



A Novel Injectable Hydrogel Incorporating Lipoxin for Treatment of Inflammation-mediated Bone Resorption

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A Novel Injectable Hydrogel Incorporating Lipoxin for Treatment of Inflammation-mediated Bone Resorption

A Thesis Presented by:

Faris Z. Jamjoom, BDS MS

To the Faculty of Medicine

In partial fulfillment of the requirements for the degree of

Doctor of Medical Sciences

Research Mentor:

Dr. Myron Spector, PhD

Professor of Orthopedic Surgery,

Brigham and Women's Hospital, Harvard Medical School

Harvard School of Dental Medicine

Boston, Massachusetts

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Dedication

To my father,

You have set the best example for me to follow. Everything I do is to be more like you.

Thank you for your constant guidance, encouragement, and support.

To my mother,

Your unconditional love and support have carried me through very challenging times.

Thank you for always being there for me.

To my wife,

You believed in me and selflessly supported me.

Thank you for your love and care. Thank you for sacrificing your dreams for me to pursue mine.

To my son,

I hope to be a great example for you, as my father was for me. This is all for you.

Thank you for giving me purpose and motivating me to be the best version of myself.

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Table of content

<u>Abstract</u>	1
<u>1. Study Overview</u>	3
1.1 Rationale of study	3
1.2 Objective of study	3
1.3 Hypothesis of study	3
<u>2. Specific aims of study</u>	4
<u>3. Background and significance</u>	5
3.1 Inflammation and bone	5
3.2 Pathophysiology of Bone Loss	6
3.3 Periodontitis and Peri-implantitis	6
3.4 Significance	8
<u>4 Innovation</u>	9
4.1 Resolution of inflammation	9
4.2 Pro-resolving Lipid Mediators Derived from Arachidonic Acids	9
4.3 Lipoxins and bone	12
4.4 Lipoxins and periodontal disease	12
4.5 Hydrogels	13
4.6 Gelatin-hydroxyphenyl propionic acid hydrogel	13
<u>5. Research Strategy</u>	15
5.1 Specific Aim 1	16
5.2 Specific Aim 2	18

5.3 Specific Aim 3 -----	19
<u>6. Results</u> -----	26
6.1 Specific Aim 1 -----	26
6.2 Specific Aim 2 -----	27
6.3 Specific Aim 3 -----	29
<u>7. Discussion</u> -----	37
<u>8. Conclusions</u> -----	42
<u>9. Future direction</u> -----	42
<u>10. References</u> -----	43

Abstract

Increased inflammation can disturb bone homeostasis leading to increased osteoclastogenesis and bone resorption. Studies have demonstrated that specialized pro-resolving lipid mediators such as lipoxins, can counteract inflammation and reduce bone loss. Despite the promising results, lipoxins may not be very stable and will require a more efficient delivery method that allows them to remain at the target site for a sufficient amount of time without disintegration. This study aims to develop a gelatin-based injectable hydrogel that will contain lipoxins for the treatment of inflammation-mediated bone resorption.

Gelatin-hydroxyphenylpropionic acid (Gtn-HPA) cross-linked using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) was used as a hydrogel delivery vehicle. Aspirin-triggered Lipoxin A_4 (ATL A_4) was incorporated in the hydrogel. The impact of Lipoxin incorporation on the cross-linking of the hydrogel was investigated using a tube tilting method. The release profile of Lipoxins from the hydrogel was studied by incubating the hydrogel in PBS and measuring lipoxin release at predetermined timepoints. Finally, a neonatal rat calvarial organ culture model was used to test the impact of Lipoxins released from hydrogel on inflammation-mediated bone resorption.

Our results indicate that Lipoxins can be successfully incorporated in the Gtn-HPA hydrogel. However, at higher doses this may result in increased gelation time. The incorporated Lipoxins can be released from the gelatin matrix and the release rate can be controlled by varying the gel weight percentage and degree of cross-linking. Softer gels release more of the original lipoxin content compared to stiffer gels. Lastly, the reduction of inflammation-mediated bone resorption from a single dose of Lipoxins released from the hydrogel was comparable to multiple

doses of Lipoxins added directly to the medium in a neonatal rat calvarial bone organ culture model. The promising data from this study provides the foundation for the development of a new therapeutic modality for the treatment of conditions characterized by inflammation-mediated bone loss.

1. Study overview

1.1 Rationale of study

Increased inflammation is detrimental to bone homeostasis and is often associated with bone loss.(1) In dentistry, this can be seen in cases of periodontitis and peri-implantitis.(2, 3) The excessive release of proinflammatory cytokines disrupts bone metabolism leading to increased osteoclastogenesis and bone resorption.(1) Lipoxins are specialized pro-resolving lipid mediators derived from arachidonic acid that can counteract inflammation and lead to resolution.(4-6) A number of studies have demonstrated the ability of lipoxins to downregulate osteoclastogenesis, reduce bone loss, and decrease inflammatory cell infiltration.(7-9) Despite the promising results, lipoxins may not be very stable and will require an efficient delivery method that allows them to remain at the target site for a sufficient amount of time without disintegration.(10) Therefore, the objective of this study is to develop a gelatin-based injectable hydrogel that will contain lipoxins for the treatment of inflammation-mediated bone resorption.

1.2 Objective of study

The objective of this study is to develop a gelatin-based injectable hydrogel that can cross-link within the body for the treatment of inflammation-mediated bone resorption. This injectable gel will contain pro-resolving lipoxin that participates in inflammation resolution and tissue regeneration.

1.2 Hypothesis of study

The hypothesis of this study is that a lipoxin A₄ (LXA₄) analog could be contained in an injectable gelatin matrix comprised of gelatin-hydroxyphenyl propionic acid (Gtn-HPA) and can subsequently be released from this matrix for the treatment of inflammation-mediated bone resorption.

2. Specific aims of study

Specific Aim 1: To determine *in vitro* the impact of lipoxin incorporation on the gelatin matrix gelation rate.

Specific Aim 2: To determine *in vitro* the rate of lipoxin release from the gelatin matrix.

Specific Aim 3: To determine *in vitro*, using a neonatal rat calvarial organ culture, the impact of the lipoxin containing gelatin matrix on inflammation-mediated bone resorption.

3. Background and significance

3.1 Inflammation and bone

Bone is a dynamic organ that undergoes constant remodeling and repair in response to specific molecules, hormones, and mechanical stress.(11, 12) Homeostasis is achieved by maintaining a balance between bone formation carried out by osteoblast and bone resorption carried out by osteoclasts.(11-13)

The interaction between bone and the immune system is of special interest particularly when dealing with chronic inflammatory conditions associated with bone loss.(11, 14) Inflammation is a natural response to stressful stimuli and injury and is essential to bone formation.(1, 15) In acute injuries such as bone fractures, the damage to local bone and vascular tissues initiates the inflammatory cascade.(1) This subsequently promotes angiogenesis and induces mesenchymal stem cell proliferation and differentiation into osteoblasts.(1) Despite the role inflammation plays in bone repair, it is generally a transient process and the environment eventually shifts to anti-inflammation.(1)

On the other hand, chronic inflammatory conditions such as rheumatoid arthritis, diabetes mellitus, and periodontitis disrupt bone homeostasis by promoting osteoclast activation and differentiation, resulting in bone loss.(1, 11, 13, 16) Age-related chronic conditions such as osteoporosis also impact bone density and turnover particularly in women, and could potentially lead to fractures.(1, 11) A persistent, low-grade pro-inflammatory status could also develop in aging individuals leading to challenges with fracture healing.(1, 14) The aging population also have an increased prevalence of osteoarthritis which is another age-related inflammatory condition that could lead to substantial joint damage and require total joint replacement (TJR) with orthopedic

implants.(1, 11) Despite the success of TJR surgery, inflammatory responses to wear particles and metal ions released into the surrounding tissues could lead to bone resorption around orthopedic implants.(1, 6) Similarly, bone loss around osseointegrated dental implants could develop from exaggerated immune responses, although bacterial causes have also been implicated.(17, 18)

3.2 Pathophysiology of Bone Loss

Normal bone remodeling is mainly controlled by receptor activator of nuclear factor- κ B ligand (RANKL)-RANK-Osteoprotegerin (OPG) system.(14, 19) RANKL expressed by a number of cells including osteoblast and osteocytes binds to RANK on the surface of osteoclast precursor cells which initiates the differentiation, maturation, and activation of osteoclasts.(12, 13, 20) OPG on the other hand, is a soluble decoy receptor for RANKL that disrupts the binding of RANKL to RANK which suppresses osteoclastogenesis.(12, 19) Therefore, the balance between RANKL and OPG is a major regulator of resorption and formation.(12, 15, 19)

In chronic inflammatory conditions, persistent inflammation leads activation of cells from both the innate and adaptive immune responses leading to the release of proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor- α (TNF- α). (1, 11, 13, 19) This results in the enhanced expression of RANKL and the stimulation of osteoclast differentiation and activation.(1, 13)

3.3 Periodontitis and Peri-implantitis

Periodontitis is a chronic multifactorial inflammatory disease characterized by loss of clinical attachment, periodontal pocketing, gingival bleeding, and radiographic bone loss.(2) The overall prevalence of periodontitis in adults aged 30 years or older is estimated to be 42.2%.(21)

On the other hand, peri-implantitis is an inflammatory disease affecting the tissues surrounding dental implants, and is characterized by progressive loss of supporting bone.(3) The estimated prevalence of peri-implantitis is 19.83% at the patient level.(22) However, inconsistencies in defining and diagnosing peri-implantitis may impact the estimated prevalence.(22)

The etiology of periodontal disease is multifactorial and related to the complex interplay between the subgingival biofilm and the host immune/inflammatory response.(16, 23) Despite the association between periodontitis and periodontal pathogens, emerging evidence suggests that the pathogenesis of this disease is inflammatory rather than infectious.(24) A host-mediated inflammatory response results in microbial dysbiosis, chronic infection, and unresolved inflammation.(16) The increased microbial virulence in the dysbiotic subgingival environment sustains the inflammatory response ultimately leading to the breakdown of periodontal tissues.(23, 24) Similar relationships between inflammation and dysbiosis have also been reported with cardiovascular disease, diabetes, and inflammatory bowel syndrome.(16, 24)

The etiology of peri-implantitis is thought to resemble that of periodontitis, in which the imbalance between host response and microbial challenge leads to an inflammatory response and tissue breakdown.(25, 26) However, there is evidence suggesting that peri-implant bone loss may be an immunological reaction unrelated to bacteria similar to aseptic loosening of orthopedic implants.(17) Furthermore, the lack of periodontal ligaments and supracrestal attachment fibers around dental implants prevents the peri-implant connective tissues from isolating the inflammatory cell infiltrate from the crestal bone.(25) This can explain the accelerated rate of progression and the more aggressive pattern of tissue breakdown compared to periodontitis.(25, 26)

Inflammation of the periodontal and peri-implant tissues are characterized by elevated proportions of neutrophils, macrophages, and lymphocytes.(23, 27) During the inflammatory process, various inflammatory mediators are produced in association with disease onset and progression.(23, 28, 29) IL-1 β and TNF- α have been reported to be substantially elevated in periodontal and peri-implant disease.(29-32) Despite advances in understanding the pathologic processes involved in periodontal disease and peri-implant disease, persistent and uncontrolled inflammation may lead to failure of therapy.(16, 23, 33)

3.4 Significance

It is relevant to understand the role of inflammation on bone homeostasis and regeneration. This is of particular importance when dealing with inflammatory conditions characterized by bone loss.(1, 11, 14) With the rise in skeletal diseases caused by immune system alterations and with a growing aging population, treatment challenges will persist.(1, 11, 14) In dentistry, the prolonged maintenance of teeth along with the rising number of individuals receiving dental implants, will ultimately lead to a rise in incidence and prevalence of periodontal and peri-implant disease.(21, 34) This points to the eminent need for the development of novel therapeutic strategies and modalities that can modulate the inflammatory response to prevent bone loss and regenerate bone.

4. Innovation

4.1 Resolution of inflammation

Distinct from the anti-inflammatory process is the recently defined resolution of inflammation.(4, 35) The resolution of inflammation is in fact an active process characterized by a switch in the predominant mediators found in the exudates.(4, 5, 36) Deficiency in any component of the resolution phase may potentially result in uncontrolled chronic inflammation, which can be responsible for the pathogenesis of a number of diseases.(5, 10, 36)

Initially, classic inflammatory mediators such as prostaglandins and leukotrienes are generated to activate the inflammatory process.(4) These mediators eventually induce the production of key enzymes that promote the synthesis of pro-resolving mediators such as lipoxins, resolvins, protectins, and maresins.(4, 5, 35-37) These pro-resolving molecules activate specific mechanisms fundamental to the resolution of inflammation that include the cessation of neutrophil and eosinophil infiltration into the afflicted tissues, counter-regulation of cytokines and chemokines, induction of apoptosis in expended neutrophils, activation of phagocytosis of apoptotic cells and microorganisms by macrophages, clearance of non-apoptotic cells through the vasculature and lymphatics, and ultimately initiating the healing process that culminates in a return to hemostasis.(4, 5, 36)

4.2 Pro-resolving Lipid Mediators Derived from Arachidonic Acids

At the start of an inflammatory response, phospholipids induced by phospholipase enzymes release polyunsaturated fatty acids (PUFA), which include arachidonic acid (AA), and omega-3 PUFAs, eicosapentaenoic acid (EPA) and decosahexaenoic acid (DHA).(5) Lipoxygenases and

cyclooxygenases are involved in a series of pathways initiated by these fatty acids that ultimately produce potent lipid mediators that influence inflammation.(5, 38) Pro-inflammatory leukotrienes (LT) and prostaglandins (PG), formed by AA, trigger the expression of inflammatory enzymes, cytokines, and chemokines that are involved in the initiation and progression of inflammation.(5) Although AA plays a major part in the initiation and progression of inflammation, it is also involved in the biosynthesis of anti-inflammatory and pro-resolving lipid mediators, namely Lipoxins (LX).(5)

First reported in 1984 by Serhan, Hamberg, and Samuelsson, lipoxins are now known to be produced in humans through multiple biosynthetic pathways.(39-41) The oxygenation of AA at 15-carbon (15C) position by the action of 15-lipoxygenase (15-LO) generates 15-hydroxyeicosatetraenoic acid (15-HPETE).(38, 40, 41) Both 15S-HPETE and the reduced alcohol form 15S-HETE can serve as substrates for 5-LO enzyme, which produces a 5S, 6S, 15S-epoxytetraene intermediate.(38, 40) The epoxytetraene intermediate is rapidly hydrolyzed to either 5S, 6R, 15S-trihydroxy-7, 9, 13-trans-11-cis-eicosatetraenoic acid, known as lipoxin A₄ (LXA₄), or 5S, 14R, 15S-trihydroxy-6, 10, 12-trans-8-cis-eicosatetraenoic acid, known as lipoxin B₄ (LXB₄).(38, 40, 41)

A second route of LX biosynthesis involves a leukotriene A₄ (LTA₄)-dependent step that occurs with the interaction of human neutrophils with platelets.(38, 40, 41) In this pathway, AA is converted to LTA₄ by the action of 5-LO.(38, 41) As platelets adhere to neutrophils, platelet 12-LO reduces hydrogen in the 13C position and inserts oxygen in the 15C position within LTA₄.(40, 41) This process converts LTA₄ into a cation intermediate, which can produce LXA₄ when reacted with water at the C6 position, or LXB₄ when reacted with water at the C14 position.(40)

A third pathway that leads to the production of aspirin-triggered lipoxins (ATL) or 15-epi-lipoxins has been discovered.(38) The anti-inflammatory activity of aspirin has long been attributed to its nonspecific inhibition of COX pathway, leading to the inhibition of PG synthesis.(40, 41) COX-2 acetylated by aspirin in endothelial and epithelial cells is still enzymatically active and able to convert AA to 15R-HPETE and 15R-HEPE.(38, 41) Extracellular release of 15-HEPE followed by 5-LO oxygenation, transforms 15-HEPE to 15R-epoxy-tetraene which in turn forms either 15-epi-LXA₄ or 15-epi-LXB₄.(38, 41)

Metabolic inactivation of LX catalyzed by 15-prostaglandin dehydrogenase (15-PGDH) suggested that their beneficial actions may be reduced.(5, 38) Furthermore, common metabolic pathways between LX, PG, and LT may suggest other mechanisms of inactivation and clearance of LX.(38) This led to the design and construction of stable LX analogs that retain the biological actions and potency of LX, with the ability to resist rapid metabolic inactivation.(5, 10, 38, 40) These analogs allowed for further investigations on the actions of LX, and lead to the development of LX-based compounds suitable for clinical trials.(5, 9)

The unique actions of LX, ATL, and their analogs influence many cell types, and have been demonstrated to play an important anti-inflammatory and pro-resolving role.(40) These actions include: Inhibition PMN infiltration and adhesion at inflammation sites, stimulation of apoptotic PMN clearance by macrophages, influencing the expression of cytokines and chemokines, and regulation of leukocyte traffic.(4, 40) In-vitro and in-vivo experimental models have produced promising evidence demonstrating the anti-inflammatory and pro-resolving actions of LX, ATL, and their analogs with inflammatory diseases and conditions such as: Angiogenesis, aseptic loosening, asthma, cystic fibrosis, periodontal disease, peritonitis, and renal disease.(6, 40, 42)

4.3 Lipoxins and bone

The treatment of osteoclast precursor cells with LXA₄ significantly downregulated the expression level of osteoclastogenesis marker genes in-vitro.(7) In addition, tartrate-resistant acid phosphatase (TRAP) enzymatic activity, often used as a marker for osteoclast activity, was reduced in a dose-dependent manner after treatment with LXA₄.(8) Ovariectomized mice simulating post-menopausal women have significant trabecular bone loss.(8) However, when these mice are treated with LXA₄ they had a visible reduction in bone loss.(8)

LXA₄ can inhibit multiple signaling pathways that impact osteoclastogenesis including nuclear factor- κ B (NF- κ B).(7, 8) This suppresses the expression of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α necessary for the regulation of osteoclastogenesis.(7, 8) Furthermore, LXA₄ can decrease the RANK/OPG ratio which is a key regulator in osteoclast formation.(8)

4.4 Lipoxins and periodontal disease

When it comes to periodontal disease, a study by Serhan et al demonstrated that transgenic rabbits overexpressing 15-LO had a marked decrease in PMN infiltration, inflammation, and associated bone loss compared to non-transgenic rabbits.(43) Moreover, transgenic rabbits had increased levels of endogenous LXA₄, which can activate anti-inflammatory responses.(43) Another study by Pouliot et al demonstrated that stable analogs of LX and of ATL were able to block *P. gingivalis* induced PMN infiltration and reduced the levels of PGE₂ in exudates.(44) Van Dyke et al constructed a nano-proresolving medicine (NPRM) that contains a stable LXA₄ analog, benzo-lipoxin A₄ (bLXA₄) and were able to demonstrate, in a large animal model, a dramatic decrease in inflammatory cells in chronic periodontitis sites and striking increase in new

periodontal bone formation and regeneration.(9) Currently, a bLXA₄ mouth rinse is under investigation for its efficacy with gingival inflammation in a randomized controlled clinical trial.(45)

4.5 Hydrogels

Hydrogels are biocompatible material that have various applications in tissue engineering and regeneration, they can be used alone or in conjunction with other materials, proteins, and cells.(46-52) Hydrogels hold the potential to modulate the environment and reestablish the structure within cavitated lesions.(48) In injectable form, hydrogels can be introduced into the lesion with little disruption to the surrounding tissues and can optimally adapt to the shape of the cavity.(48) Hydrogels can also serve as biodegradable drug delivery systems.(46, 53, 54) In dentistry, hydrogels have been investigated for use in periodontal therapy, and bone healing/regeneration.(50, 51, 54-56)

Lipoxins have been incorporated in hydrogels for potential use in periodontal therapy.(50, 51, 57) These injectable hydrogels demonstrated sustained release of lipoxins over time while maintaining biological activity.(57-59) Furthermore, the hydrogels demonstrated cytocompatibility in-vitro, an no inflammatory responses in-vivo.(57-59)

4.6 Gelatin-hydroxyphenyl propionic acid hydrogel

A gelatin-hydroxyphenyl propionic acid (Gtn-HPA) based hydrogel is formed by the oxidative coupling of the phenol moieties catalyzed by hydrogen peroxide (H₂O₂) and horseradish peroxidase (HRP).(48, 50) This biopolymer can be fine-tuned by controlling the degree of cross-linking and gelation, which ultimately affects its physical properties and degradation rate, in

addition to influencing cell migration and proliferation.(48-50) Various cells, growth factors, and peptides have been incorporated in the Gtn-HPA hydrogel for the treatment of different conditions.(52, 60, 61) Dromel et al, incorporated human epidermal growth factor (hEGF) in the Gtn-HPA hydrogel and verified sustained release over time.(60) Alshihri et al, demonstrated the beneficial effects of platelet-derived growth factor (PDGF)-BB incorporated into and released from the Gtn-HPA hydrogel on bone marrow-derived mesenchymal stem cells (BM-MSC)s proliferation, migration, and differentiation.(61) In-vitro studies by Lim et al on the use of Gtn-HPA hydrogel for brain lesions showed promising results, and demonstrated that the hydrogel can act as a scaffold with compatible structural support, and provided sustained release of incorporated proteins, and influenced stem cell differentiation.(48, 49) Furthermore, Wang et al demonstrated that varying the degree of stiffness of the Gtn-HPA hydrogel can influence stem cell differentiation into specific lineages.(50)

Gtn-HPA hydrogel may have prospective uses with inflammation-mediated bone lesions. Due to its cross-linking capacity, this hydrogel has the potential for providing a maintainable scaffold that can conform to the shape of the lesion without the risk of deformation, wash out, or dislodgement while promoting cell migration and proliferation. Furthermore, Gtn-HPA hydrogel scaffolds can provide sustained release of regenerative or anti-inflammatory substances to resolve any ongoing inflammatory processes.

5. Research Strategy

Gel Preparation

- Lyophilized Gelatin-hydroxyphenylpropionic acid (Gtn-HPA) polymer was dissolved in PBS at either: 2 wt.% or 8 wt.%.
- Cross-linking was done using enzyme-catalyzed oxidation, as previously described (50, 51) The 2 wt.% gel was cross-linked using 0.1U/ml horseradish peroxidase (HRP) as a catalyzer and 1.2mM H₂O₂ as a crosslinker. While the 8 wt.% gel was cross-linked using 0.1U/ml HRP and 6.8mM H₂O₂.

Lipoxin

- Aspirin-triggered lipoxin A4 (ATL A4) dissolved in ethanol at a concentration of 100 µg/ml.
- ATL A4 was further diluted in ethanol to 25 µg/ml.

Sample size

The objective was to determine as significant a difference between the means of the test and control values of at least 30%. Assuming that there would be a 15% standard deviation in the mean values for each group and considering that the goal is to have alpha=0.05 and beta=0.20 (i.e., a power of 80%), it would be necessary to have $n=6$.

5.1 Specific Aim 1: To determine *in vitro* the impact of lipoxin incorporation on the gelatin matrix gelation rate.

Experimental design:

Tube Tilting Method (56, 62, 63)

- 200 μl of hydrogel was cross-linked in Eppendorf tubes.
- Aspirin-triggered lipoxin (ATL) A₄ [15-epi-LXA₄] added to each wt.% solution at either 50ng [=2.84 μM] or 500ng [=28.4 μM] concentration prior to adding the HRP and H₂O₂ for hydrogel cross-linking.
- After uniformly mixing the solutions using pipette, gelation was monitored by tilting the tube to observe the flow of the solution (Figure 1).
- Gelation time was recorded (in seconds) from the moment of mixing to the point at which the solution does not tilt or flow with the tube.
- Gelation of hydrogels containing ATL A₄ was compared to lipoxin-free hydrogels
- Each group contained $n=6$ samples

Statistical Analysis

- All data were reported as mean \pm standard error mean and analyzed using two-way analysis of variance (ANOVA).
- If statistical significance was found when the degrees of freedom are greater than one, the Tukey HSD test was used to determine the statistically significant differences between the compared pairs (overall $\alpha = 0.05$).
- Statistical software (JMP 16.0.0) was used for the analysis.

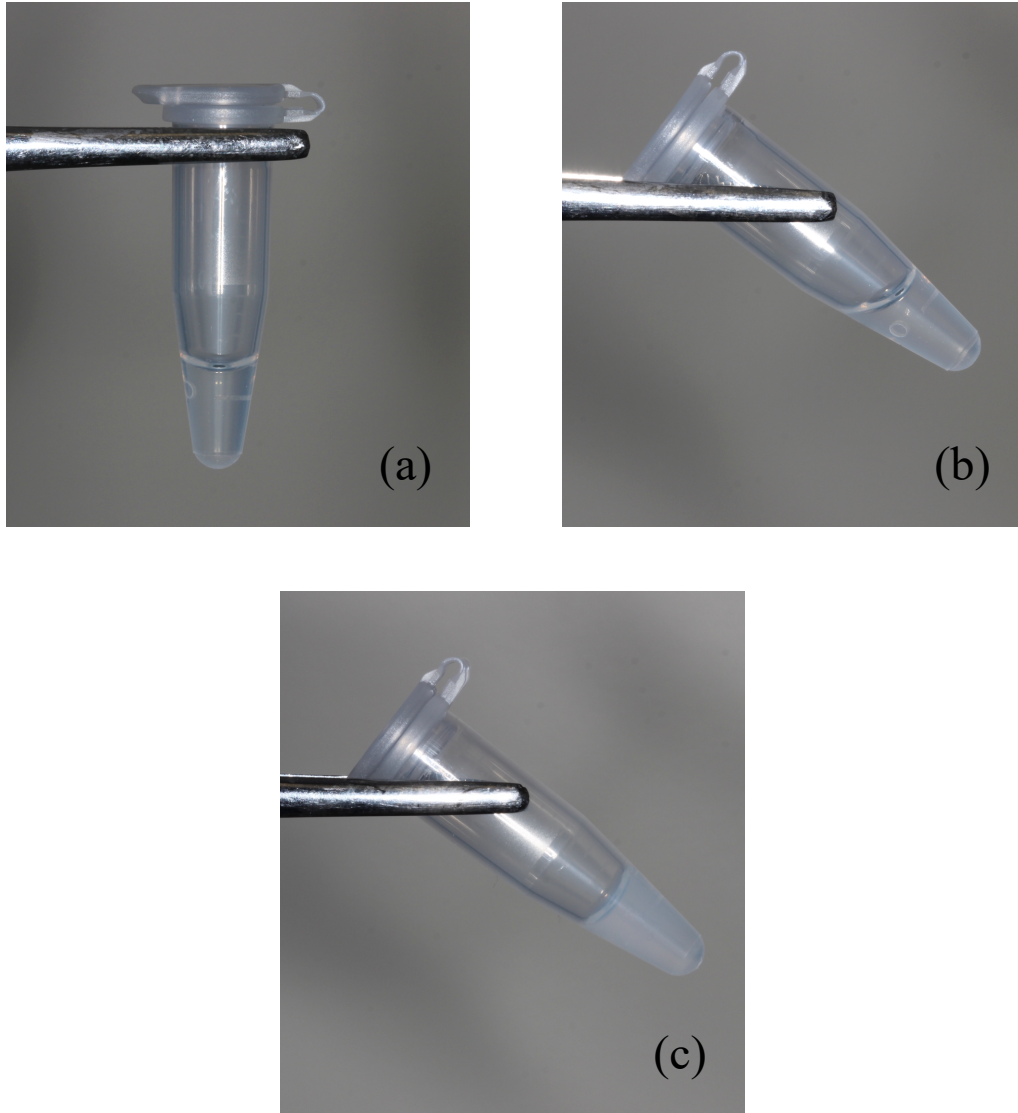


Figure 1 – (a) Gtn-HPA conjugate dissolved in PBS prior to addition of HRP and H_2O_2 . (b) Tilted tube demonstrating flow of Gtn-HPA solution. (c) Tilted tube after gelation demonstrating lack of flow of Gtn-HPA solution.

5.2 Specific Aim 2: To determine in vitro the rate of lipoxin release from the gelatin matrix.

Experimental design:

In Vitro ATL A₄ Release

(All samples of hydrogel containing ATL A₄ from Specific Aim 1 were used)

- 200 µl of hydrogel was cross-linked in Eppendorf tubes.
- Aspirin-triggered lipoxin (ATL) A₄ [15-epi-LXA₄] added to each wt.% solution at either 50ng [=2.84 µM] or 500ng [=28.4 µM] concentration prior to adding the HRP and H₂O₂ for hydrogel cross-linking.
- 1 ml of PBS added to each tube and incubated at 37°C on an orbital shaker at 100 rpm.
- 500 ml PBS was withdrawn for evaluation of ATL release from the Gtn-HPA gel at the end of days 1, 4, 7, and 14. The withdrawn PBS was replaced with fresh PBS.
- The amount of ATL A₄ released in the solution was measured using ELISA.

Statistical Analysis

- All data were reported as mean ± standard error mean and analyzed using two-way analysis of variance (ANOVA).
- If statistical significance was found when the degrees of freedom are greater than one, the Tukey HSD test was used to determine the statistically significant differences between the compared pairs (overall $\alpha = 0.05$).
- Statistical software (JMP 16.0.0) was used for the analysis.

5.3 Specific Aim 3: To determine *in vitro*, using a neonatal rat calvarial organ culture, the impact of the lipoxin containing gelatin matrix on inflammation-mediated bone resorption.

Experimental design:

Calvarial bone organ culture (64-71)

- 1-day old Sprague-Dawley rat calvaria.
- Using scissors, the scalps were excised exposing the calvaria.
- Calvaria were sectioned as seen in Figure 2.



Figure 2 – (a) Diagram showing cuts for hemi-calvaria preparation. (b) Live sample of prepared hemi-calvarium.

- Each hemi-calvarium was cultured in a separate well of a 24-well plate with 2 ml culture medium.

- Stainless steel mesh was used to fabricate grids to support the hemi-calvaria at the air/liquid interface (Figure 3, 4).

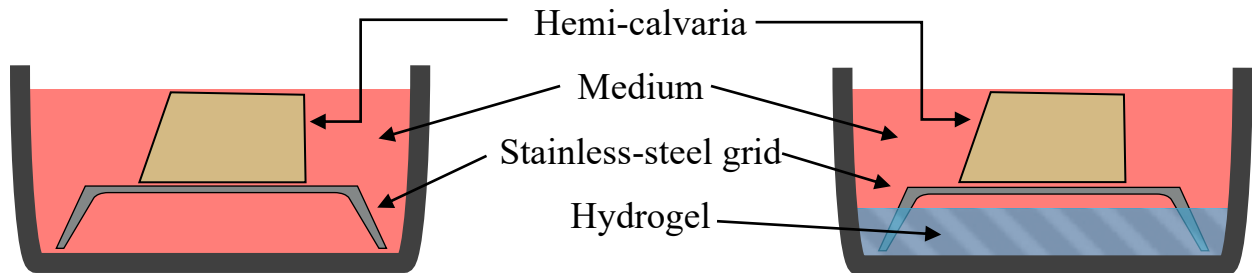


Figure 3 - Diagram of hemi-calvaria incubated in medium on stainless-steel grids with hydrogel (R) and without (L).

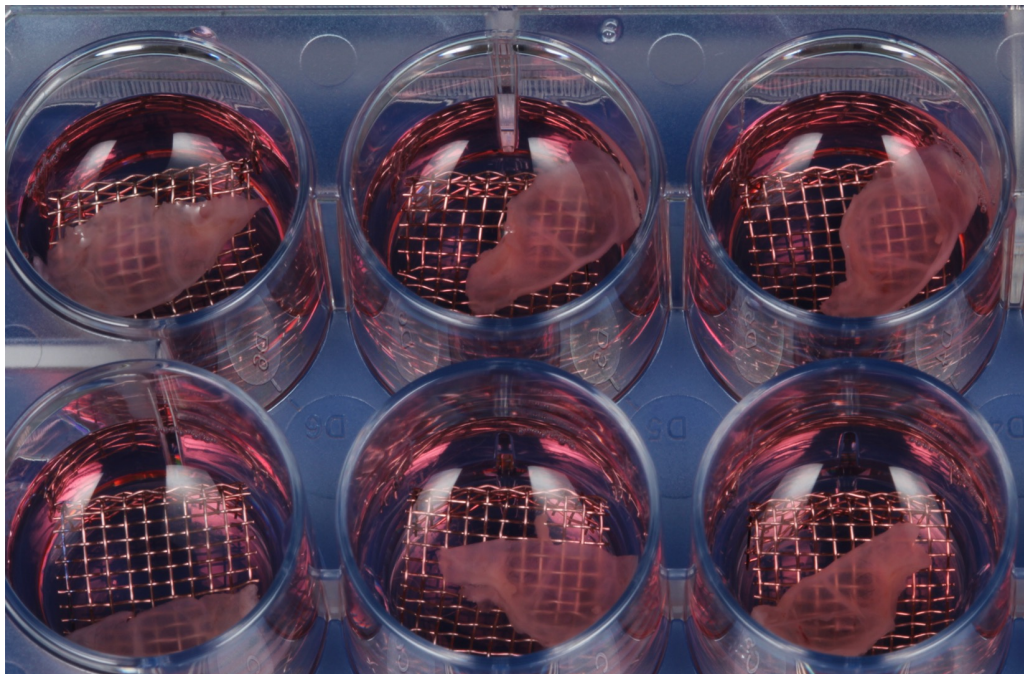


Figure 4 – Cultured hemi-calvaria samples

- Bone organ culture medium: DMEM supplemented with 5 mg/ml bovine serum albumin (BSA), fraction V (5 mg/ml, Sigma Co.), 100 U/ml Penicillin, and 100 µg/ml Streptomycin.
- Resorption medium: To stimulate bone resorption 10 ng/ml IL-1β and 10 ng/ml TNF-α were added to the bone organ culture medium
- Cultured hemi-calvaria were pre-incubated in a humidified atmosphere of 5% CO₂ at 37°C for 24 hours.
- Hemi-calvaria were then divided into the following groups, with 6 calvaria per group:
 1. Negative control - Bone organ culture medium only.
 2. Positive control – Resorption medium only.
 3. Vehicle – Resorption medium + 2% Gtn-HPA
 4. Lipoxin 1 – Resorption medium + 50 ng exogenous ATL A₄ (added directly to medium)
 5. Lipoxin 2 – Resorption medium + 500 ng exogenous ATL A₄ (added directly to medium)
 6. Treatment 1 – Resorption medium + 2% Gtn-HPA containing 50 ng ATL A₄
 7. Treatment 2 – Resorption medium + 2% Gtn-HPA containing 500 ng ATL A₄
- All samples containing hydrogel will have the gel cross-linked at base of culture dish.
- Assay duration: 7-days (Figure 5).
- Medium was collected on days 3, 5, and 7, and stored in -20^o for analysis.

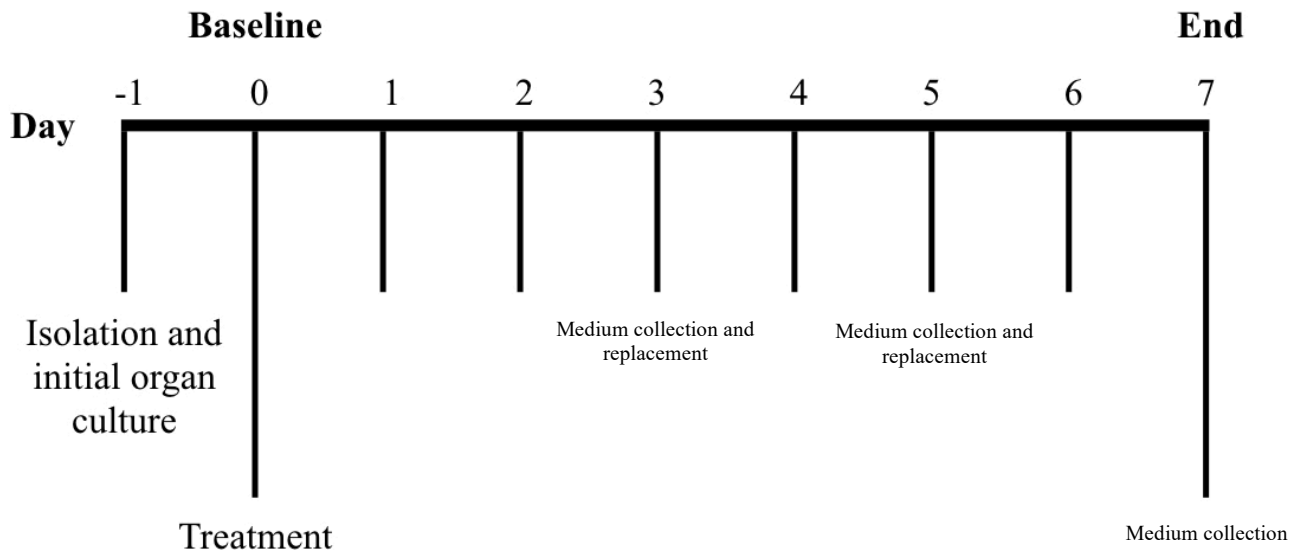


Figure 5 – Assay timeline.

Global Visualization of Osteoclastic Activity (67-70)

- Neutral red (NR) is a vital stain used for global observation of multinucleated osteoclasts (MN-OSC) under light microscope.
- Hemi-calvaria from each group were incubated for 45 minutes at 37^o in culture medium with 70µg/ml neutral red (NR) stain.
- Samples were visualized under light microscopy.

Global Histomorphometric Analysis (67-70)

- Silver nitrate stain is used to reveal gross mineral loss and areas of bone resorption.
- After NR staining, same 4 samples were fixed in 10% formalin overnight.
- Samples were counterstained with 2% silver nitrate for 20 minutes.
- Samples were visualized under low magnification light microscopy.

- Digital images will be converted to grayscale (4 distinct values: black and dark grey = no resorption, light gray and white = resorption) (Figure 6).
- Resorption area-to-total area (RA/TA) ratio was calculated.

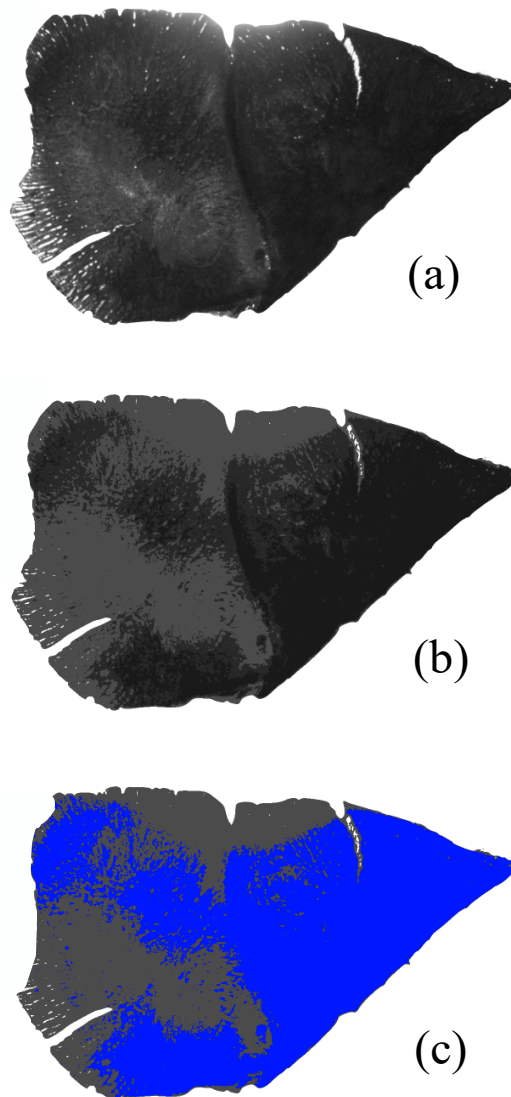


Figure 6 – (a) Grayscale image of hemi-calvarium. (b) 4 distinct values defined (black and dark grey = no resorption, light gray and white = resorption). (c) Calculation of RA/TA ratio.

Calcium Release Assay

- Used media were stored at -20 for calcium analysis.
- A colorimetric calcium assay was used according to manufacturer instructions to assess the amount of calcium released from the hemi-calvaria into the medium.

Statistical Analysis

- All data from the silver nitrate staining and calcium release assay were reported as mean \pm standard error mean.
- Considering the number of variables involved in the groups (type of medium, presence of ATL A₄, and presence of Gtn-HPA hydrogel) multiple t-tests were used to compare the means of different pairs of groups (overall $\alpha = 0.05$).
- The negative control group (Group 1) was compared to the positive control (Group 2), the positive control group (Group 2) was compared to the vehicle group (Group 3), the positive control group (Group 2) was compared to the exogenous lipoxin groups (Groups 4, 5), and the lipoxin loaded hydrogel groups (Group 6, 7) were compared to the vehicle group (Group 3).
- Statistical software (JMP 16.0.0) was used for the analysis.

Effects of Gtn-HPA Hydrogel on Medium Calcium Concentration

- 2 wt.% Gtn-HPA was cross-linked at the base of culture dish as describe in the earlier aims.
- 2 ml resorption medium added to culture dish and replaced according to Aim 3 protocol: day 3, 5, and 7

- After day 7, 1 ml of dH₂O was added to culture dish. The dish was placed on orbital shaker for 30 minutes.
- After 30 minutes dH₂O was collected and another 1 ml of dH₂O was added. This process was repeated four times.
- After the final repetition, the culture dish was incubated at 37° overnight.
- dH₂O was collected after each wash.
- Calcium in the medium and dH₂O was measured using the same Calcium Assay used in Aim 3.
- Samples of stock resorption medium were used as control.

6. Results

6.1 Specific Aim 1:

Data on gelation rate for each group is presented in Figure 7. ANOVA revealed an interaction between ATL A₄ dose and gel wt.% ($p < .0001$). The main effect of ATL A₄ dose was significant ($p < .0001$), but the main effect of gel wt.% was not significant ($p = 0.31$).

The addition of 50 ng did not seem to have a significant effect on gelation rate ($p = 0.72$). The addition of 500 ng ATL A₄ resulted in a significant increase in gelation time ($p < .0001$). Furthermore, when looking at the impact of adding 500 ng of ATL A₄ on each gel wt.% we can see that the 8wt.% gel had a 109% increase in gelation time whereas the 2wt.% gel had a 51% increase in gelation time, the difference was statistically significant ($p < .0001$).

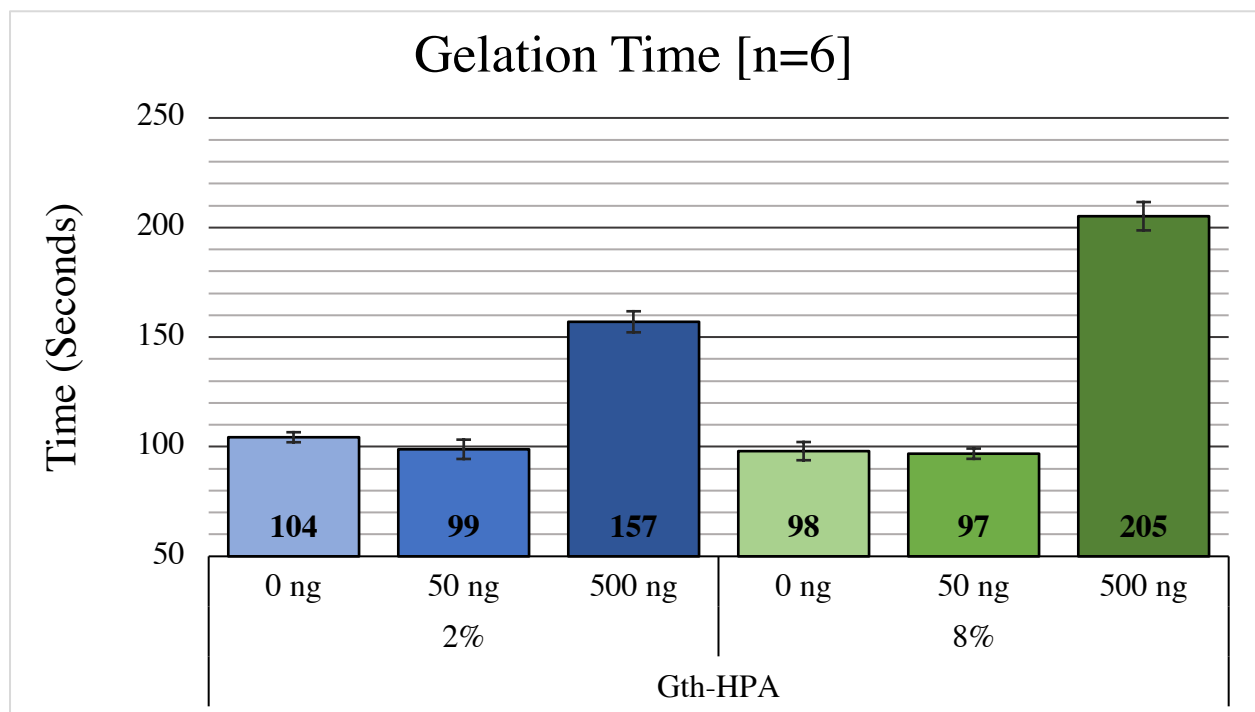


Figure 7 – Gelation rate (in seconds) for each gel wt.% with different ATL A₄ doses. Mean value reported at the base of each bar. Error bars = \pm standard error mean.

6.2 Specific Aim 2:

The release profile of ATL A₄ from the Gtn-HPA hydrogel over the 14-day period is demonstrated in Figure 8. As expected with hydrogels, majority of the release occurred in the first 24 hours. The main effect for gel wt.% had a significant effect on the release rate (P=.0008). The 2wt.% released approximately 25% of the initial ATL A₄ content while the 8wt.% gel released 7% of the initial ATL A₄ content over 14 days. The main effect for ATL A₄ dose had no significant effect on the release rate (p=0.89), nor was there a significant interaction between gel wt.% and ATL A₄ dose (p=0.92). Although the overall percentage of ATL A₄ released from the gel varied depending on the gel wt.%, the percentage of ATL A₄ released remained constant regardless of the initial ATL A₄ dose. Table 1 presents the specific amount of released ATL A₄ (in nanograms) at the different timepoints for all groups.

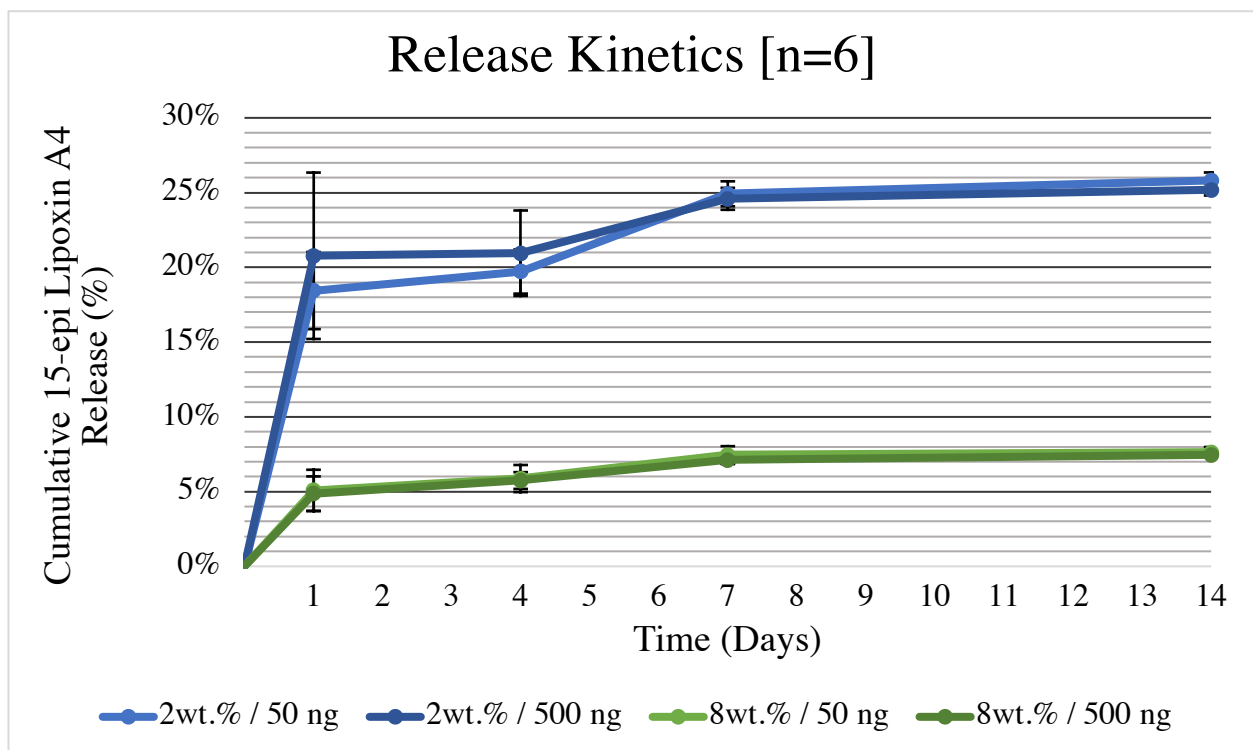


Figure 8 – Sustained release of ATL A₄ from Gtn-HPA hydrogel over 14 days. Error bars = \pm standard error mean.

	2% Gtn-HPA		8% Gtn-HPA	
	50 ng	500 ng	50 ng	500 ng
Day 1	9.22 ng	103.9 ng	2.54 ng	24.32 ng
Day 4	9.86 ng	104.7 ng	2.94 ng	28.69 ng
Day 7	12.46 ng	122.9 ng	3.73 ng	35.56 ng
Day 14	12.91 ng	125.9 ng	3.82 ng	37.28 ng

Table 1 – Cumulative amount of ATL A₄ released (in nanograms) at each specified timepoint for each group over the 14-day period.

6.3 Specific Aim 3:

Global Visualization of Osteoclastic Activity

After NR staining, MN-OSCs were absent in the parietal region of the unstimulated hemi-calvaria (negative control) (Figure 9; Panel 1a). In contrast, MN-OSCs were visible when the hemi-calvaria were stimulated (positive control) (Figure 9; Panel 1b). The Gtn-HPA matrix did not seem to have an effect on MN-OSCs as they were still visible even in the presence of the blank hydrogel (vehicle group) (Figure 9; Panel 1c).

When the hemi-calvaria were treated with exogenous ATL A₄ either dispensed directly in the medium or released from the hydrogel, there seemed to be no evidence of MN-OSCs (Figure 9; Panel 1d-g).

Global Histomorphometric Analysis

The mean RA/TA ratios for each group are presented in Figure 10. When comparing unstimulated hemi-calvaria (negative control) to stimulated hemi-calvaria (positive control) the mean RA/TA ratios were significantly different ($p=0.0002$) (Figure 9; Panel 2a, b). In the presence of the blank hydrogel (vehicle), the mean RA/TA was not different from that of the positive control ($p=0.67$) (Figure 9; Panel 2c). ATL A₄ dose did not have an impact on the outcome regardless of whether it was added directly to the medium or released from the hydrogel. This was noted by the lack of statistically significant differences between groups 4 and 5 ($p=0.75$) and groups 6 and 7 ($p=0.73$). As such, the data from groups 4 and 5 were pooled together and the data from groups 6 and 7 were also pooled together for the statistical analysis. The addition of exogenous ATL A₄ directly to the medium resulted in a 17.5% decrease in mean RA/TA ratio compared to the positive control, which was statistically significant ($p=0.0093$) (Figure 9; Panel 2d, e) (Figure 11). ATL A₄ released from

the hydrogel also resulted in a 23.1% decrease in mean RA/TA ratio compared to the blank hydrogel (vehicle), which was statistically significant ($p=0.039$) (Figure 9; Panel 2f, g) (Figure 12).

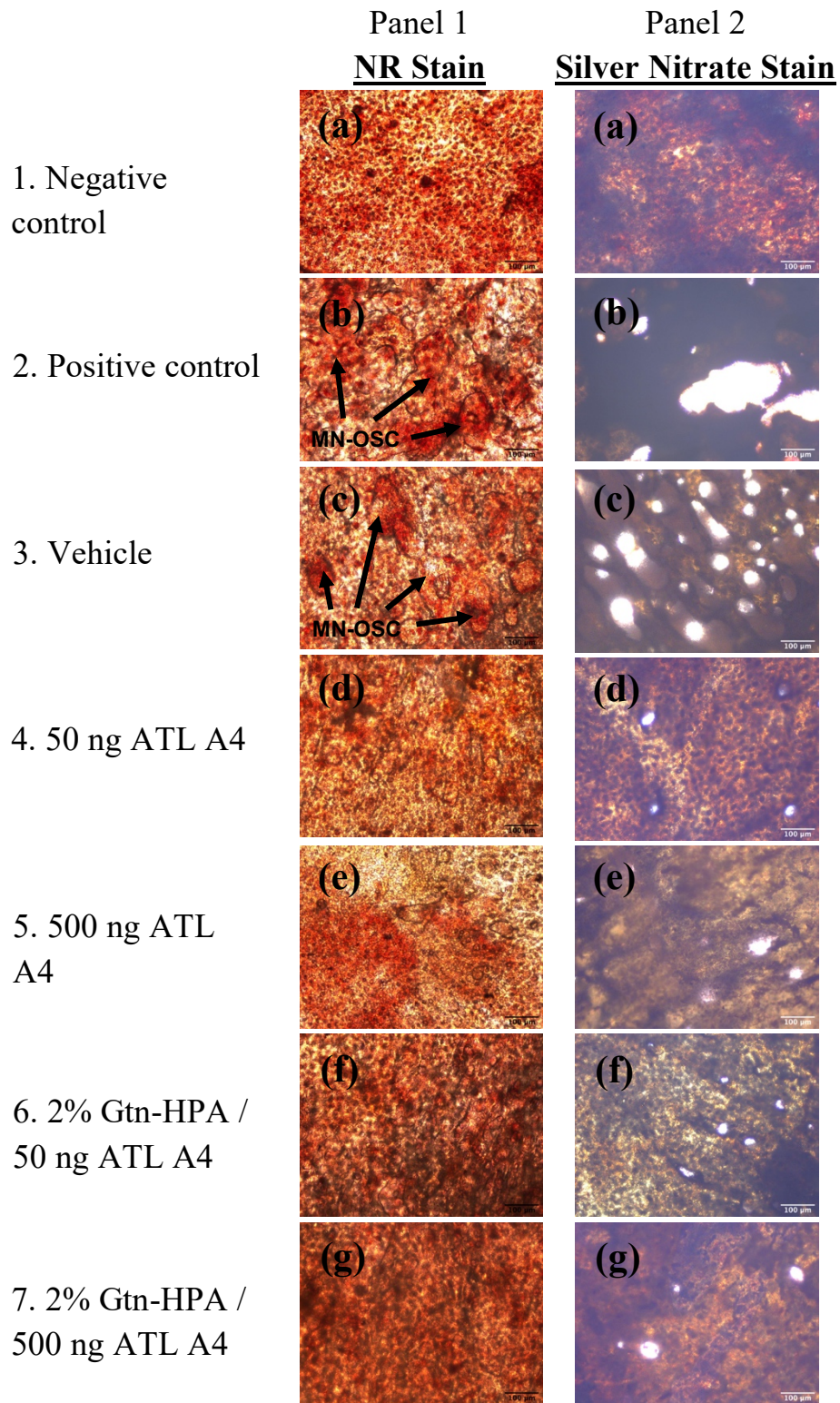


Figure 9 – Sample hemi-calvaria from each group. Panel 1 shows NR stain, and panel 2 shows silver nitrate stain. Scale bar 100 μ m

Silver Nitrate Staining [n=6]

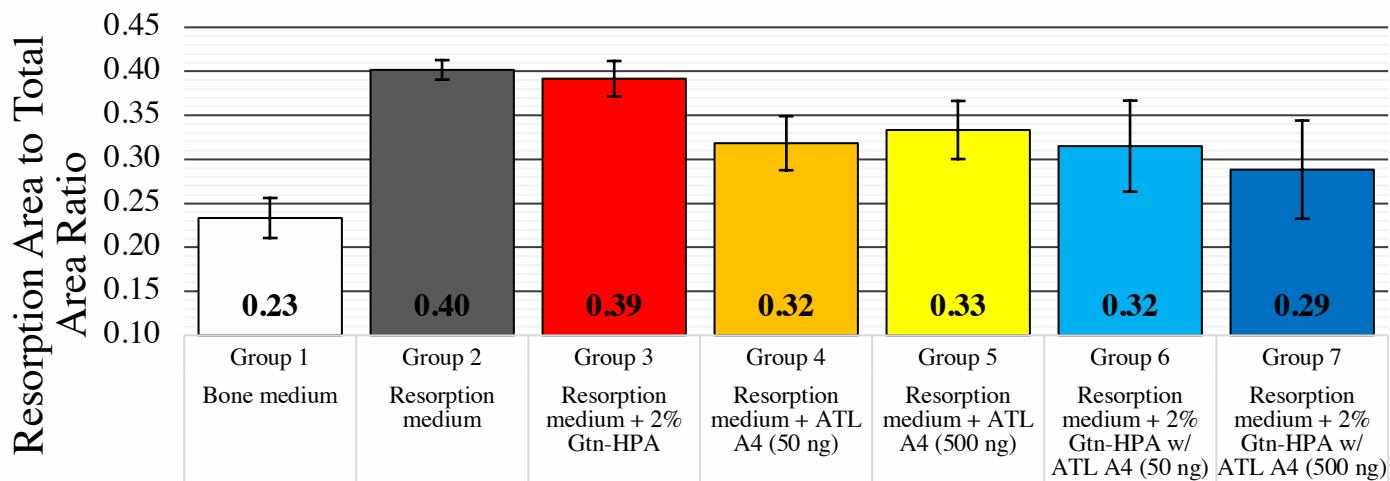


Figure 10 – Mean RA/TA ratio for each group. Error bars = \pm standard error mean.

Silver Nitrate Staining

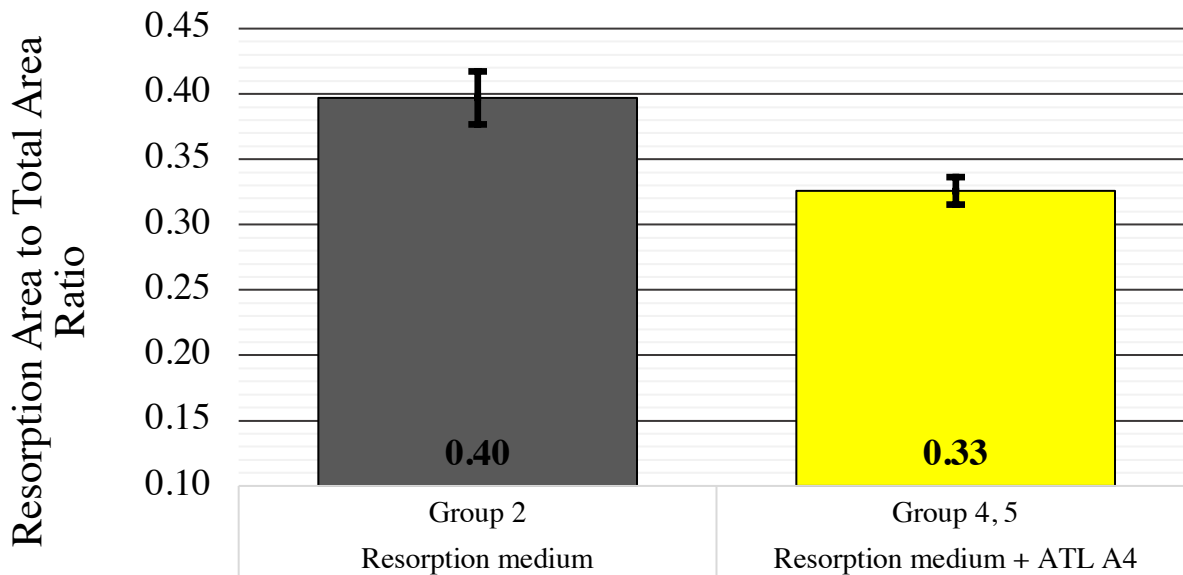


Figure 11 – Mean RA/TA ratio for positive control group and exogenous Lipoxin group. Error bars = \pm standard error mean.

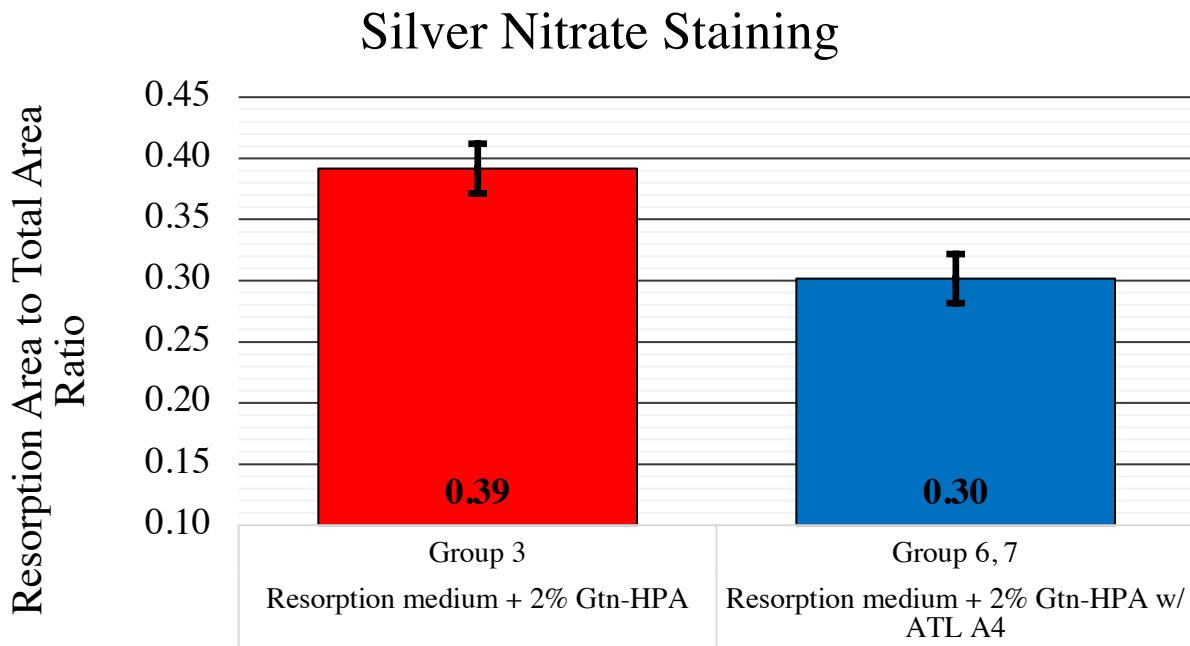


Figure 12 – Mean RA/TA ratio for blank Gtn-HPA (vehicle) group and Lipoxin loaded Gtn-HPA. Error bars = \pm standard error mean.

Calcium Release Assay

The mean medium calcium concentration for each group is presented in Figure 13. When comparing unstimulated hemi-calvaria (negative control) to stimulated hemi-calvaria (positive control) the mean calcium concentration was significantly different ($p=0.0009$). In the presence of the blank hydrogel (vehicle), the mean calcium concentration was reduced by 30.4% compared to the positive control, which was statistically significant ($p=0.0039$). ATL A₄ dose did not have an impact on the outcome regardless of whether it was added directly to the medium or released from the hydrogel. This was noted by the lack of statistically significant differences between groups 4 and 5 ($p=0.91$) and groups 6 and 7 ($p=0.99$). As such, the data from groups 4 and 5 were pooled

together and the data from groups 6 and 7 were also pooled together for the statistical analysis. The addition of exogenous ATL A₄ directly to the medium resulted in a 29.4% decrease in mean calcium concentration compared to the positive control, which was statistically significant (p=0.0091) (Figure 14). ATL A₄ released from the hydrogel resulted in a 29.6% decrease in mean calcium concentration compared to the blank hydrogel, which was statistically significant (p=0.031) (Figure 15).

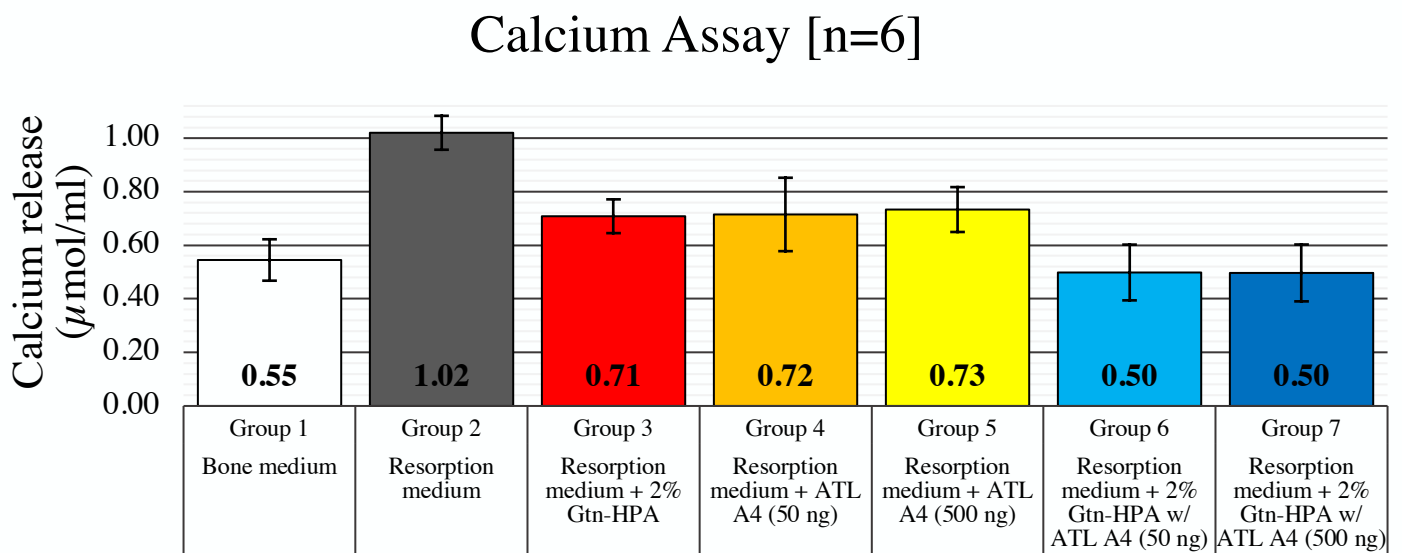


Figure 13 – Mean calcium concentration (in µmol/ml) for each group. Error bars = ± standard error mean.

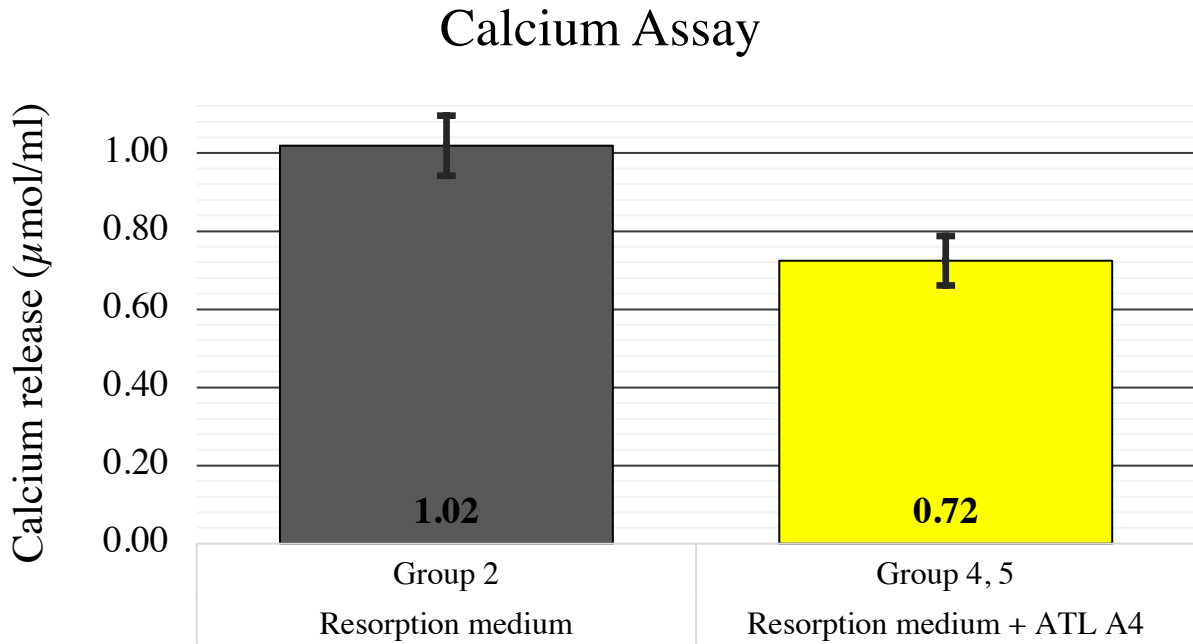


Figure 14 – Mean calcium concentration (in $\mu\text{mol/ml}$) for positive control group and exogenous Lipoxin group. Error bars = \pm standard error mean.

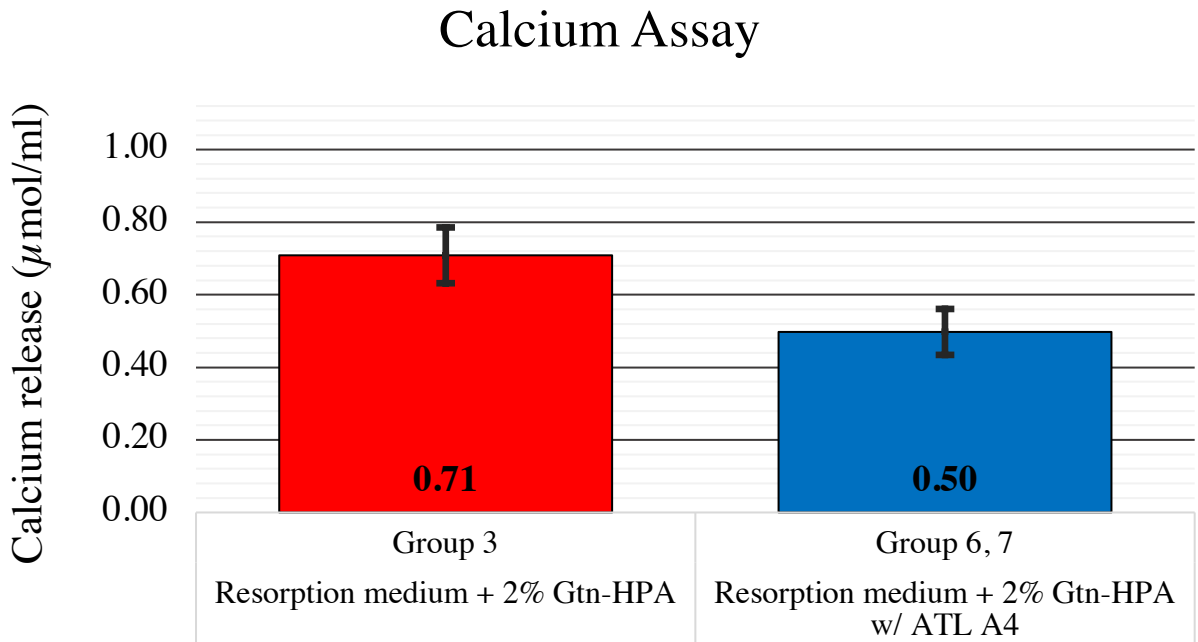


Figure 15 – Mean calcium concentration (in $\mu\text{mol/ml}$) for blank Gtn-HPA (vehicle) group and Lipoxin loaded Gtn-HPA. Error bars = \pm standard error mean.

Effects of Gtn-HPA Hydrogel on Medium Calcium Concentration

After the 7-day culture period, it was noted that the Gtn-HPA had changed color from transparent to slightly pink as the medium was absorbed in the hydrogel. Medium incubated with Gtn-HPA hydrogel had a lower calcium concentration compared to control medium as measured using the calcium assay. The Gtn-HPA hydrogel lost the pink tint after washing with distilled water, indicating that the medium absorbed was removed from the hydrogel. After measuring the calcium content of the wash, it was observed that the calcium concentration in the incubated medium combined with the calcium concentration of the wash was comparable to the control medium calcium concentration (Figure 16).

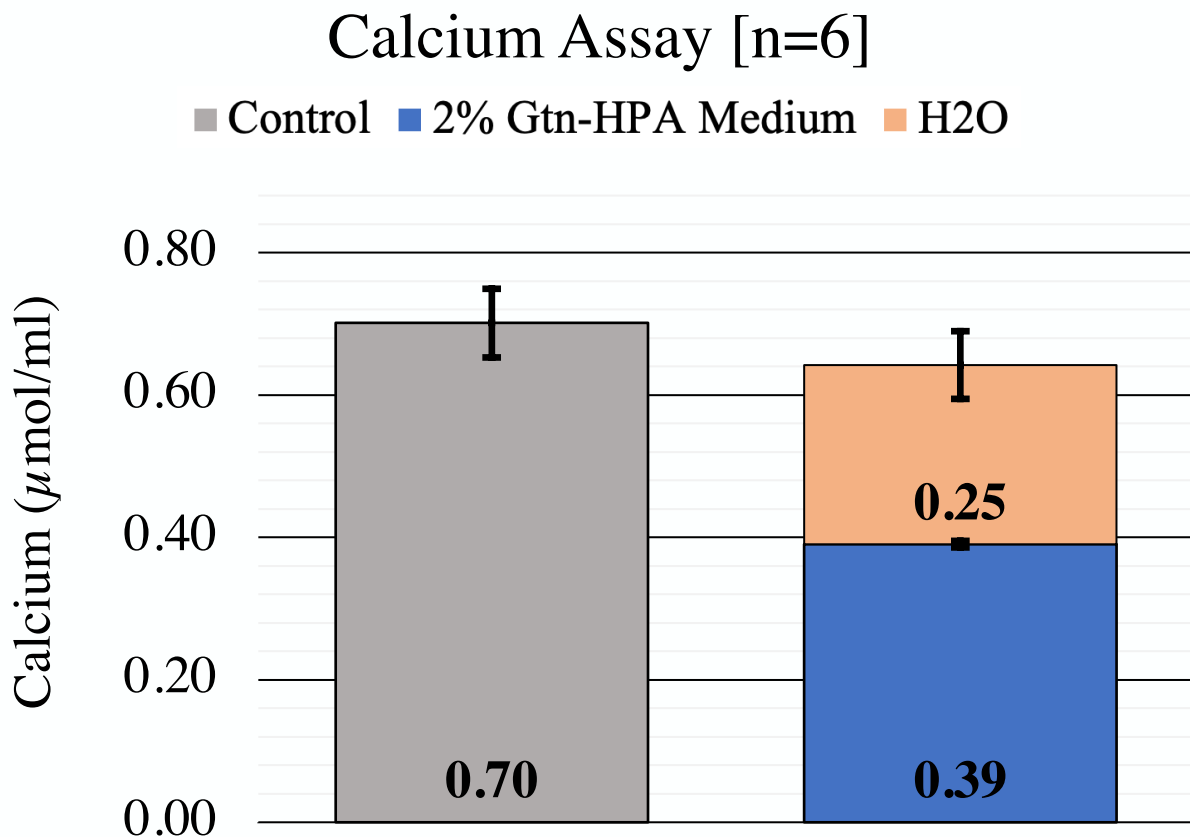


Figure 16 – Mean calcium concentration (in $\mu\text{mol/ml}$) for control medium, incubated medium, and H₂O wash. Error bars = \pm standard error mean.

7. Discussion

The data regarding the gelation rate shows that the addition of 500 ng of ATL A₄ to the Gtn-HPA matrix can significantly increase the gelation time. Furthermore, there was a significant interaction between the gel wt.% and the amount of added lipoxins. This led to a more pronounced increase in gelation time for the 8wt.% gel compared to the 2wt.% gel.

The Gtn-HPA hydrogel gelation process is controlled by the HRP independently from H₂O₂, with an inversely proportional relationship between gelation rate and HRP concentration.(51, 53) Nevertheless, a rise in gelation rate could occur due to HRP deactivation by excess H₂O₂.(50, 51, 53) This may not be the case in this study, as the higher amounts of H₂O₂ used to cross-link the 8wt.% gel did not appear to have an impact on gelation rate without the addition of 500 ng of ATL A₄. Previous studies have reported that enzyme activity may be affected by organic solvents.(72) Since ATL A₄ was supplied by the manufacturer dissolved in ethanol, there was concern that the ethanol would interfere with the action of HRP. However, it seems that methanol might have a more pronounced effect on HRP activity compared to ethanol.(73)

Precise rheological measurements including the storage modulus and gel point could be obtained using a rheometer.(48, 50, 53) Although this information is useful to understand the viscoelastic behavior the hydrogel, a more basic visual approach was preferred as it could more accurately resemble the clinical handling of the Gtn-HPA matrix.

The diffusive release of drugs from hydrogels is largely dependent on mesh size.(46, 47) Larger meshes contain more spaces between the cross-linked polymer networks which allow rapid diffusion of the incorporated drugs.(46, 47) The cross-link density of Gtn-HPA can be increased by using higher concentrations of Gtn-HPA conjugate and H₂O₂.(50, 51) This slows down the degradation of the hydrogel and the diffusive release of the drugs.(46, 47, 50, 51)

The high amount of water in hydrogels often results in early burst release of drugs over a few hours or days.(47, 60) A recent study by Dromel *et al* demonstrated that hEGF can be incorporated into and subsequently released from different hydrogels including Gtn-HPA, hyaluronic acid-tyramine (HA-TYR) hydrogel, and copolymeric networks of both for potential use with retinal disease.(60) Varying the copolymeric network affected the free water content and stiffness of the hydrogel which, in turn, impacted the diffusion rate suggesting that controlled release could be optimized without compromising physical properties.(60)

Our results agree with previous studies that report higher initial release, as the highest amount of ATL A₄ released occurred during the first 24 hours. Furthermore, the less dense 2wt.% gel had a significantly higher release rate of incorporated ATL A₄ compared to the denser 8wt.% gel. The percentage of ATL A₄ released from each wt.% gel was not significantly different regardless of the initial ATL A₄ concentrations. Given that a consistent percentage of the added ATL A₄ diffuses out of the hydrogel, the added amount of ATL A₄ could be determined by the intended application. In other words, if the inflammation is extensive and a more potent anti-inflammatory action is needed a higher dose of lipoxin could be incorporated in the gel, and vice versa. A limitation to this release kinetics assay is that it does not take into account the degradation of the Gtn-HPA matrix. As the hydrogel network degrades the mesh size increases, allowing additional drug diffusion out of the hydrogel.(46) Factoring in the hydrogel degradation may result in a different ATL A₄ release profile.

A major advantage to bone organ cultures is that they maintain bone cells in their natural extracellular matrix which maintains the cellular composition and arrangement, and the native tissue architecture.(71) This can be advantageous when investigating the biological processes and interactions of bone cells and tissue.(71) In our study, MN-OSCs were clearly visible in the

positive control samples but were absent in the negative control samples. Furthermore, significant differences were found between the negative control and positive control samples in both RA/TA ratio and medium calcium concentration, which validates the bone resorption organ culture model.

Lipoxins have an inhibitory effect on osteoclastogenesis and osteoclast activity in-vitro as demonstrated by Ali et al and Liu et al.(7, 8) The addition of exogenous lipoxins to the medium resulted in the absence of observable MN-OSCs and a significant reduction in both RA/TA ratio and medium calcium concentration compared to the positive control samples, which confirms that lipoxins are capable of reducing inflammation-mediated bone resorption. In a similar organ culture model, Li et al demonstrated that lipoxins are able to reduce bone resorption especially with higher doses.(6) We were unable to find significant differences between 50 ng and 500 ng of ATL A₄ and this can be due to the short-acting nature of lipoxins as they are rapidly rendered inactive by dehydrogenation.(6) Due to the differences in SPMs and animal models, the dosage of SPMs vary a lot in the literature with little details on the rationale behind the dose.(10)

The addition of the blank Gtn-HPA hydrogel to the organ culture did not have a significant effect on the RA/TA ratio compared to the positive controls, MN-OSCs were also visible. However, the hydrogel significantly reduced the medium calcium concentration. When lipoxins were incorporated into the hydrogel the MN-OSCs were not visible and the mean RA/TA ratio was significantly reduced compared to the positive control samples. Although the blank hydrogel significantly reduced the medium calcium concentration compared to the positive control, the incorporation of lipoxins lead to further reduction in mean calcium concentration compared to the blank hydrogel. It is worth noting that the single dose of ATL A₄ incorporated into the Gtn-HPA hydrogel and slowly released over time had similar effects to multiple doses added directly to the

medium. Taken together these results confirm the Gtn-HPA hydrogel as a potential carrier for the local delivery and release of lipoxins.

As noted earlier, the presence of the blank Gtn-HPA reduced the calcium concentration in the medium compared to the positive control despite no reduction in RA/TA ratio and visible osteoclastic activity. Since no active substances that could impact bone resorption were present or incorporated in the blank hydrogel, further investigation into this phenomenon was warranted. After culturing the medium with the Gtn-HPA hydrogel the mean calcium concentration of the culture medium was reduced compared to the control medium. Washing the hydrogel with distilled water lead to the release of calcium which we were able to measure in the wash. The combined calcium concentration in the cultured medium and the wash was comparable to that of the control medium indicating some calcium uptake by the hydrogel. Hydrogels have the capacity to swell by absorbing water.(46, 51) For the Gtn-HPA hydrogel swelling is related to stiffness, with softer gels absorbing more water than stiffer gels.(51) As the Gtn-HPA hydrogel absorbs water it is possible that dissolved calcium is binding to the gel. A similar phenomenon was observed by Alshihri et al, when attempting to quantify the calcium content of BM-MSCs seeded in the gel.(61) They noted that although the BM-MSCs were undifferentiated and kept in expansion medium, a certain amount of calcium was measured in the gel.(61) A likely source of the calcium could be the medium.

The effect of this observation is yet to be determined in a clinical or in-vivo situation. Should there be a similar uptake of calcium in-vivo, this calcium will likely be released upon degradation of the hydrogel. Calcium may have direct effects on osteoblast and osteoclast function.(74, 75) As osteoclast resorb bone, calcium is released into the surrounding which can be

sensed by osteoblast via calcium-sensing receptors.(75) This promotes osteoblast differentiation, proliferation, and function, and inhibits osteoclast activity.(74, 75)

There may be some benefits to exogenous calcium or calcium-containing biomaterials in bone regeneration.(76) Aquino-Martinez et al, demonstrated that BM-MSCs cultured in a 3D gelatin scaffold with added exogenous calcium had upregulated expression of osteogenic markers in-vitro.(76) Furthermore, the combination of calcium and BMP-2 lead to an added expression of osteogenic markers, and greater bone formation in-vivo.(76) Jo et al, used the same Gtn-HPA hydrogel used in this study to develop a novel calcium-accumulating peptide/gelatin hydrogel that was capable of increasing osteogenic expression in human periodontal ligament stem cells (hPDLSC)s and increasing bone volume and mineral density in calvarial defects in-vivo.(52)

Intraorally, calcium plays a role in the reduction of dental demineralization particularly during a pH drop.(77) This led to the development of restorative materials that release calcium particles to aid in the remineralization of tooth lesion.(78) On the other hand, calcium is a major constituent of dental calculus.(77, 79) Supersaturation of saliva and plaque fluid with calcium salts contributes to dental plaque mineralization and calculus formation.(79) As the Gtn-HPA matrix could potentially be used intraorally, the uptake of calcium and subsequent release needs to be investigated with regards to calculus formation.

8. Conclusions

- ATL A₄ can be successfully incorporated in the Gtn-HPA matrix. However, the addition of 500 ng of ATL A₄ can increase gelation time.
- ATL A₄ can be released from the Gtn-HPA matrix. The release rate can be controlled by varying the gel weight percentage.
- ATL A₄ was capable of reducing inflammation-mediated bone loss. The reduction in inflammation-mediated bone resorption by a single dose of ATL A₄ released from Gtn-HPA hydrogel was comparable to multiple doses of ATL A₄ added directly to the medium in a bone organ culture model.

9. Future direction

- In-vivo experiments for the preventive and regenerative potential of Gtn-HPA incorporating Lipoxins.
- The incorporation of other SPMs and growth factors such as RvE₁ and BMP-2 into the Gtn-HPA hydrogel, either alone or in conjunction with ATL A₄.
- Experimental models for the use of Gtn-HPA incorporating Lipoxins for other sites or organs.

10. References

1. Gibon E, Lu L, Goodman SB. Aging, inflammation, stem cells, and bone healing. *Stem Cell Res Ther.* 2016;7:44.
2. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, et al. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol.* 2018;89 Suppl 1:S173-s82.
3. Schwarz F, Derks J, Monje A, Wang HL. Peri-implantitis. *J Periodontol.* 2018;89 Suppl 1:S267-s90.
4. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol.* 2008;8(5):349-61.
5. Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. *Chem Rev.* 2011;111(10):5922-43.
6. Li G, Wu P, Xu Y, Yu Y, Sun L, Zhu L, et al. The effect of Lipoxin A4 on the interaction between macrophage and osteoblast: possible role in the treatment of aseptic loosening. *BMC Musculoskelet Disord.* 2009;10:57.
7. Ali M, Kucko N, Jansen JA, Yang F, Walboomers XF. The effect of lipoxin A4 on E. coli LPS-induced osteoclastogenesis. *Clin Oral Investig.* 2021;25(3):957-69.
8. Liu C, Guan H, Cai C, Li F, Xiao J. Lipoxin A4 suppresses osteoclastogenesis in RAW264.7 cells and prevents ovariectomy-induced bone loss. *Exp Cell Res.* 2017;352(2):293-303.
9. Van Dyke TE, Hasturk H, Kantarci A, Freire MO, Nguyen D, Dalli J, et al. Proresolving nanomedicines activate bone regeneration in periodontitis. *J Dent Res.* 2015;94(1):148-56.
10. Osorio Parra MM, Elangovan S, Lee CT. Specialized pro-resolving lipid mediators in experimental periodontitis: A systematic review. *Oral Dis.* 2019;25(5):1265-76.
11. Dar HY, Azam Z, Anupam R, Mondal RK, Srivastava RK. Osteoimmunology: The Nexus between bone and immune system. *Front Biosci (Landmark Ed).* 2018;23:464-92.
12. Lerner UH, Kindstedt E, Lundberg P. The critical interplay between bone resorbing and bone forming cells. *J Clin Periodontol.* 2019;46 Suppl 21:33-51.
13. Tanaka Y. Clinical immunity in bone and joints. *J Bone Miner Metab.* 2019;37(1):2-8.
14. Gruber R. Osteoimmunology: Inflammatory osteolysis and regeneration of the alveolar bone. *J Clin Periodontol.* 2019;46 Suppl 21:52-69.
15. Adamopoulos IE. Inflammation in bone physiology and pathology. *Curr Opin Rheumatol.* 2018;30(1):59-64.
16. Van Dyke TE, Sima C. Understanding resolution of inflammation in periodontal diseases: Is chronic inflammatory periodontitis a failure to resolve? *Periodontol 2000.* 2020;82(1):205-13.
17. Albrektsson T, Becker W, Coli P, Jemt T, Mölne J, Sennerby L. Bone loss around oral and orthopedic implants: An immunologically based condition. *Clin Implant Dent Relat Res.* 2019;21(4):786-95.
18. Albrektsson T, Jemt T, Mölne J, Tengvall P, Wennerberg A. On inflammation-immunological balance theory-A critical apprehension of disease concepts around implants: Mucositis and marginal bone loss may represent normal conditions and not necessarily a state of disease. *Clin Implant Dent Relat Res.* 2019;21(1):183-9.
19. Sharaf-Eldin WE, Abu-Shahba N, Mahmoud M, El-Badri N. The Modulatory Effects of Mesenchymal Stem Cells on Osteoclastogenesis. *Stem Cells Int.* 2016;2016:1908365.
20. Tanaka Y, Ohira T. Mechanisms and therapeutic targets for bone damage in rheumatoid arthritis, in particular the RANK-RANKL system. *Curr Opin Pharmacol.* 2018;40:110-9.

21. Eke PI, Borgnakke WS, Genco RJ. Recent epidemiologic trends in periodontitis in the USA. *Periodontol* 2000. 2020;82(1):257-67.
22. Lee CT, Huang YW, Zhu L, Weltman R. Prevalences of peri-implantitis and peri-implant mucositis: systematic review and meta-analysis. *J Dent*. 2017;62:1-12.
23. Sima C, Viniegra A, Glogauer M. Macrophage immunomodulation in chronic osteolytic diseases-the case of periodontitis. *J Leukoc Biol*. 2019;105(3):473-87.
24. Van Dyke TE. Shifting the paradigm from inhibitors of inflammation to resolvers of inflammation in periodontitis. *J Periodontol*. 2020.
25. Berglundh T, Jepsen S, Stadlinger B, Terheyden H. Peri-implantitis and its prevention. *Clin Oral Implants Res*. 2019;30(2):150-5.
26. Salvi GE, Cosgarea R, Sculean A. Prevalence and Mechanisms of Peri-implant Diseases. *J Dent Res*. 2017;96(1):31-7.
27. Belibasakis GN. Microbiological and immuno-pathological aspects of peri-implant diseases. *Arch Oral Biol*. 2014;59(1):66-72.
28. Severino VO, Napimoga MH, de Lima Pereira SA. Expression of IL-6, IL-10, IL-17 and IL-8 in the peri-implant crevicular fluid of patients with peri-implantitis. *Arch Oral Biol*. 2011;56(8):823-8.
29. Van Dyke TE, van Winkelhoff AJ. Infection and inflammatory mechanisms. *Journal of Clinical Periodontology*. 2013;40(s14):S1-S7.
30. Emecen-Huja P, Hasan I, Miller CS. Biologic markers of failing implants. *Dent Clin North Am*. 2015;59(1):179-94.
31. Hultin M, Gustafsson A, Hallström H, Johansson LA, Ekfeldt A, Klinge B. Microbiological findings and host response in patients with peri-implantitis. *Clin Oral Implants Res*. 2002;13(4):349-58.
32. Duarte PM, de Mendonça AC, Máximo MB, Santos VR, Bastos MF, Nociti Júnior FH. Differential cytokine expressions affect the severity of peri-implant disease. *Clin Oral Implants Res*. 2009;20(5):514-20.
33. Fu JH, Wang HL. Can Periimplantitis Be Treated? *Dent Clin North Am*. 2015;59(4):951-80.
34. Romanos GE, Javed F, Delgado-Ruiz RA, Calvo-Guirado JL. Peri-implant diseases: a review of treatment interventions. *Dent Clin North Am*. 2015;59(1):157-78.
35. Bartold MP, Van Dyke TE. Host modulation: controlling the inflammation to control the infection. *Periodontol* 2000. 2017;75(1):317-29.
36. Headland SE, Norling LV. The resolution of inflammation: Principles and challenges. *Semin Immunol*. 2015;27(3):149-60.
37. Serhan CN, Levy BD. Resolvins in inflammation: emergence of the pro-resolving superfamily of mediators. *J Clin Invest*. 2018.
38. Petasis NA, Akritopoulou-Zanze I, Fokin VV, Bernasconi G, Keledjian R, Yang R, et al. Design, synthesis and bioactions of novel stable mimetics of lipoxins and aspirin-triggered lipoxins. *Prostaglandins Leukot Essent Fatty Acids*. 2005;73(3-4):301-21.
39. Serhan CN, Hamberg M, Samuelsson B. Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes. *Proc Natl Acad Sci U S A*. 1984;81(17):5335-9.
40. Serhan CN. Lipoxins and aspirin-triggered 15-epi-lipoxins are the first lipid mediators of endogenous anti-inflammation and resolution. *Prostaglandins Leukot Essent Fatty Acids*. 2005;73(3-4):141-62.

41. Kantarci A, Van Dyke TE. Lipoxin signaling in neutrophils and their role in periodontal disease. *Prostaglandins Leukot Essent Fatty Acids*. 2005;73(3-4):289-99.
42. McMahon B, Mitchell S, Brady HR, Godson C. Lipoxins: revelations on resolution. *Trends Pharmacol Sci*. 2001;22(8):391-5.
43. Serhan CN, Jain A, Marleau S, Clish C, Kantarci A, Behbehani B, et al. Reduced inflammation and tissue damage in transgenic rabbits overexpressing 15-lipoxygenase and endogenous anti-inflammatory lipid mediators. *J Immunol*. 2003;171(12):6856-65.
44. Pouliot M, Clish CB, Petasis NA, Van Dyke TE, Serhan CN. Lipoxin A(4) analogues inhibit leukocyte recruitment to *Porphyromonas gingivalis*: a role for cyclooxygenase-2 and lipoxins in periodontal disease. *Biochemistry*. 2000;39(16):4761-8.
45. Safety and Preliminary Efficacy of Lipoxin Analog BLXA4-ME Oral Rinse for the Treatment of Gingivitis [Available from: <https://ClinicalTrials.gov/show/NCT02342691>].
46. Li J, Mooney DJ. Designing hydrogels for controlled drug delivery. *Nat Rev Mater*. 2016;1(12).
47. Hoare TR, Kohane DS. Hydrogels in drug delivery: Progress and challenges. *Polymer*. 2008;49(8):1993-2007.
48. Lim TC, Toh WS, Wang LS, Kurisawa M, Spector M. The effect of injectable gelatin-hydroxyphenylpropionic acid hydrogel matrices on the proliferation, migration, differentiation and oxidative stress resistance of adult neural stem cells. *Biomaterials*. 2012;33(12):3446-55.
49. Lim TC, Rokkappanavar S, Toh WS, Wang LS, Kurisawa M, Spector M. Chemotactic recruitment of adult neural progenitor cells into multifunctional hydrogels providing sustained SDF-1alpha release and compatible structural support. *Faseb j*. 2013;27(3):1023-33.
50. Wang LS, Boulaire J, Chan PP, Chung JE, Kurisawa M. The role of stiffness of gelatin-hydroxyphenylpropionic acid hydrogels formed by enzyme-mediated crosslinking on the differentiation of human mesenchymal stem cell. *Biomaterials*. 2010;31(33):8608-16.
51. Wang LS, Chung JE, Chan PP, Kurisawa M. Injectable biodegradable hydrogels with tunable mechanical properties for the stimulation of neurogenic differentiation of human mesenchymal stem cells in 3D culture. *Biomaterials*. 2010;31(6):1148-57.
52. Jo BS, Lee Y, Suh JS, Park YS, Lee HJ, Lee JY, et al. A novel calcium-accumulating peptide/gelatin in situ forming hydrogel for enhanced bone regeneration. *J Biomed Mater Res A*. 2018;106(2):531-42.
53. Lee F, Chung JE, Kurisawa M. An injectable enzymatically crosslinked hyaluronic acid-hydrogel system with independent tuning of mechanical strength and gelation rate. *Soft Matter*. 2008;4(4):880-7.
54. Utari TR, Ana ID, Pudyani PS, Asmara W. The intrasulcular application effect of bisphosphonate hydrogel toward osteoclast activity and relapse movement. *The Saudi Dental Journal*. 2020.
55. Li X, Liu X, Ni S, Liu Y, Sun H, Lin Q. Enhanced osteogenic healing process of rat tooth sockets using a novel simvastatin-loaded injectable microsphere-hydrogel system. *J Craniomaxillofac Surg*. 2019;47(7):1147-54.
56. Li D, Zhao L, Cong M, Liu L, Yan G, Li Z, et al. Injectable thermosensitive chitosan/gelatin-based hydrogel carried erythropoietin to effectively enhance maxillary sinus floor augmentation in vivo. *Dent Mater*. 2020;36(7):e229-e40.
57. Wang B, Shao J, Jansen JA, Walboomers XF, Yang F. A Novel Thermoresponsive Gel as a Potential Delivery System for Lipoxin. *J Dent Res*. 2019;98(3):355-62.

58. Wang B, Wang J, Shao J, Kouwer PHJ, Bronkhorst EM, Jansen JA, et al. A tunable and injectable local drug delivery system for personalized periodontal application. *J Control Release*. 2020;324:134-45.
59. Wang B, Booi-Vrieling HE, Bronkhorst EM, Shao J, Kouwer PHJ, Jansen JA, et al. Antimicrobial and anti-inflammatory thermo-reversible hydrogel for periodontal delivery. *Acta Biomater*. 2020;116:259-67.
60. Dromel PC, Singh D, Christoff-Tempesta T, Martheswaran T, Alexander-Katz A, Spector M, et al. Controlling Growth Factor Diffusion by Modulating Water Content in Injectable Hydrogels. *Tissue Eng Part A*. 2021.
61. Alshihri A, Niu W, Kämmerer PW, Al-Askar M, Yamashita A, Kurisawa M, et al. The effects of shock wave stimulation of mesenchymal stem cells on proliferation, migration, and differentiation in an injectable gelatin matrix for osteogenic regeneration. *J Tissue Eng Regen Med*. 2020;14(11):1630-40.
62. Peng Z, She Y, Chen L. Synthesis of poly(glutamic acid)-tyramine hydrogel by enzyme-mediated gelation for controlled release of proteins. *J Biomater Sci Polym Ed*. 2015;26(2):111-27.
63. Gao Q, Jiang Y, Li X, Chen H, Tang S, Chen H, et al. Intratumoral Injection of Anlotinib Hydrogel Combined With Radiotherapy Reduces Hypoxia in Lewis Lung Carcinoma Xenografts: Assessment by Micro Fluorine-18-fluoromisonidazole Positron Emission Tomography/Computed Tomography Hypoxia Imaging. *Front Oncol*. 2021;11:628895.
64. Stern PH, Krieger NS. Comparison of fetal rat limb bones and neonatal mouse calvaria: effects of parathyroid hormone and 1,25-dihydroxyvitamin D3. *Calcif Tissue Int*. 1983;35(2):172-6.
65. Sakai R, Eto Y, Hirafuji M, Shinoda H. Activin release from bone coupled to bone resorption in organ culture of neonatal mouse calvaria. *Bone*. 2000;26(3):235-40.
66. Curtin P, McHugh KP, Zhou HY, Flückiger R, Goldhaber P, Oppenheim FG, et al. Modulation of bone resorption by phosphorylation state of bone sialoprotein. *Biochemistry*. 2009;48(29):6876-86.
67. Curtin P, Youm H, Salih E. Three-dimensional cancer-bone metastasis model using ex-vivo co-cultures of live calvarial bones and cancer cells. *Biomaterials*. 2012;33(4):1065-78.
68. Liu J, Czernick D, Lin SC, Alasmari A, Serge D, Salih E. Novel bioactivity of phosvitin in connective tissue and bone organogenesis revealed by live calvarial bone organ culture models. *Dev Biol*. 2013;381(1):256-75.
69. Dibart S, Alasmari A, Zanni O, Salih E. Effect of Corticotomies with Different Instruments on Cranial Bone Biology Using an Ex Vivo Calvarial Bone Organ Culture Model System. *Int J Periodontics Restorative Dent*. 2016;36 Suppl:s123-36.
70. Salih E. Ex-Vivo Model Systems of Cancer-Bone Cell Interactions. *Methods Mol Biol*. 2019;1914:217-40.
71. Staines KA, Brown G, Farquharson C. The Ex Vivo Organ Culture of Bone. *Methods Mol Biol*. 2019;1914:199-215.
72. Ryu K, Dordick JS. How do organic solvents affect peroxidase structure and function? *Biochemistry*. 1992;31(9):2588-98.
73. Straus W. Letter: Cleavage of heme from horseradish peroxidase by methanol with inhibition of enzymic activity. *J Histochem Cytochem*. 1974;22(9):908-11.
74. Sugimoto T, Kanatani M, Kano J, Kaji H, Tsukamoto T, Yamaguchi T, et al. Effects of high calcium concentration on the functions and interactions of osteoblastic cells and monocytes and on the formation of osteoclast-like cells. *J Bone Miner Res*. 1993;8(12):1445-52.

75. Sharan K, Siddiqui JA, Swarnkar G, Chattopadhyay N. Role of calcium-sensing receptor in bone biology. *Indian J Med Res.* 2008;127(3):274-86.
76. Aquino-Martínez R, Artigas N, Gámez B, Rosa JL, Ventura F. Extracellular calcium promotes bone formation from bone marrow mesenchymal stem cells by amplifying the effects of BMP-2 on SMAD signalling. *PLoS One.* 2017;12(5):e0178158.
77. Leitão TJ, Cury JA, Tenuta LMA. Kinetics of calcium binding to dental biofilm bacteria. *PLoS One.* 2018;13(1):e0191284.
78. Cheng L, Weir MD, Xu HH, Antonucci JM, Lin NJ, Lin-Gibson S, et al. Effect of amorphous calcium phosphate and silver nanocomposites on dental plaque microcosm biofilms. *J Biomed Mater Res B Appl Biomater.* 2012;100(5):1378-86.
79. Jin Y, Yip HK. Supragingival calculus: formation and control. *Crit Rev Oral Biol Med.* 2002;13(5):426-41.