THE ANTI-INFLAMMATORY EFFECTS OF SMALL MOLECULE MEDIATOR (R)-PFI-2 IN THE TEMPOROMANDIBULAR JOINTS OF RATS AND MICE EXPOSED TO COMMON INFLAMMATORY MEDIATORS

A Thesis

By

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ABSTRACT

Introduction**.** TMD is a complex multi-factorial chronic inflammatory condition involving a host of life altering symptoms. The etiology of TMD is not well understood. To treat these chronic inflammatory conditions, small molecule mediators, such as (R)-PFI-2 have been selected to target robust protein kinase methyltransferases such as SETD7 in an effort to stop and even reverse the effects of chronic inflammation. Further study is needed to better understand its in vivo and in vitro potential. The aim of this investigation is to (1) establish a protocol for in-vitro and in-vivo (R)-PFI-2 concentrations that demonstrate anti-inflammatory effects in the TMJs of rats and mice and (2) show a predictable anti-inflammatory effect of (R)-PFI-2 both in-vitro and in-vivo in the TMJs of rats and mice.

Material and Methods. A pilot study was completed to demonstrate predictable production of inflammation in the temporomandibular joints of rats and mice and establish an inflammatory/rescue protocol for future investigation. The in-vitro arm of this study used mice TMJs which were harvested, organ cultured and injected with IL-6 to demonstrate inflammation for both test groups, with only one test group receiving the rescue PFI-2 for comparison. Tissues were subjected to H/E analysis.

Results. Rats: X-ray analysis demonstrated that the radio-opaque intertrabecular matter [proteoglycans or mineral formation] returned to the condylar bone following PFI-2 application. Micro-CT analysis demonstrated that inflammatory conditions resulted in an irregular rough, condylar surface. Following PFI-2 application, a partially rescued pathological TMJ phenotype could be observed. Histological staining demonstrated a

connective tissue lining of the articular fossa and the condylar fibrous perichondrium that was fibrotic in inflammatory TMJs [CFA mice] and consisted of loose connective tissue in the healthy TMJs [control mice]. PFI-2 application resulted in a partial rescue. Immunohistochemistry demonstrated inflammatory conditions that resulted in an increased level of IL-1B expression and PFI-2 application that reduced the number of IL-1B positive cells. Mice: Histological slides demonstrated that PFI-2 restored bone and cartilage morphology and promoted muscle fiber growth.

Conclusions. Our data suggest that PFI-2 may be useful as an anti-inflammatory mediator to reverse the effects of inflammatory TMD in rats and mice.

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NOMENCLATURE

PMN	Polymorphonuclear leukocyte
IL-6	Interleukin 6
TNF-α	Tumer necrosis factor-alpha
DNMT	DNA methyltransferase
SETD7	Set domain-containing lysine methyltransferase 7
(R)-PFI-2	R – partition fraction - 2
TMJ	Temporomandibular Joint
TMD	Temporomandibular Joint Disorder

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1. INTRODUCTION

1.1 Purpose

To demonstrate the anti-inflammatory/rescue effects of small molecule mediator, PFI-2, on the inflamed TMJ tissues in rats and mice using common inflammatory mediators such as: CFA and IL-6.

1.2 Temporomandibular Joint Disorders

1 in 10 women will suffer from Temporomandibular Joint Disorder [TMD] in their lifetimes. 25% of these women will seek professional help and only 10% of those women will have a positive outcome/resolution of their TMD. Looking at the population as a whole, TMD affects approximately 25% of the population and may be severe in a small subgroup as described above. The etiology is unknown but some studies have looked at arthrtitis, disc displacement, infection, trauma, and bruxism as likely etiologic factors. [1] TMD is much more common in females. Mandibular dislocation is one of the described disorders associated with this group of TMD issues.

Mandibular dislocation also known as subluxation of the temporomandibular joint will occur as the condyle translates in an anterior direction down the articular eminence. During dislocation, the patient's mouth may appear at max opening, as the condyle is located completely anterior to the eminence. Clinically, this can be palpated as a space or depression at the posterior most part of the temporomandibular joint. These joint

dislocations can be spontaneous from a yawn or be the direct result of extended dental treatment. If the latter occurs, patients may have trouble communicating their problem with the clinician since their jaw is locked in a depressed and anterior position. Relocation techniques have been described in the literature, but vary clinically between patients. [2] In general, the technique includes depressing the condyle in a posterior direction around and up the articular eminence where it can rest in its natural position while the patient closes.

Arthritis and ankylosis have also been described in the literature as part of this group of temporomandibular diseases. Arthritis is the most common cause of pathologic changes in the temporomandibular joint. For example, with our rheumatoid arthritis patients, these patients may have bilateral involvement of their temporomandibular joints before or after other joint problems have manifested. Radiological analysis of these patients temporomandibular joints will show decreased joint space without osseous changes initially, while in later stages of the disease, osseous changes are more easily identified including ankylosis of the temporomandibular joint itself. While the etiology still remains unclear, some possible etiological sources include bruxism, trauma, and even normal wear. Patients symptoms have a range from no pain to mild to highly severe pain that effects daily function and has been associated with suicidal thoughts/actions. [3]

There are certain bones involved in the articulation of the cranium and the upper facial skeleton known as the temporal bone and mandible. Therefore, the joint that connects these two is called the temporomandibular joint. During development in other vertebrates, the mandible is several pieces compounded together including bones that

house teeth [dentary] and Meckel's cartilage remnants [articular bone]. [4] Together these bones would articulate with the quadrate bone posteriorly on the skull and represent hinge only movement for these vertebrates. As evolution continued, mammals evolved from a compound jaw into a single jaw bone called the mandible which house teeth and have a part of the mandible [the condyle] that would articulate with the cranium making the TMJ a secondary joint leaving the primary joint still evolutionarily conserved as the malleus and incus of the middle ear.

There are three main types of joints: fibrous joints, cartilaginous joints, and synovial joints. Fibrous joints typically connect two bones via three types of fibrous joints: sutures, gomphosis, or syndesmosis. [5,6] A suture permits very little movement and it functions to permit growth with several histological studies demonstrating a osteogenic layers over the suture. A good example of a fibrous joint connected with a suture would be the skull bones on a developing brain. A gomphosis is a socketed attachment of tooth to bone found in the fibrous periodontal ligament. Limited movement is allowed in this joint, more like a shock absorber for internal, functional movement during mastication. Finally, a syndesmosis represents joints between the fibula and tibia as well as the radius and ulna. Despite their limited distance apart, the two bones are joined by an interosseous ligament permitting very small movement. Cartilaginous joints can be divided into primary and secondary joints. Primary cartilaginous joints have bone and cartilage in direct apposition, like in a costochondral junction compared to a secondary cartilaginous joint where tissues of the articulation occur in the order of bone-cartilage-fibrous tissue-cartilage-bone. A good example of a secondary cartilaginous joint is the pubic symphysis.

There is very limited movement allowed with cartilaginous joints. Finally, there are synovial joints, which will permit the most movement between two bones. Typically these two bones are covered in hyaline cartilage and surrounded by a capsule filled with synovial fluid. [7,8]

A variety of ligaments are typically associated with these joints to limit excursive movements and stabilize the joint. Therefore, we can begin to describe the temporomandibular joint for mammals as a synovial joint, however since differences in masticatory requirements will differ among mammals a single description is challenging to ascertain. For example, movements are restricted to hinge motions only in carnivores. In humans, this is no the case, since there is a demand for movement not just restricted to hinge axis forces but also protrusive and lateral forces dictating both translational and rotational forces be applied to this synovial sliding-ginglymoid joint. The temporomandibular joint begins development at 3 months of gestation. [9] At this time, the secondary jaw joint of the temporomandibular joint begins formation. The primary evidence of the TMJ is the appearance of 2 specific areas of mesenchymal gatherings called the temporal and condylar blastema. The condylar blastema grows quickly dorso-laterally to close the gap between the two areas. Ossification begins in the temporal blastema. The condylar blastema, still condensed mesenchyme, becomes the inferior joint space and then differentiates into condylar cartilage followed by a second cleft forming the superior joint cavity. Then the primitive articular disc forms.

The temporomandibular joint [TMJ[is the articulation between the squamous portion of the temporal bone and the condyle of the mandible. It has the following structural

components. The TMJ comprises 2 types of synovial joints – sliding and hinge – and consists of ligaments, which serve as boundaries, squamous portion of the temporal bone, condyle of the mandible, and articular disc which is contained within the TMJ. The TMJ articulation is located on the squamous portion of the temporal bone and has an avascular articular surface composed of fibrous connective tissue as opposed to hyaline cartilage. [10].

Areas which are primarily responsible for bearing the main load of the TMJ are on the lateral aspect of the squamous portion of the condyle and articular disc. There is dense, fibrous connective tissue in the thickest and most critical of these load bearing areas as evidence by the perichondrium. The perichondrium which will be described later in the embryology section, serves as a possible etiology and point of resolution in the pathogenesis of TMD. Anatomic relations concerning the squamous portion of the temporal bone include an anterior-articular eminence becoming the articular tubercle, a posterior tympanic plate tapering to the postglenoid tubercle and an intermediate glenoid fossa. [11] The articular eminence represents the strong bony protuberance on the base of the zygomatic process. The articular tubercle is located on the lateral part of the articular eminence and provides the capsular attachment and ligament for the lateral temporomandibular ligament. There is also a depression called the glenoid fossa where the condyle is primary located. Just superior to this glenoid fossa, is a thin plate of bone in the middle cranial fossa.

There is a tympanic plate which is a vertical plate located anterior to the external auditory meatus. [12] The posterior glenoid tubercle which is an inferior extension of the

squamous portion of the temporal bone, comprises the posterior aspect of the glenoid fossa and provides connection for the retrodiscal pad and TMJ capsule. The mandibular condyles measure approximately 20mm in a mediolateral direction and 10mm anterio-posteriorly. The articular surface of the condyles is avascular fibrous connective tissue instead of hyaline cartilage and mainly loads on its lateral aspect. Also composed of dense fibrous connective tissue is the articular disc. The disc is located between the squamous portion of the temporal bone and the condyle and is aneural and avascular towards the middle aspect but transitions to highly vascularized and innervated on its posterior aspect. This is mainly because the posterior aspect has minimal load bearing.

An area of potential tearing for the articular disc would be its lateral areas, where most of the load occurs. This articular disc can be divided into three areas or bands: anterior, intermediate and posterior. [13, 14] The anterior area is a thick band that lies just in front of the condyle when the mouth is closed. The thinnest part, the intermediate band, is located along the eminence while the mouth remains closed. Finally, the posterior section, which is a thick band much like the anterior section, is located above the disc while the mouth remains closed. There are additional lateral ligaments which provide limited stability and function to the joint. Medially and laterally there are collateral ligaments that tend to anchor the condyle with the disc. Anteriorly, the disc is connected to the capsule and the superior head of muscle called the lateral pterygoid, however here the condyle is not attached. This allows the articular disc to have a rotational movement over the condyle when moving anteriorly or posteriorly. At its

posterior-most site, the disc is continuous with the bil-aminar zone that will blend with the joint capsule. [15]

The bi-laminar zone also known as the posterior attachment complex is a two-layered structure that is located behind the articular disc it can be highly distorted especially when the mouth is opening. This structure is composed of a retrodiscal pad, superior lamina and inferior lamina. The retrodiscal pad is highly innervated and vascularized and made of elastic fibers, collagen, nerves, blood vessels and fatty tissue. For example, when the condyle shifts forward in function, a large venous plexus within the retrodiscal pad will fill with blood. The superior lamina which also contains elastic fibers, will anchor the upper most portion of the most posterior aspect of the disc to the bone and capsule at the tympanic plate and post glenoid tubercle. The inferior lamina, which alternatively contains collagen fibers, will anchor the lowest part of the most posterior portion of the articular disc to the condyle. [16]

The TMJ has several compartments. The articular disc will divide these compartments into an upper [superior] and lower [inferior] area. The internal surface of both these compartments will house specialized endothelial cells, forming a synovial lining producing synovial fluid which is the reason the TMJ is considered a synovial joint. Synovial joints have synovial fluid which will work as a lubricant for the joint and also serve as an instrument for the metabolic requirements to the articular surface. The superior compartment contains a volume of approximately 1.2mL and provides movement in the translational direction for the TMJ. Alternatively, the inferior compartment contains a volume of approximately 0.9mL. [17,18] Residing between the

condyle and articular disc, this inferior space is responsible for movement of a rotational sense for the TMJ.

The TMJ's capsule will completely surround the articular surface of the temporal bone as well as the mandibular condyle. This capsule is composed of fibrous connective tissue and has been stabilized on its lateral and medial aspects by accessory ligaments. It is also lined by a highly vascularized synovial membrane. There are also a variety of sensory components such as nociceptors that are associated with this capsule. Forwarding our discussion to the capsular support via ligaments, there are four main groups of ligaments associated with the TMJ: collateral ligaments, the temporomandibular or lateral ligament, the stylomandibular ligament, and the sphenomandibular ligament. The collateral ligaments also known as the discal ligaments, are formed by 2 main ligaments: the lateral collateral ligament and the medial collateral ligament. [19] The lateral collateral ligament will connect the lateral part of the disc to the lateral aspect of the condyle, while the medial collateral ligament tends to connect the medial part of the disc to the medial wall of the condyle.

These ligaments are composed of collagenous connective tissue, meaning that they will not stretch. The temporomandibular or lateral ligament is a single thickened ligament located on the lateral surface of the capsule and functions in the prevention of lateral and posterior displacement. There are 2 different and separated bands associated with the temporomandibular ligament. The outer oblique part which is the largest portion, is attached to the articular tubercle and travels posterior and inferior to connect immediately inferior to the condyle which will limit opening of the mouth. The second band is the

inner horizontal part which is a smaller band, attached to the articular tubercle and runs horizontal to connect with the lateral part of the condyle and the articular disc, thereby limiting posterior movement of the disc and condyle. The stylomandibular ligament helps limit the anterior protrusion of the lower mandible. This ligament is composed of a thickened deep cervical fascia extending from the styloid process to the posterior margin of the angle and ramus of the mandible. [20]

Finally, the sphenomandibular ligament is actually a remnant of Meckel's cartilage, extending from the spine of the sphenoid to the lingual of the mandible. Studies have shown that the sphenomandibular ligament takes a part in the pivoting action of the mandible while maintaining tension during opening and closing of the mouth. The TMJ has an abundant arterial supply comprising of the superficial temporal, deep auricular and anterior tympanic arteries. The superficial temporal artery comes from a terminal branch of the external carotid artery, it begins in the parotid gland and at its beginning, is located posterior to the mandible providing slight branches to the TMJ. [21] Arising from the maxillary artery, the TMJ has arterial supply from both the deep aruicular, which also lies in the parotid gland, giving posterior branches to the TMJ along with the anterior tympanic artery which passes superiorly and posteriorly to the TMJ, entering the tympanic cavity through the petrotympanic fissure, finally giving off branches to the TMJ. The venous draining from the TMJ is accomplished via the superficial temporal vein which ultimately joins the maxillary vein to for the retromandibular vein.

The TMJ is innervated by the auriculotemporal n, the masseteric n., and the posterior deep temporal n. The auriculotemporal n. arises from the mandibular division [V3] of the

trigeminal n, splitting around the middle meningeal a and passing between the sphenomandibular ligament and condylar neck. This nerve supplies sensory branches all along the capsule. This nerve also will carry autonomic supply to the parotid gland. Both the masseteric and posterior deep temporal nerves arise from the anterior division of the mandibular trigeminal n. These nerves both lie anterior to the TMJ and provide branches to the joint. [22] Slightly before innervating the joint, the masseteric n. passes over the masseteric notch to innervate the masseter muscle while the posterior deep temporal nerve innervates the temporalis muscle and provides sensory branches that aid the auriculotemporal nerve in the supply of the anterior joint. This branch is mainly motor in function but also has some sensory functions as well.

The temporomandibular joint has a complex and multifaceted embryology when looking at comparative anatomy among humans and reptiles. For example, the hinge only action from reptilian species denotes the simplistic physiology of the temporomandibular joint that has complicated with the development of an articular disc in humans, allowing greater range of movement [23] and at the same time greater complexity of etiology when discussing pathophysiology of the joint.

Mandibular movement during normal function and during abnormal function also known as parafunction [Ie. Bruxism], likewise involves a complex neuromuscular pattern originating in a pattern generator in the brainstem with modified influences from higher centers of the cerebral cortex, basal ganglia, and peripheral influences from the Periodontium and muscles of mastication among other environmental factors. During opening, in health there is a complicated series of movements that occur. The initial most

movement is pure rotation, occurring in the inferior temporomandibular joint space. In this movement, the lateral pterygoid, specifically the inferior head, initiates opening. As the jaw begins to open and the mandible is depressed, the medial and collateral ligaments support the connection of the condyle to the disc allowing for this pure rotational movement. [24]

At some point during mandibular depression, those ligaments reach their maximum capacity and become taut, stopping the rotation. In general, pure rotation occurs until approximately 20mm of space occurs between maxillary and mandibular teeth. After this pure rotation, translation must occur for further opening. This translational movement occurs in the superior joint space, allowing for maximum opening. For translation to occur, the disc and condyle together slide down the articular eminence, allowing for maximum opening of the mandible. This rotation and translational movement allow for complete opening and closing of the mandible and are responsible for the primary functions of mastication.

1.3 Epigenetics: Phenotypic Expression of DNA

Immune response to oral bacterial pathogens and following activation of the immune system via inflammatory signaling does not solely rely on genetic factors [25]. Highlights importance of epigenetics and its role specifically on chronic inflammatory mechanisms, specifically to TMD and periodontitis, among other chronic inflammatory systemic conditions. Epigenetics is described as “changes in gene expression that are not encoded

in the DNA sequence itself and include chemical alterations of DNA and its associated proteins..these changes lead to remodeling of chromatin and subsequent activation or inactivation of a gene" [26]. This unwinding of histones and exposure of chromatin, lead to subsequent activation [turning on], or inactivation [turning off] of genes. Epigenetics has been shown to contribute to a variety of diseases including inflammatory, autoimmune diseases, and even cancer [27, 28]. Epigenetic modifications include chemical alterations of DNA and associated proteins via small molecule mediators [29].

The role of SET D7 as a target for small molecule mediators, like (R)-PFI-2 in a mouse TMD model demonstrates the role epigenetics can play in chronic inflammatory disease and has been studied in periodontal disease as well. This host inflammatory response, induced by oral bacteria are influenced in an epigenetic sense by both genetic and environmental factors [30, 31].

Gene expression is dependent on how tightly coiled/packed or loosely packed a histone may become during transcription. A nucleosome makes DNA accessible for transcription factors by unwinding of the histones, making transcription of DNA possible- hence, gene expression. Alternatively, when histones become tightly coiled, transcription can on longer occur, leading to silencing of a gene altogether despite the gene being a part of a patient's DNA. [32] That gene will not be expressed which can be beneficial in some cases and detrimental in others. For example, in chronic inflammatory diseases, such as TMD or periodontitis, inflammation targets specific tissues leading to clinical observation of tissue breakdown and eventual organ dysfunction.

Some epigenetic modifications are actually able to be reversed, and can even be modified by a patient's environment which leads into the link between the genetic make up of the patient and the patients environment [33, 34]. Generally speaking, the two major epigenetic modifications are DNA methylation and histone acetylation and methylation [35]. In order to understand epigenetics, we have to look at the building blocks of chromatin, which are nucleosomes. Just one nucleosome consists of 146 base pairs of DNA and a core histone complex [includes 2 copies each of histones: H2B, H4, H2A, and H3, along with a linker histone [H1], connecting the nucleosomes. This unit forms the 'primary chromatin structure', commonly known as 'beads on a string' [36]. Histones have these amino acid tails that can be methylated or acetylated as they protrude from the nucleosome.

Acetylation is regulated by HATs [Histone acetyltransferases] and HDACs [histone deacetylases]. [37, 38] Classically, the addition of methyl groups to cytosine bases next to a guanine base happens to occur at critical sites in the DNA via DNMTs [DNA methyltransferases] which alters the configuration of the NDA and binding of transcription factors which lead to a change in gene expression. Different methylation patterns have been associated with different regulatory pathways such as lippolysaccharide [LPS] mediated signaling, apoptosis, oncogenesis, cell differentitation, and cell adhesion – all found within inflamed tissues. Active genes tend to be correlated with low levels of DNA methylation. Some studies have shown that epigenetic changes increase during ones lifetime, however age itself is a risk factor for epigenetic changes. These differences in epigenetic changes may explain why some patients respond

differently to treatment and why some patients are more susceptible to chronic inflammatory and autoimmune diseases and for that matter, cancer.

The idea here is to focus on epigenetics as it relates to the targeting of SETD7 with small molecule mediator PFI-2 in the presence of pro-inflammatory molecules, CFA [complete freund's adjunctive] and IL-6 [interleukin 6]. Secondarily, the aim is to correlate our findings with previous studies linking small molecule mediator, [39] PFI-2 with epigenetic mechanisms related to periodontitis, drawing a link between two chronic inflammatory conditions.

SETD7 is a histone lysine methyltranserfase with a variety of cellular functions specifically inflammation, oncogenesis and metabolism. SETD7 inhibition has been recently investigated resulting in the identification of (R)-PFI-2: a potent and selective inhibitor of SETD7. SET D7 is a histone H3K4 lysine methyltransferase [40] with a variety of cellular functions involved in human gene regulation including transcriptional regulation, differentiation, DNA repair, cell cycle control and DNMT1 [41]. As a methyltransferase for H3K4, SETD7 (also known as SET7, SET9, or SET7/9) belongs to the SET domain-containing proteins, [defining methylation] which can change the chromatin state by influencing the binding abilities of the cofactor to the histone via direct histone methylation, which is associated with demethylation of H3K4 (H3K4me2) and promotes downstream gene expression [epigenetics part] [42]. SET D7 is also known to have a multitude of substrates which contributes to its role in a variety of diseases.

Over-expression of SETD7 has an important role in inflammation, metabolism-associated diseases, viral infection, and oncogenesis. [42]. For example, when

considering its role in type 2 diabetes mellitus, SET D7 is upregulated in the presence of hyperglycemic conditions, leading to methylation of H3K4, ultimately contributing to vascular dysfunction via NF-kB promoter. [43, 44]. Therefore several attempts have been made to discover inhibitors of SETD7, however most inhibitors have been too weak to display any observable benefit on the inhibition of SETD7 with the exception of R-PFI-2. SET D7 is the most abundant and well-studied modification on histones. Its robust monomethyltransferase activity allows it to have several substrates and it can be localized in both the nucleus and cytoplasm of cells making its nuclear localization easily regulated and ideal for study.

1.4 Small Molecule Mediator: (R)-PFI-2: Background and Significance of SAM

R-PFI-2 is a potent and selective inhibitor of SETD7 [45] and is more potent than its enantiomer S-PFI-2. (R)-PFI-2 shows a much higher inhibiting activity (IC50 ≈ 2.0 ± 0.2 nM) with respect to the (S)-PFI-2(IC50 ≈ 1.0 ± 0.1 μM). (R)-PFI-2 is the first SETD7 inhibitor with nanomolar inhibitory potency and a known mechanism. Although there is no definitive criteria for what constitutes a cut-off point for a “good” vs “bad” inhibitor [IC50] due to several compounding factors [ie. Family of enzymes the inhibitor targets, concentrations of inhibitor, substrates, biochemical vs cellular assays, etc.], in general the significance of nanomolar inhibitory potency for (R)-PFI-2 is key to setting itself apart from weaker inhibitors with micromolar inhibitory potency.

Therefore, the purpose of this study is to expand upon the knowledge base of (R)-PFI-2 and confirm its role as an anti-inflammatory agent in the mouse and rat models both in-vitro and in-vivo. SAM serves as a co-substrate during methylation of histones which is party how the body regulates gene expression. SAM is involved in anabolic reactions throughout the body and is the most prolific donor of one carbon groups in biosynthetic reactions. SAM is important because it donates methyl groups [-CH3s] to a large number of acceptors [for example: DNA, RNA Phospholipids, and proteins].

SAM has been shown to lower LPS induced expression of pro-inflammatory cytokines such as TNF-alpha and increase expression of anti-inflammatory cytokines IL-10 in macrophages. SAM is a naturally occurring substance and is necessary for binding of small molecule mediator PFI-2 in the binding groove of SET-D7 during DNA histone methylation.

1.5 Mouse TMJ as a model for inflammation

The TMJ is a synovial joint necessary for sliding and hinge movements of the jaw. TMD occurs when the muscles around the TMJ become disrupted in structure or physiology. This can be evidenced by pain, limited jaw opening and clicking/popping. The TMJ is one of the most frequently used joints in the human body and has unique features that separate it from other joints. For instance, the cartilage of the mandibular condyle is a secondary cartilage [independent from the chondroskeleton] and has a

different embryonic origin [derived from cranial NCC] compared to the articular cartilage of the knee. [46]

Another unique feature is that the condyle of the mandible has a lower amount of collagen type I (COLIA1) compared to the other synovial joints [47]. Finally, the articular surfaces are not composed of hyaline but of fibrous tissue [48]. Development. Developmentally, animal models [including rats and mice] are useful because the process and molecular mechanisms are conserved. However, it is challenging to attempt in vivo studies in rodents, where TMD is the target objective of study, because of morphological differences in the TMJ among rats/mice and humans [49].

In Figure 1 some of the differences in morphology of the TMJ between rodents and humans can be observed. For instance in rodents, the glenoid fossa is shallow and flattened, there is no articular eminence, and the lateral pterygoid muscle is less in the volume.

1.6 Linking epigenetic mechanisms to periodontal inflammation via DMKTs

According to Bartold 2013, periodontal diseases are no longer considered a simple bacterial infection. They are complex, multifactorial diseases involving an ‘interplay’ between the subgingival microbiota, the host immune and inflammatory responses and environmental modifying factors. [50] Therefore, anti-inflammatory agents could be beneficial towards the control and ultimately reversal of periodontal inflammation. Periodontitis is no longer considered a 'simple' infection leading to the destruction of the

periodontium. Instead, a new etiology has been proposed involving the interactions amongst the host response, sub gingival oral environment and external environmental factors. Typically, a periodontitis patient will present clinically with sub gingival calculus and plaque.

These patients follow more of a 'normal' paradigm in the etiology of periodontitis because the increase of plaque and calculus is associated with an increase in the severity of the disease. However, there are also a great number of patients that present to clinic with very small amounts of deposits with a large amount of periodontal destruction. The latter patient population has provoked some to reconsider the etiology of periodontitis specifically the role of bacteria and oral hygiene. In some studies, scientists have called into question past concepts where the primary focus is only plaque levels and less-than-perfect oral hygiene as the primary etiology of periodontitis. Also, in a study by Grossi et.al.(1994) it was well established that plaque only accounts for as little as 20% of the rest for developing periodontitis. This supports further the fact that oral hygiene, plaque formation and calculus do not constitute the total etiology of periodontal disease.

Therefore, periodontitis is considered a multifactorial disease entity and has several contributing etiologic factors. Observations like these, among others, formed the basis of a model for the pathogenesis of periodontitis in which not only was the microbial challenge considered important, but those response factors, genetic risk factors and environmental and acquired risk factors are important as well. [51, 52] This is what changed the focus from solely plaque as the primary etiologic agent to a more 'holistic' approach. Several models have been proposed to capture this relationship; the newest

ones published today accentuate the use of host modulation to target pathways of inflammation in the development of periodontitis.

One model also stressed the importance of incorporating genes, proteins, and metabolite data into dynamic biologic networks that include inflammation-mediated disease initiation and disease resolving mechanisms. In several papers, Van Dyke [53] stated that although periodontal inflammation is initiated by the sub gingival biofilm, it is the production and release of mediators generated by the host response that are primarily responsible for the periodontal breakdown. Thus shifting the paradigm from a 'bug of the month' to host response as the primary etiologic agent for periodontitis, because it isn't the pathologic bacteria directly responsible for the destruction of bone and soft tissue but more so an abnormal immune response to normal biofilm thus changing the sub gingival biome into a pathogenic environment which ultimately creates an enlarged immune response leading to periodontal destruction.

It must be accepted that the host inflammatory response is what largely drives the pathological process along with new concepts in the treatment of periodontitis. In this particular model, it is also accepted that gingivitis is a mandatory initiating condition for the subsequent development of periodontitis. Gingivitis results from a non-specific inflammatory reaction in the gingival tissues to supragingival plaque accumulation. [54] The resultant inflammation changes the Subgingival due to an increase concentration of host inflammatory mediators and byproducts of connective tissue and collagen breakdown in the gingival crevicular fluid. These are the ideal conditions that provide an acceptable environment for the overgrowth of periodontal pathogens within the

subgingival biofilm. The host inflammatory response must be sufficient alongside a favorable genetic and environmental influence to proceed to periodontitis. If one of these factors is not compliant, then the gingivitis will resolve due to the host's ability to 'contain' the infection toward microbiota compatible with health and no periodontal destruction will occur. Adversely, if all of these factors are present, the likely result is periodontal breakdown.

To link periodontitis with epigenetic mechanisms, we have shown that there is a chronic inflammatory component ongoing following exposure with bacteria to a susceptible host. However, we will now discuss where these epigenetic changes are thought to occur as a way to target periodontal disease by targeting the changes to the epigenetic mechanisms occurring within the inflamed tissues. One area of study: the biofilm-gingival interface around each tooth, roughly where we see the beginnings of gingivitis and eventual periodontitis when bone loss has occurred. Previous epigenetic studies including the oral cavity have shown that the epigenomes of patients with and without inflammation will show statistical differences, not only between patients but even between sites [inflamed vs uninflamed]. [55]. Several inflammatory cytokines and DNA methylation have been studied as well, including IL-8, IL-6, IFN-y, and TNF-alpha.

In general, studies show that IL-8 and IL-6 promoters tend to be hypomethylated in patients with chronic inflammatory diseases such as chronic periodontitis versus healthy controls [55]. This shows that methylation of certain cites of DNA may influence cytokine production and therefore turn on and off disease. There has also been proposed a link between DNA methylation, the chronic inflammatory process and cancer via

methylation alterations in histones due to the aberrant methylation in tissues. In general, when tissue becomes inflamed, eosinophils and neutrophils in the area produce substance s that DNMTs cannot distinguish from methylated DNA. This is a problem because it results in de novo methylation in cells of the inflamed tissues [56].

2. MATERIALS AND METHODS

2.1 Rats [In-Vivo Arm]

Our test groups were divided into Test group 1: CFA, Test Group 2: CFA + (R)-PFI-2 [rescue], and then a control group. Rats served as our initial in-vivo animal model to our study. We used adult male Sprague-dawley rats and injected rats bilaterally with 50ug of CFA [complete Freund's adjunct] dissolved in 50uL of paraffin oil into the TMJ of anesthetized rates. Rats were either rescued with PFI-2 [10uM] via bilateral injections or injected with saline at the time of the rescue intervention. The control rats were given bilateral injections of saline at the time of inflammatory injections and rescue injections and served as uninjected controls. Based on in-vivo enzymatic assay, the IC50 110nM + (R)-PFI-2 does not affect the viability of cell lines when used at a concentration of <50uM.

CFA was used and not some other noxious stimulant due to the well tested model of CFA to induce inflammation in the TMJs of rats. Several studies have supported the efficacy of the CFA model. Also reports in the literature investigating the pro-inflammatory cytokine TNF-alpha, caspases or apoptosis may have a role in the inflammatory process. Rats were sacrificed using carbon dioxide inhalation and decapitation 2-6days after PFI-2 injected. The TMJ tissues [synovium, retrodiscal tissues, articular disc] extirpated bilaterally and either immediately frozen in liquid nitrogen or prepared for histology, immunohistochemistry, or radiographic analysis. TMJs from both

CFA-injected and un-injected controls were removed, rinsed in phosphate buffered saline [PBS].

Our analysis included sample preparation for micro-computed tomography with a voxel size of 10um, voltage of 50-70kV, current of 115-150uA and 0.5 aluminum filter which is protocol standard for microCT data analysis based on our literature review. Part of our fixed samples were placed in paraffin wax and histology was completed according to standardized protocols for Hematoxylin and eosin staining, Mallory's or Masson's Trichrome. The remaining fixed samples were prepared for immunohistochemistry and used IL-1B as an antibody to IL-6. For accurate images of trabecular microarchitecture, voxel size of <20um in rats or 10um in mice is recommended. Voltage optimal for establishing high contrast images of bone with minimal beam hardening compared to lower voltage may be appropriate for mouse neonates. Aluminum filter used for absorbing low energy x-ray before and after passing through the sample.

2.2 Mice [In-Vitro Arm]

Our test groups were broken up into Test group 1: IL-6, and test group 2: IL-6 + (R)-PFI-2 [rescue]. There was no control in the mice branch of our study due to sufficient control in our rat arm. A pilot study was carried out prior to beginning the formal research project on (R)-PFI-2 to demonstrate predictability in causing inflammation in a mouse model in vitro with TNF-alpha and IL-6. Various concentrations of inflammatory promoters and rescue [(R)-PFI-2] were used in culture to demonstrate an effect on the

TMJ's of infant mice [2-3d old]. These inflammatory and rescue effects were observed under light microscopy after paraffin sections were stained in Trichrome and H/E.

Analysis was carried out with observing coloring and cell counts under magnification [see Figure 7]. The experimental protocol for organ cultures was as follows:

- Day 0: 1 day old pups → TMJ harvested by single trained researcher [TD]
- Day 1: Organ culture TMJs in Regular Media for 24hrs
- Day 2, 3, 4: Addition of IL-6 [5ng/ml] to Groups 1 & 2 for 72hrs w. media changed q24hrs
- Day 5: Replace media with regular media and culture for 24hrs
- Day 6, 7, 8: Addition of PFi2 [10um] to Group 1 cultures and culture for 72hrs
- Day 9: Begin analyses of specimen

This experimental protocol was carried out for each group. Data analysis followed. Histology was performed concomitantly with paraffin wax samples stained with TriChrome and H/E stains.

Media preparation was completed using a Nutritive 'Regular' media: BGJb Medium (1x) Fitton-Jackson Modification [+] L – Glutamine. REF: 12591-038. LOT: 1921430. EXP: 2018-10-30. 500mL. Gibco by Life technologies. IL-6 was prepared with 5ng/mL of regular media.

3. RESULTS

3.1 Rats [In-Vivo Arm]

As seen in Figure 2, the radiographic analysis demonstrated that the radio-opaque intertrabecular matter [possibly proteoglycans or mineral formation] returned to the condylar bone following PFI-2 application [seen in Figure 2B, 2C]. It is also apparent where demineralization has occurred following CFA application [Figure 2D] and where a healthy control condylar bone as a baseline was analyzed with optimal radio-opacity of condylar bone [Figure 2A].

As seen in Figure 3, our MicroCT analysis showed that inflammatory conditions resulted in an irregular rough condylar surface shown in figure 3D. When PFI-2 was applied, a partial rescue can be observed in the pathological TMJ phenotype [Figures 3B and 3C]. Looking closely at figures 3B and 3C, the rescue has smoothed the irregularities from inflammation shown in 3D. compared to a healthy control in figure 3A where we see a smooth condylar surface, free from roughened and demineralized condylar bone.

As seen in Figure 4, our H/E staining showed a connective tissue lining of the articular fossa and the condylar fibrous perichondrium, which was fibrotic in inflammatory TMJs [Figure 4D] and consisted of loose connective tissue in the healthy TMJs [Figure 4A]. PFI-2 application resulted in a partial rescue with a partial return of that loose connective tissue [Figure 4C] however this loose connective tissue was only partially returned, as evidenced in figure 3B where more of a fibrotic inflammatory tissue

remains with scant evidence of loose inflammatory tissue. Here the perichondrium can be easily observed which is a layer of dense, irregular connective tissue that surrounds the cartilage and consists of an outer fibrous layer and an inner chondrogenic layer. The dense fibrotic tissue observed in figure 4D is synonymous with inflammation.

As seen in Figure 5, our Masson's stained slides, supported our findings from H/E histological sections. Again, we see the connective tissue lining of the articular fossa and the condylar fibrous perichondrium, which was fibrotic in inflammatory TMJs [Figure 5D] and consisted of loose connective tissue in the healthy TMJ controls [Figure 5A]. When PFI-2 was applied to the specimens, partial rescue from inflammation resulted as evidenced by a change to loosened connective tissue and partial dense fibrotic inflammatory sequelae [Figure 5B and 5C].

As seen in Figure 6, our immunohistochemistry was completed with IL-B antibody used to test for our CFA and IL-6 inflammatory mediators. Here, the inflammatory conditions, evidenced in figure 6D, resulted in an increased level of IL-1B expression and PFI-2 application reduced the number of IL-1B positive cells shown in figures 6B and 6C. Inflammatory staining of IL-1B was produced, even in the control sections [figure 6A] however this was hypothesized to be a normal reaction to the trauma from saline injection into the joint. The main idea was IL-1B cells stained approximated the articulation of the joint to demonstrate acute inflammation of the joint and replicate most closely TMD in rats. Therefore, figure 6D showed inflammatory IL-1B cells within the articulation point and at the point of injection compared to our control and rescue

slides [6A, 6B, and 6C] which showed minimal to very few IL-1B stained cells at the point of articulation.

3.2 Mice [In-Vitro Arm]

As seen in Figure 7, PFI-2 showed restored bone and cartilage morphology and promoted muscle fiber growth of the lateral pterygoid demonstrated in figure 7B. In Figure 7A, inflammatory tissue destruction can be observed despite the lack of inflammatory cells in the tissue section.

In our Masson's staining, we observed muscle regeneration of the lateral pterygoid despite the inflammatory destruction created with IL-6 [Figure 7C]. In figure 7D we see new muscle fibers stained red/brown indication partial reversal of inflammation with our small molecule mediator PFI-2.

4. DISCUSSION AND CONCLUSIONS

Using two different models to demonstrate efficacy of small molecule mediator PFI-2. PFI-2 demonstrated partial efficacy in both the rat and mouse model to reverse the harmful tissue effects of induced inflammation in the TMJs with both CFA and IL-6 pro-inflammatory agents. Rats were used as the initial animal model as a trial for efficacy in vivo. Once positive effects of PFI-2 were observed in vivo, the mouse model was an addition to the study to show specifically how the TMJ cells would respond in an isolated environment with an organ culture. Organ cultures with mice are more suitable due to the smaller organs [TMJs] of pups [mice] compared to larger joints of rats which are more suited for in-vivo study.

Use of IL-6 compared to another inflammatory mediator. IL-6 has been identified in several studies as a pro-inflammatory mediator involved in the inflammatory mechanisms and epigenetic mechanisms of chronic inflammatory diseases including TMD and periodontitis. IL-6 for this reason seamed an ideal pro-inflammatory agent for use in our organ culture mouse model.

Our inflammatory rat TMD model was associated with fibrotic connective tissues in the condylar fibrous perichondrium and the articular fossa. There was also evidence of bone resorption and a roughened articular condyle surface following exposure to CFA. (R)-PFI-2 application resulted in a partial reversal of the deleterious effects of inflammatory conditions of the TMJ. Specifically, smoothness of the condylar surface was restored and fibrotic tissue was replaced by loose connective tissue. In the organ

cultures, PFI-2 application resulted in new bone and hyaline cartilage formation as well as new formation of muscle tissue.

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APPENDIX

FIGURES

FIGURE 1: Human TMJ vs. Mouse TMJ – comparative anatomy

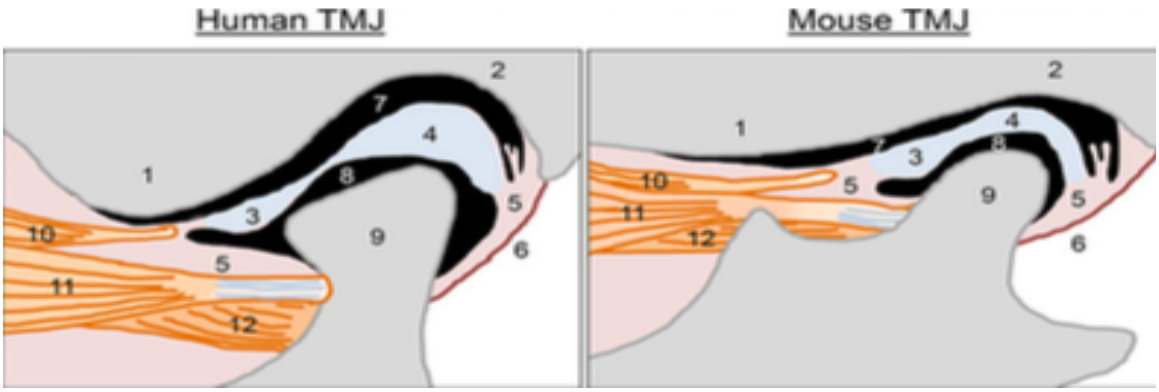

Figure 1 Description: Comparison of the structure of the TMJ between humans and mice. 1: the articular eminence of the temporal bone, 2: the glenoid fossa of the temporal bone, 3: anterior band of the articular disk, 4: posterior band of the articular disk, 5: connective tissue, 6: the posterior joint capsule, 7: the upper articular cavity, 8: the lower articular cavity, 9: mandibular condyle, 10: a part of upper head of the lateral pterygoid muscle, associated with the articular disk, 11: upper head of the lateral pterygoid muscle, connected with the mandibular condyle, 12: lower head of the lateral pterygoid muscle, connected with the mandibular condyle . Reprinted from [Sukuki and Iwata, 2016].

FIGURE 2: X-ray Analysis [RATS]

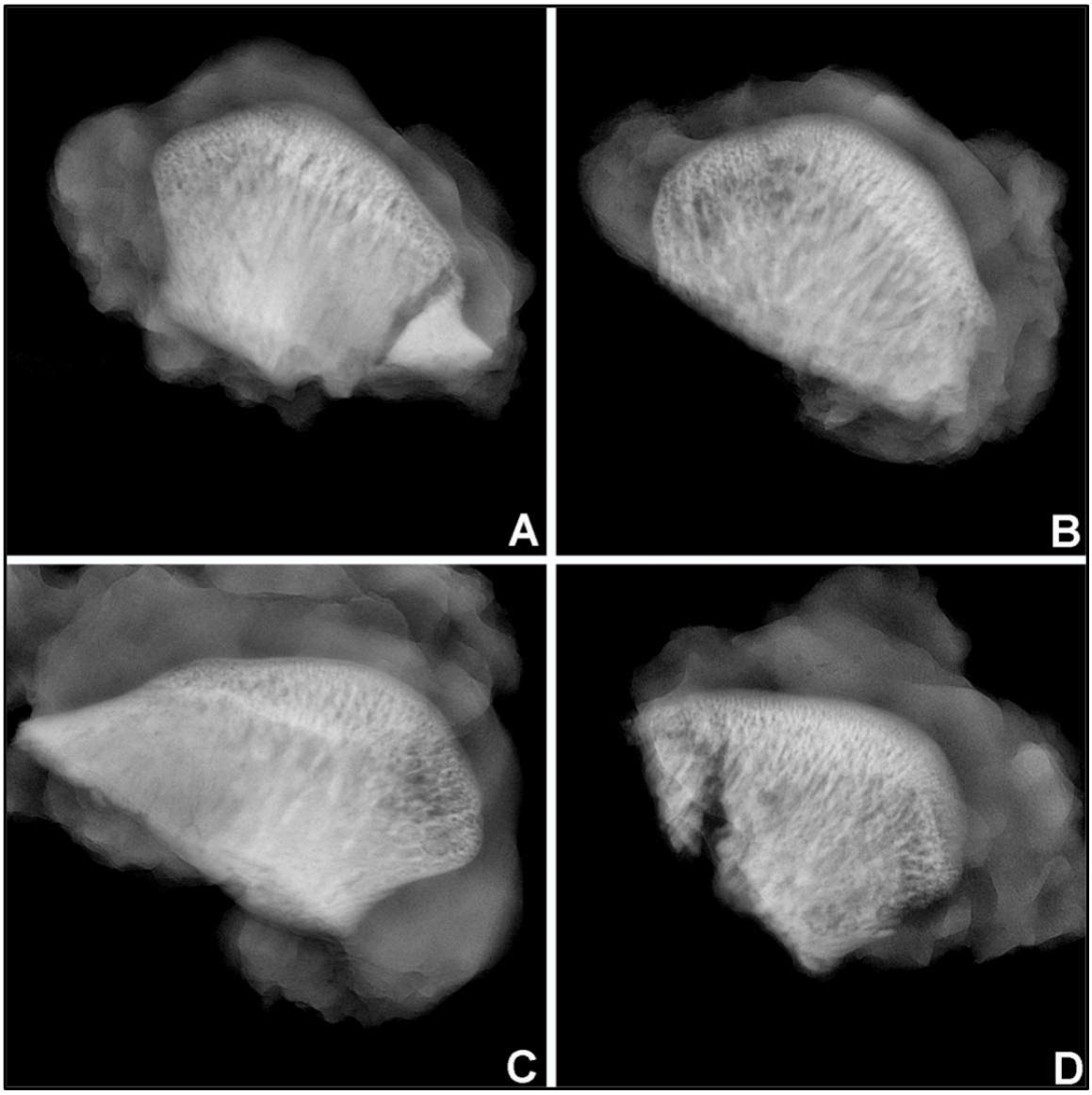

Figure 2 Description: A. Control, B. Partial rescue with PFI-2, C. Partial rescue with PFI-2, D. CFA

FIGURE 3: Micro-CT Analysis [RATS]

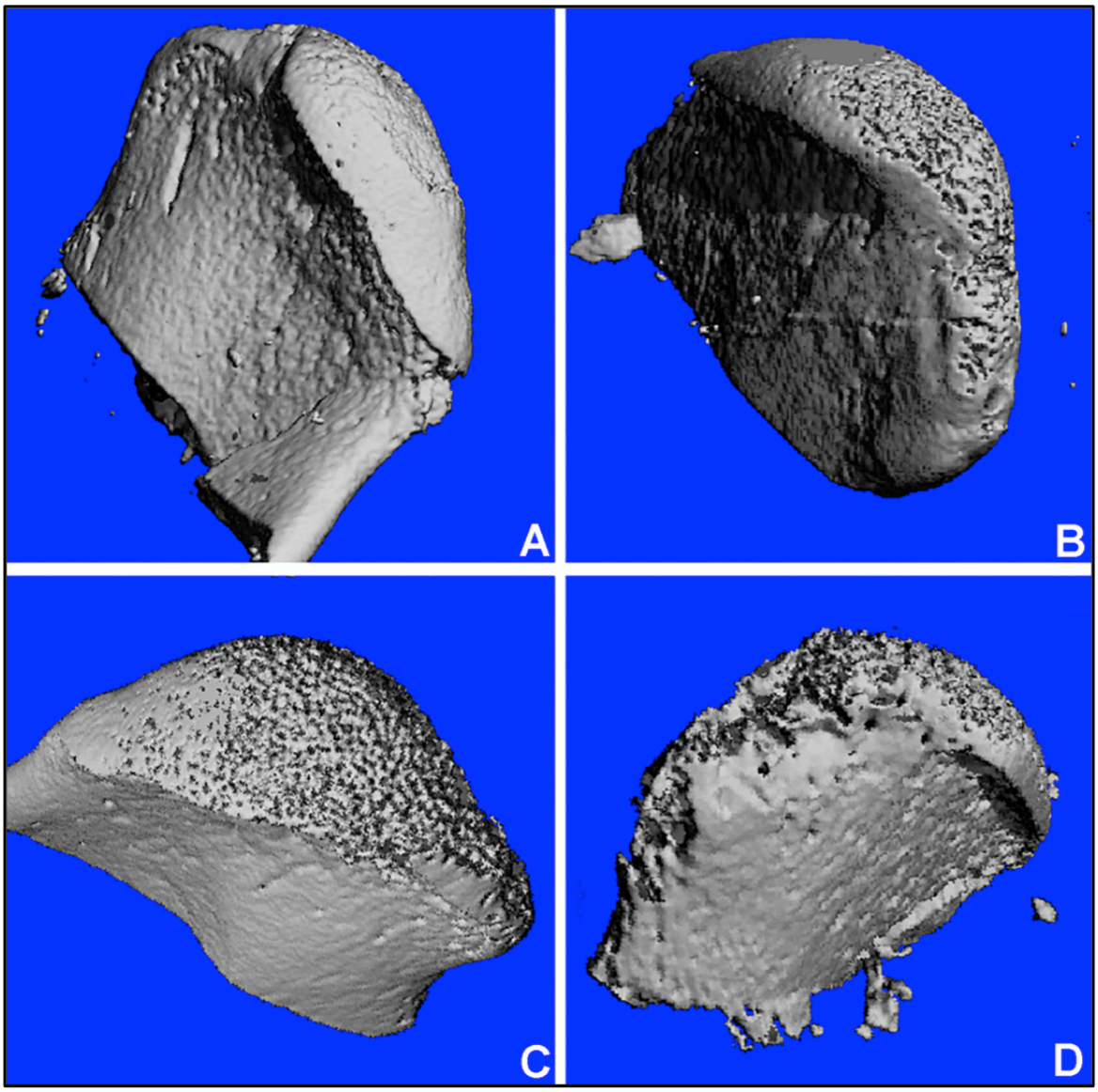

Figure 3 Description: A. Control, B. Partial rescue w. PFI-2, C. Partial Rescue with PFI-2, D. CFA

FIGURE 4: H/E Analysis [RATS]

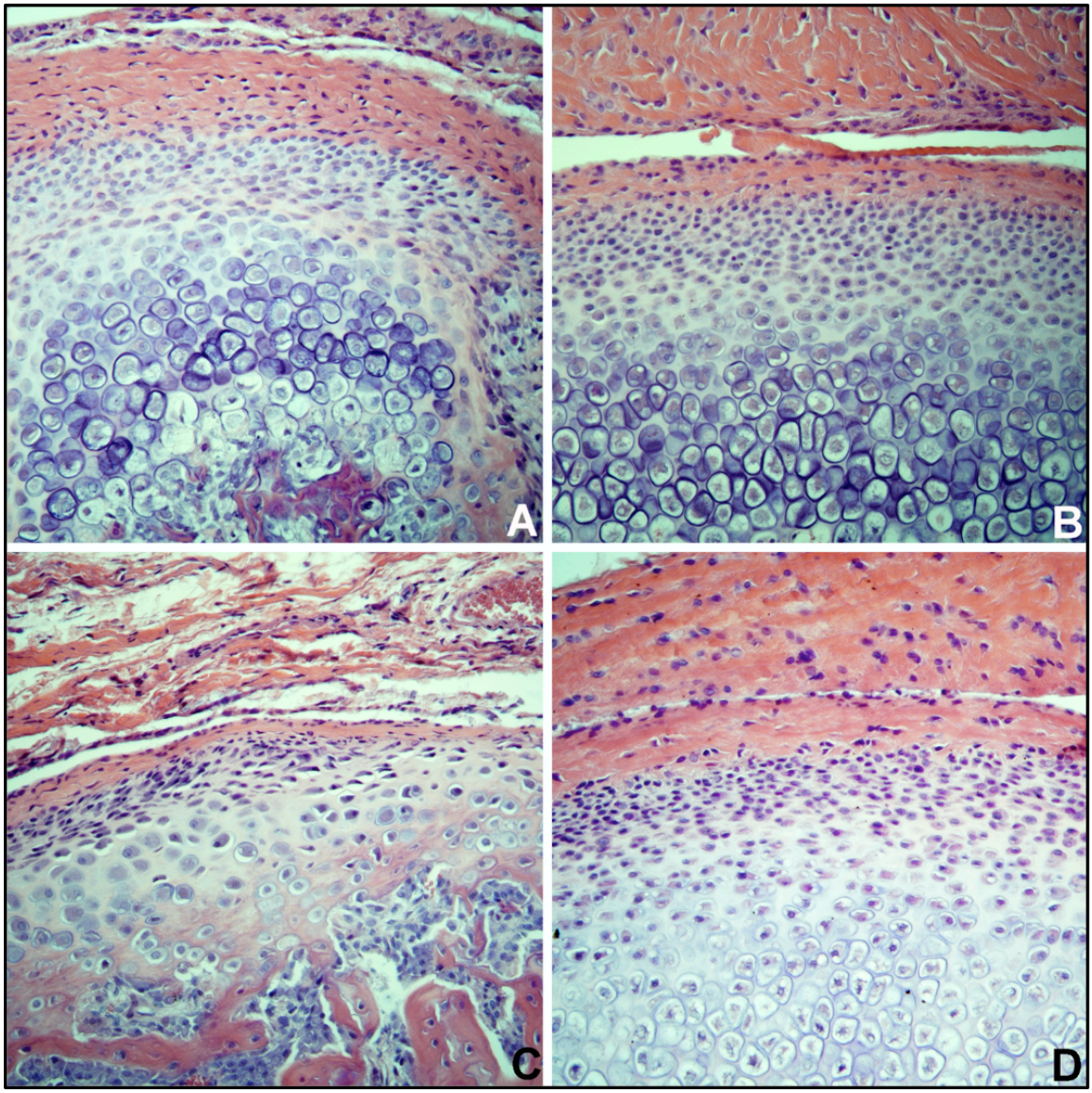

Figure 4 Description: A. Control, B. Partial rescue w. PFI-2, C. Partial Rescue with PFI-2, D. CFA

FIGURE 5: Masson's Trichrome Analysis [RATS]

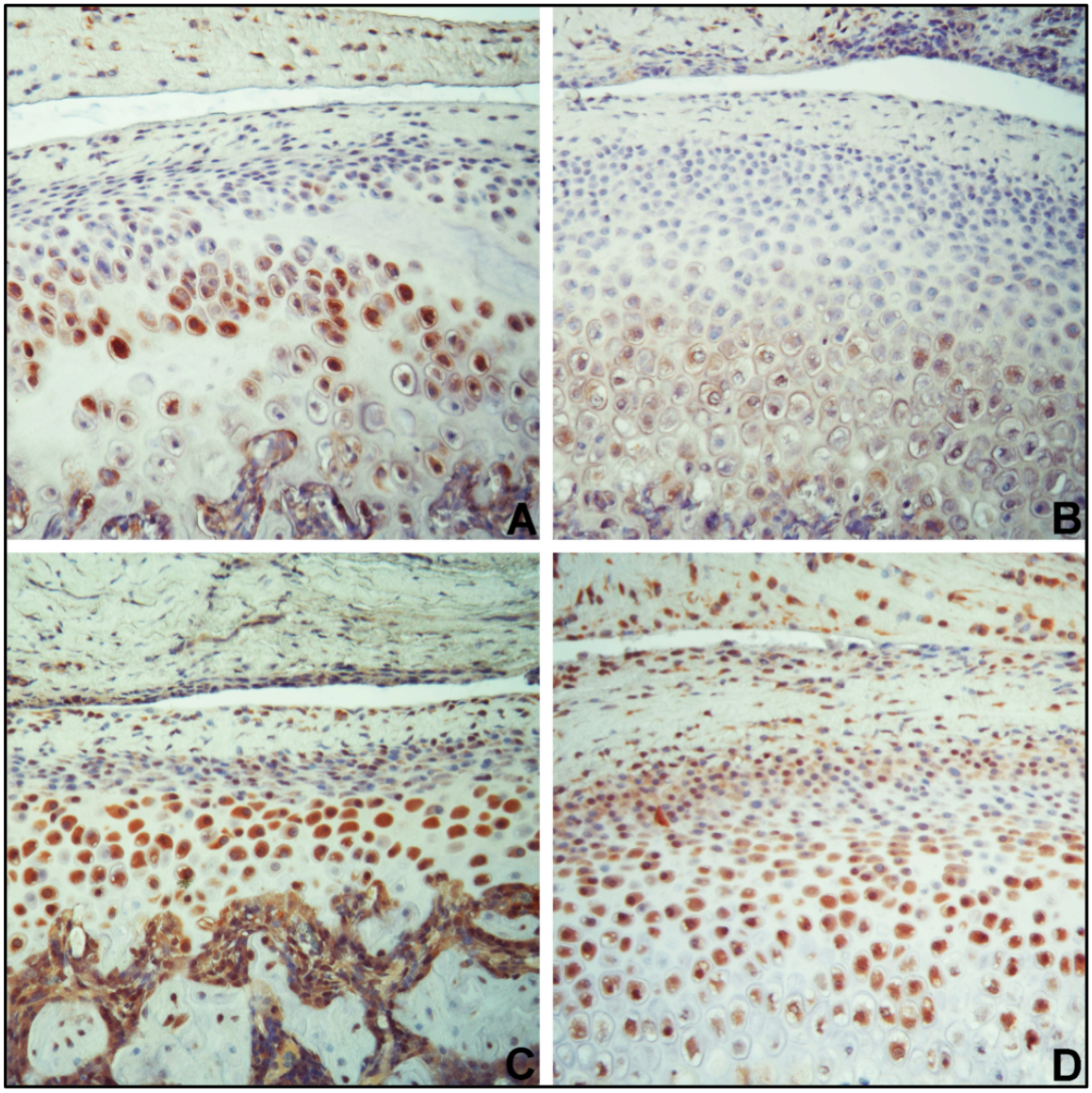

Figure 5 Description: A. Control, B. Partial rescue w. PFI-2, C. Partial Rescue with PFI-2, D. CFA

FIGURE 6: Immunohistochemistry Analysis with IL1-B [RATS]

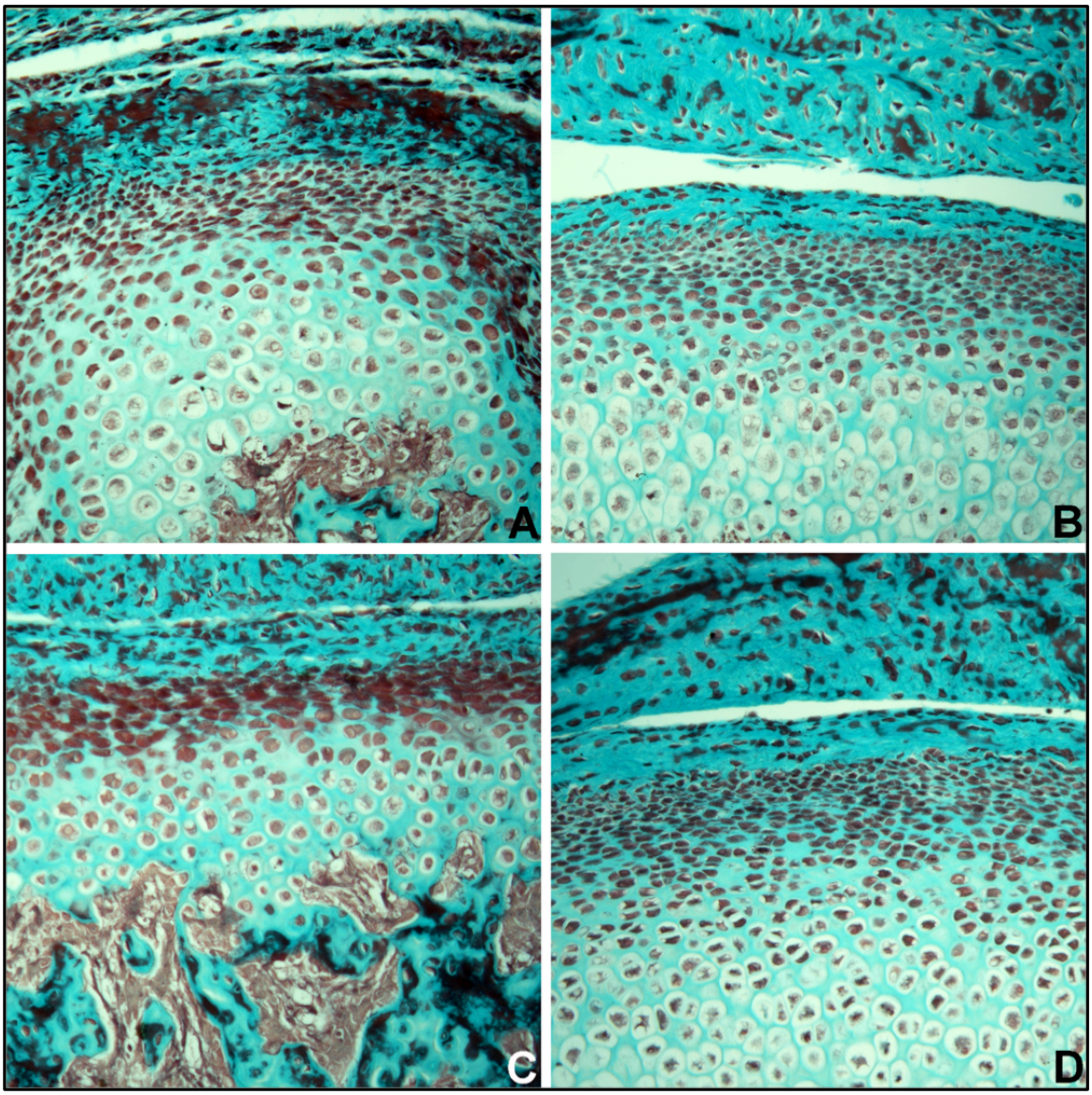

Figure 6 Description: A. Control, B. Partial rescue w. PFI-2, C. Partial Rescue with PFI-2, D. CFA

FIGURE 7: H/E [A+B] + Masson's Trichrome [C+D] in [MICE]

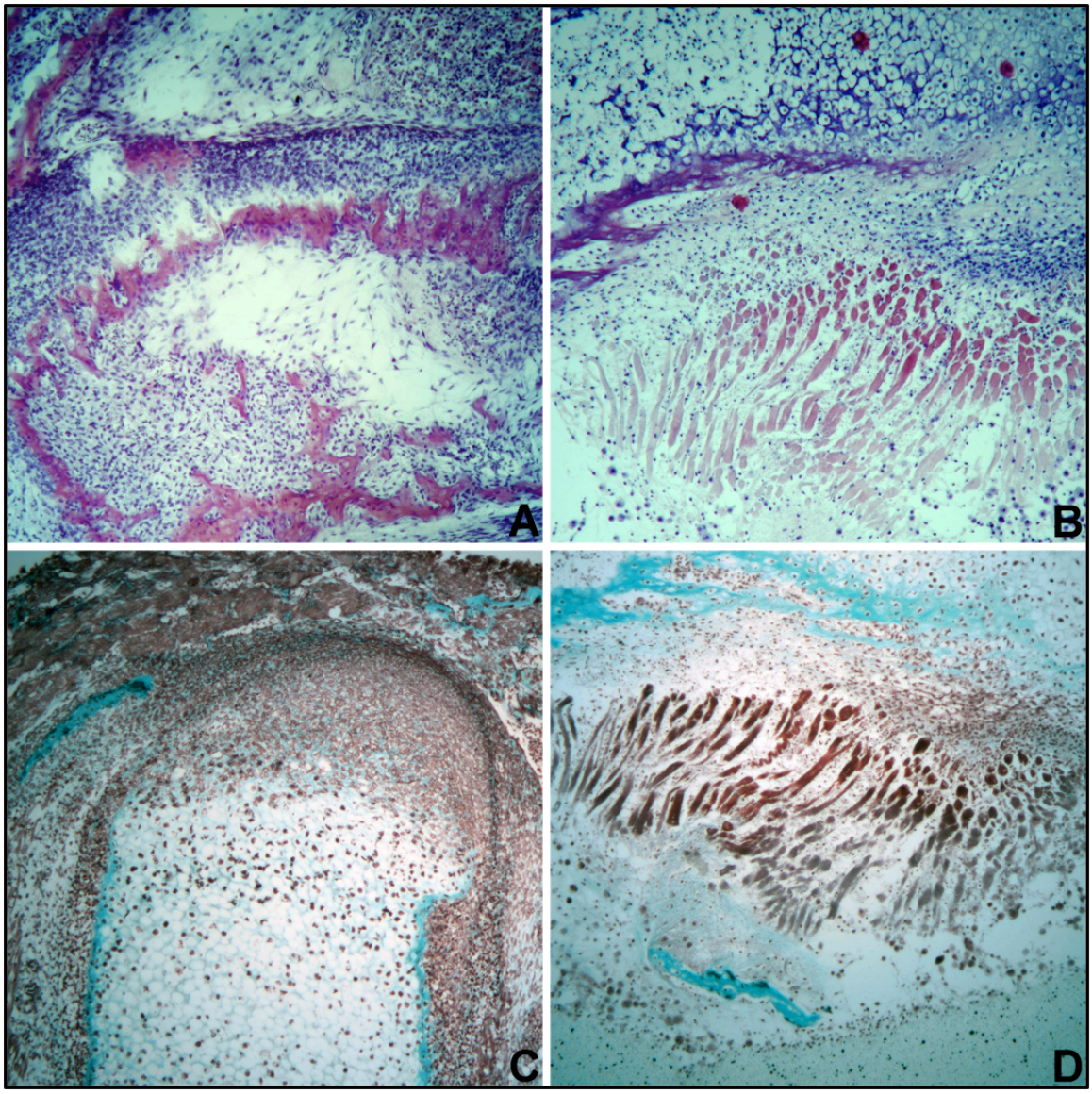

Figure 7 Description: A. Inflammation, B. Partial rescue w. PFI-2, C. Inflammation, D. Partial rescue w. PFI-2