th percentile (Q1), median, 75th percentile (Q3) and maximum. ANOVA statistical test followed by Tukey's HSD test were used. *P*value <0.05.

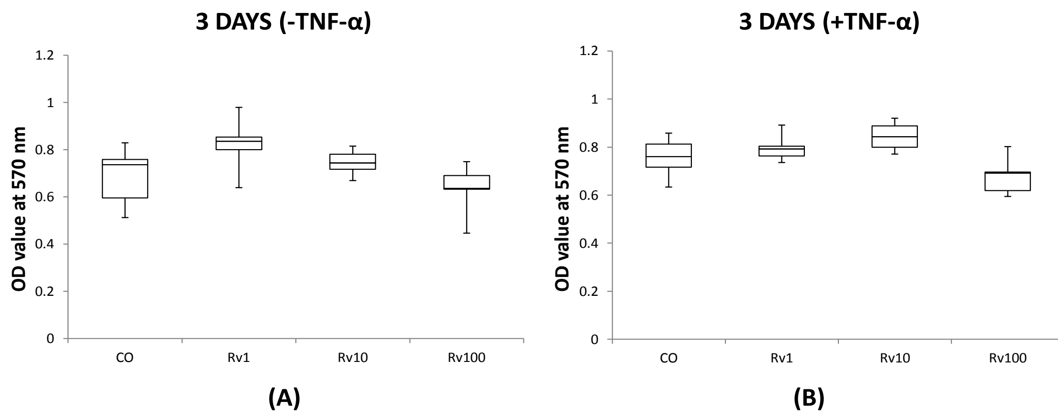


Fig. 9 Effect of different doses of Resolvin on proliferation of periodontal ligament fibroblasts (donor B) on three days time point in the absence **(A)** or presence **(B)** of TNF-α (I ng/ml). Optical density (OD) values of five replicates are presented as box plots (showing minimum, 25th percentile (Q1), median, 75th percentile (Q3) and maximum. ANOVA statistical test followed by Tukey's HSD test were used. *P*value <0.05.

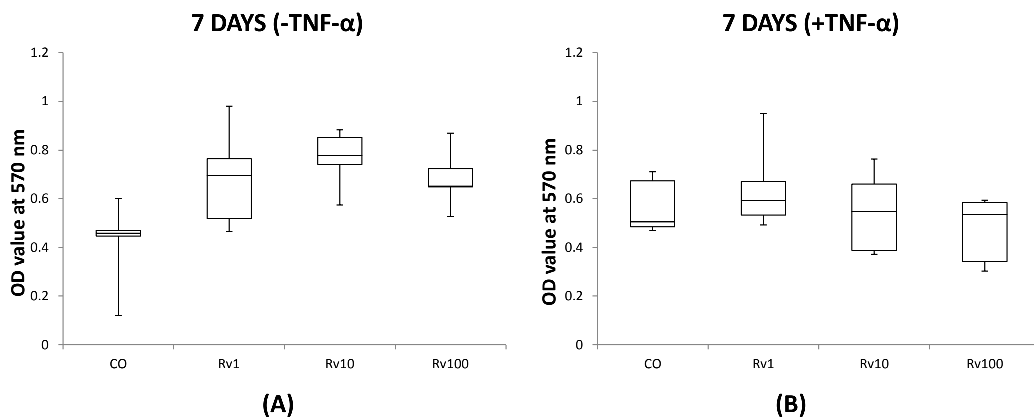


Fig. 10 Effect of different doses of Resolvin on proliferation of periodontal ligament fibroblasts (donor B) on seven days time point in the absence (A) or presence (B) of TNF-α (I ng/ml). Optical density (OD) values of five replicates are presented as box plots (showing minimum, 25th percentile (Q1), median, 75th percentile (Q3) and maximum. ANOVA statistical test followed by Tukey's HSD test were used. *P*value <0.05.

Effects of RvD1 on gene expression

PCR test was done to examine the role of RvD1 on the expression of osteogenic markers (ALP, Col-1 and OC) in PDL cells in the presence or absence of 1ng/ml TNF-α. Effects were seen as follows:

ALP

The lower dose of RvD1 (10 ng/ml) induced statistically significant up-regulation of ALP when compared to control group. In contrast, the higher dose of RvD1 (100 ng/ml) induced down-regulation of ALP in both presence and absence of TNF-α but it was not statistically significant (Fig. 11).

Col-1

Col-1 gene expression showed a prominent and significant up-regulation associated with the lower dose of Resolvin (10ng/ml) in both absence and presence of TNF-α in all donors. There was no effect seen in association with the higher dose (100ng/ml) in the presence and absence of TNF-α in all donors (Fig. 12).

OC

No effect of RvD1 was observed in the expression of OC gene as there was a slight up-regulation of the gene expression in association with both doses of Resolvin in all donors in both absence and presence of TNF-α (Fig. 13).

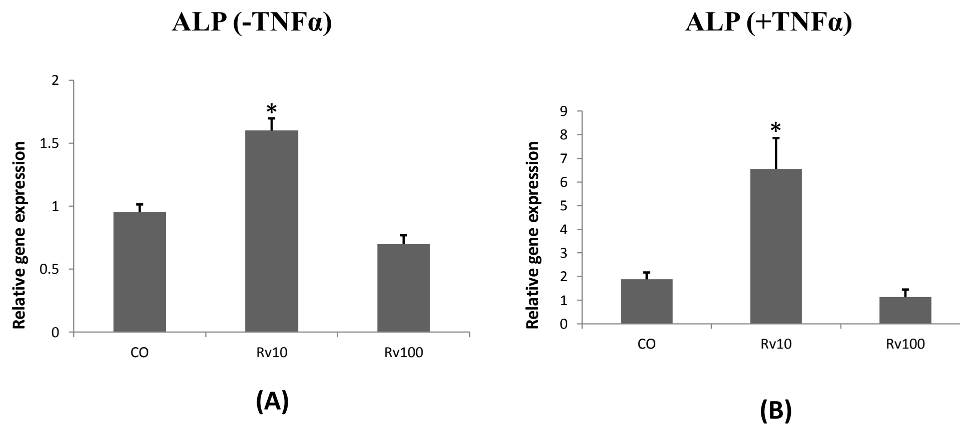


Fig. 11 Effect of RvD1 on Alkaline Phosphatase (ALP) gene expression by periodontal ligament fibroblasts in the absence (A) or presence (B) of TNF-α (1 ng/ml).Relative gene expression values are presented as mean ± SD of five replicates of samples compared to control (GAPDH). ANOVA was used for statistical analysis,* p value < 0.05

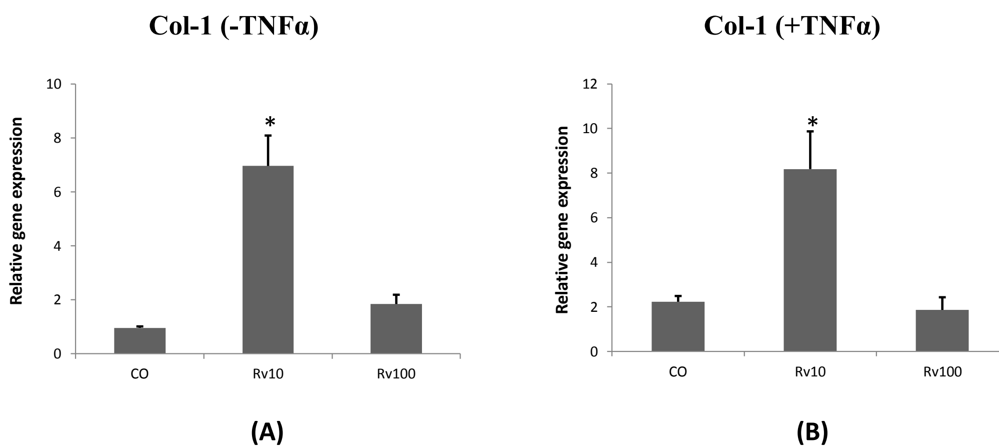


Fig. 12 Effect of RvD1 on Collagen-1 (COL-1) gene expression by periodontal ligament fibroblasts in the absence (A) or presence (B) of TNF-α (1 ng/ml).Relative gene expression values are presented as mean ± SD of five replicates of samples compared to control (GAPDH). ANOVA was used for statistical analysis,* p value < 0.05

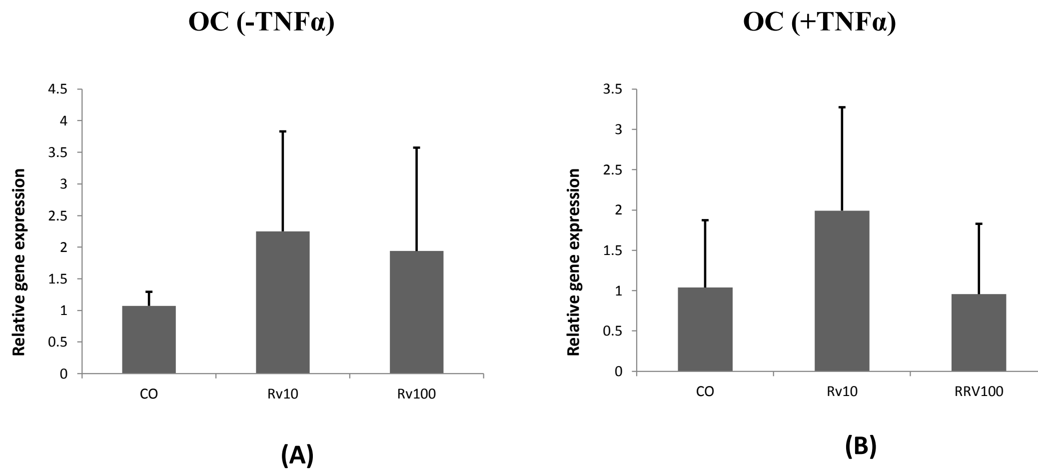


Fig. 13 Effect of RvD1 on Osteocalcin (OC) gene expression by periodontal ligament fibroblasts in the absence (A) or presence (B) of TNF-α (1 ng/ml).Relative gene expression values are presented as mean ± SD of five replicates of samples compared to control (GAPDH). ANOVA was used for statistical analysis,* p value < 0.05

In-vivo

In the present investigation a pilot study was performed to assess the effect of RvD1 on periapical lesion development in a murine model of apical periodontitis.

Effects of RvD1 on osteoclast number

Number of osteoclasts (TRAP-positive cells) was counted (fig. 14). Results showed that the number of TRAP-positive cells was greater in both the saline treated group (32,56 ± 2,985) as well as the Resolvin treated group (19,24 ± 4,523) compared to the control group (13,13 ±

3,966). There was a tendency for higher numbers of TRAP-positive cells in the saline-treated group compared to the Resolvin-treated group (Fig. 15). The difference was statistically significant; however, the results are only indicative as the number of sections evaluated was very small in the saline group due to technical difficulties.

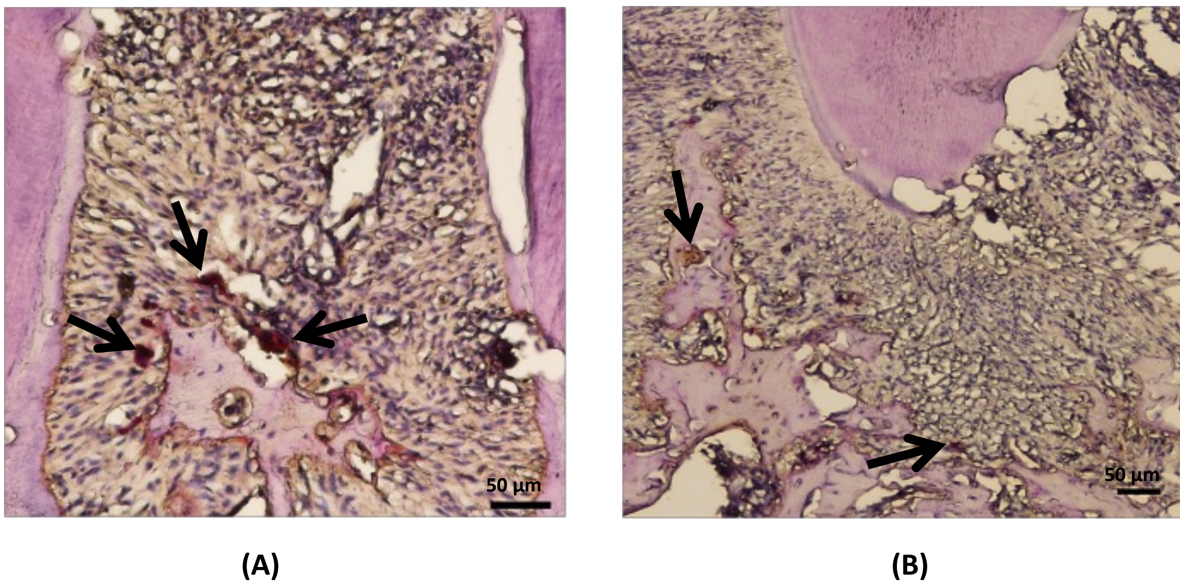


Fig. 14 TRAP-positive multinucleated cells (arrows) in exposed molar teeth viewed under the photomicroscope. **(A)** at the furcation area and **(B)** in the periapical area.

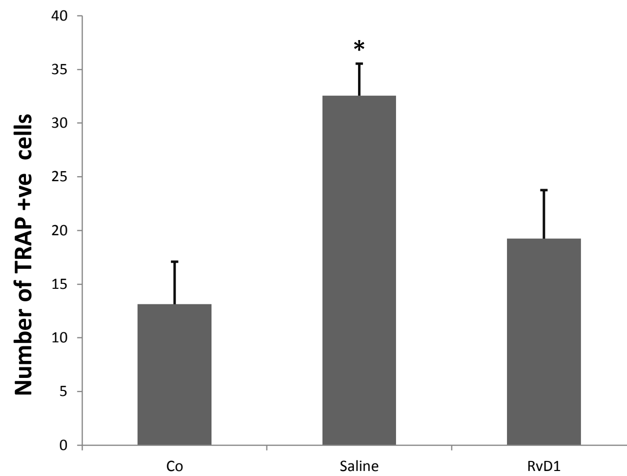


Fig. 15 TRAP-positive cells count presented as mean +/- StD. ANOVA was used for statistical analysis, * p value <0.05

Effects of RvD1 on bone volume

Effects of RvD1 on periapical inflammation were evaluated using a micro-CT scans (Fig. 16 and 17). Bone volume at the area of interest was measured in the untreated controls. When periapical inflammation was induced by pulp exposure, the bone volume was reduced in both the saline-treated group and the Resolvin-treated group, meaning that there was bone resorption at the periapical area of the 1st mandibular molars. Reduction was greater in the saline treated group, but no statistical differences were observed due to low sample size (pilot study). Results are presented in figures 18 and 19.

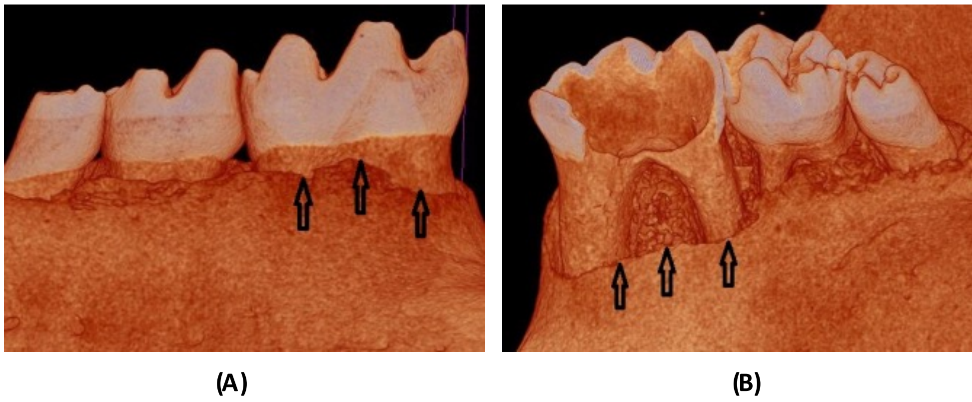


Fig. 16 Micro CT images for mandibular sections from **(A)** a control mouse and **(B)** a RvD1 treated mouse. Arrows pointing to the alveolar bone. Note the resorption of alveolar bone in the RvD1 treated mouse.

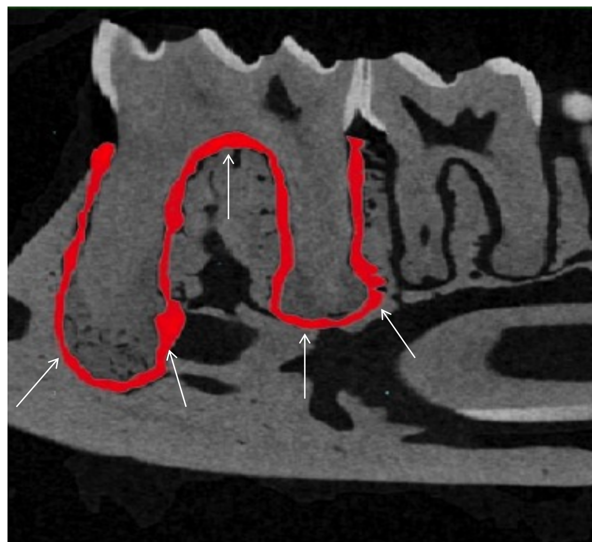


Fig. 17 Region of interest selected (red area) and bone volume in this area calculated. Arrows pointing to ROI.

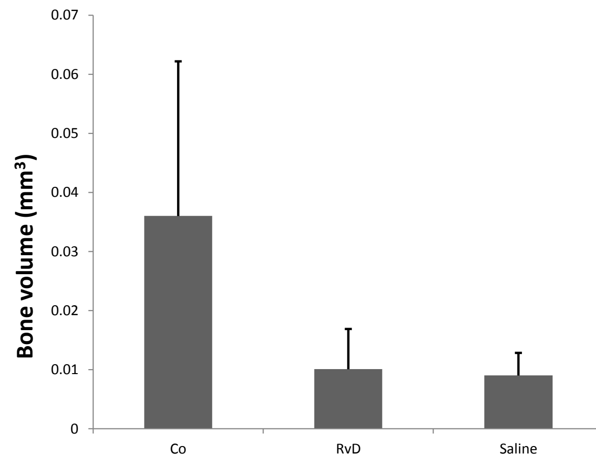


Fig. 18 Bone volume measurements for all groups. Values are presented as mean +/- StD.

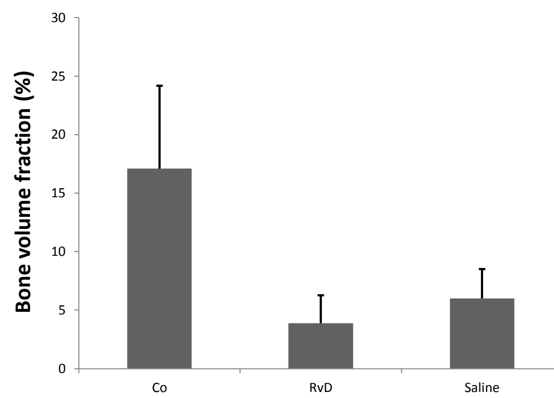


Fig. 19 Bone volume fraction measurements for all groups. Values are presented as mean +/- StD.

DISCUSSION

Resolvins are a new group of bioactive lipid mediators produced naturally in the human body from ω3-polyunsaturated fatty acids (ω3-PUFA). They have a significant role in resolution of inflammation and in wound healing [40]. They actively aid in the resolution process by reducing both the leukocyte adhesion and the endothelial-leukocyte interactions [76]. Several studies have reported their role in resolution of many inflammatory conditions including periodontal diseases [7, 13]. The current work was performed to study the role of Resolvin D1 on the bone preservation and wound healing in periodontitis lesion (both in vitro and in vivo).

Effects of Resolvins on cell proliferation

In the current study, we wanted to assess the effects of RvD1 on periodontal fibroblast proliferation. We used different doses of RvD1 (1, 10 and 100ng/ml) in the presence and absence of inflammatory molecules (+/- TNFα). RvD1 at 10 ng dose induced the proliferation of PDL fibroblasts both at 3 and 7 days time points. This came in accordance with Mustafa et al who have studied the effects of RvD1 on periodontal fibroblastic proliferation (injury model) [62]. They found that RvD1 (10ng/ml) had improved cellular proliferation and hence injury closure. Zhang et al has also arrived to the same conclusion when they studied the effects of RvE1 in human corneal endothelial cells [78]. They also used a scratch model and found that the migratory rates were increased in cells stimulated with RvE1 leading to faster wound closure. The effect was dose dependent (i.e. the higher the dose, the higher cell migration). In contrast to this, Qu, X. and co-workers studied the role of RvE1 (300ng/ml) in an irreversible fibrosis model [79]. They found that there was a reduction in fibroblasts proliferation in both in-vivo and in-

vitro models. Interesting results were provided by Bohr and co-workers where they introduced a burn injury into mice and then investigated the therapeutic effects of intravenous administered RvD2 [80]. They found that RvD2, during the early phase of injury, prevented thrombosis of the deep dermal venous network and reduced depth of necrosis. Interestingly, it leads to delayed wound contraction and increased scar area.

In 2013, Mihyahara, T. studied the effects of both RvD1 and RvD2 on vascular smooth muscle phenotype (determined by cell proliferation) and vascular injury [81]. Results showed that both RvD1 and RvD2 produced a dose-dependent inhibition of vascular smooth muscle cell proliferation and hence a modulation of vascular injury. In accordance with this, Ho, K. J. and co-researchers [82] have found that Aspirin triggered Lipoxins and Resolvins lead to a decreased vascular smooth muscle cell proliferation. This leads to protection of the vessel wall decreasing the disease severity. Interestingly, Ramon et al have studied the effects of 17-DHDA on B-cell proliferation on the spleen [83]. Their results showed that 17-DHDA had no effects on that type of cells (neither toxic nor proliferative effects). An earlier report demonstrated that PDL proliferation can also be obtained when the periodontal ligament is treated with enamel matrix protein derivative (EMDOGAIN) [77].

Effects of Resolvins on gene expression

We have examined, in this work, the effects of different doses of RvD1 (10 and 100ng/ml) on the expression of some osteogenic markers *in vitro*. There was an increase in the expression of ALP and Col-1 in association with the lower dose (10ng/ml). A previous study showed that stimulation of intestinal epithelium with RvE1 elicited production of intestinal alkaline

phosphatase (ALPI) enzyme which in turn aided in inflammatory resolution. This production was ChemR23 mediated and it was not observed in ChemR23-knockedout intestinal epithelial cells [84]. In a previous in vitro experiment, administration of IL-6 and its receptor (IL-6R) into bone cultures decreased the expression of RANKL and OPG. Stimulation of these cultures with RvE1 significantly increased the levels of OPG without causing a change in RANKL levels. [85]. The levels of other osteogenic markers (ALP, BSP and RunX2) were not changed. In a previous in vitro experiment, osteoblasts from neonatal calvariae were treated with 10nM testosterone [86]. This caused a significant reduction on OC, OPG and RANKL levels. Treatment of these osteoblasts with RvD2 restored back these markers into their baseline levels. This shows that RvD1, by inducing both proliferation and bone osteogenic molecules expression in PDL fibroblasts may have an impact on bone remodeling in the periapical tissues.

Effects of Resolvins on osteoclasts

We tested the effects of RvD1 on osteoclastic activity in experimentally induced apical periodontitis (*in vivo* pilot study). Our results showed that there were less TRAP positive cells in the RvD1 treated group, when compared with the saline treated group and the difference was statistically significant. Herrera et al have found that RvE1 led to decreased osteoclastic differentiation and led to reduction in bone resorption in experimental periodontitis model in vitro [38]. McCauley and co-workers have found that human and murine primary macrophages have ingested apoptotic osteoblasts in vitro. Treatment with both RvD1 and RvD2 has enhanced this process providing a feedback system for the inflammatory resolution and maintenance of the skeletal homeostasis [87]. In 2014, Steffens et al studied the role of RvD2 on testosterone

induced osteoblast function [86]. They found that it had a significant role in improvement in osteoblastic functions and hence inflammation resolution.

Effects of Resolvins on bone volume

In this study we assessed the role of RvD1 and its effect on preservation of bone volume in vivo (pilot study). Our results showed that there was no statistical difference in bone volume measurement between the saline treated mice and Resolvin treated mice. A tendency for lower number of osteoclasts was observed in the Resolvin treated mice (when tested with TRAP), but that does not reflect on bone volume, which means the lesions are established anyway. Further experiments are needed because conclusions are difficult to be drawn at this stage due to the small number of mice used. Tests were performed three weeks after exposure. In fact the lesion was established at this point, but, maybe after that (in the chronic phase of inflammation), the bone resorption is affected by RvD1 treatment and possibly RvD1 treatment can have a potential therapeutic effect in the treatment of apical periodontitis. In a previous study, alveolar bone loss was introduced into a group of transgenic mice over expressing ChemR23 receptor [85]. RvE1 treatment reduced this bone loss. A uniform craniotomy defect was also introduced into the parietal bone of these mice and local treatment with RvE1 showed an enhancement in the regeneration of the bony defect. Steffens et al. studied the response of alveolar bone to testosterone stimulation (different doses) on male rats. They further evaluated the impact of RvD2 treatment on testosterone-induced osteoblast function [86]. As the testosterone stimulation increased inflammatory bone loss, RvD2 treatment was seen to mitigate the testosterone-induced down-regulation of OC, OPG and RANKL. Bendyk et al. induced periodontitis into a group of

mice [88]. Dietary supplementation of these mice with ω-3 PUFAs (tuna oil) reduced the severity of alveolar bone loss suggesting a preventive role of ω-3 PUFAs in periodontitis.

CONCLUSIONS

1. Addition of Resolvin D1, in vitro, in the absence of inflammation, leads to proliferation of periodontal ligament fibroblasts in three and seven days. This indicates an improvement in wound healing.

2. Treatment with Resolvin D1 also leads to expression of early bone formation markers in vitro (ALP and Col-1). This indicates that treatment of these inflamed cells with Resolvin D1 leads to reduction in inflammation and stimulation of bone formation (acceleration of wound healing).

3. Resolvin D1, in vivo, leads to reduction of osteoclastic cells around teeth with apical periodontitis.

4. 10ng/ml dose showed the best response in both periodontal fibroblasts proliferation and expression of bone formation markers.

5. Further investigations are needed to study the effects of Resolvin D1 on inflammation and wound healing.

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