Halofuginone Prevents the Progression of Osteoarthritis

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Halofuginone prevents the progression of osteoarthritis

A Thesis Presented by

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to

The Faculty of Medicine in partial fulfillment of the requirements for

the degree of

Doctor of Medical Sciences

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ACKNOWLEDGEMENTS

“This thesis is dedicated to my family, for their endless love and support, and to all the individuals whom I learned from and helped me to have a productive, rewarding and enjoyable four years of residency at Harvard School of Dental Medicine. “
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ABSTRACT

**Background:** Halofuginone (HF) is a natural product that has been shown to have therapeutic benefits in a variety of pathologic conditions, from cancer to autoimmune diseases. These beneficial effects are mainly through inhibition of pro-inflammatory cytokines. Inflammatory cytokines have been recently shown to exert a crucial role in development of osteoarthritis and one of their most important targets is Matrix metallopeptidase-13 (MMP-13). In this preclinical study, we investigated the effect of HF on the progression of Osteoarthritis.

**Methods:** The effect of adding HF ± IL-1β/TNF-α on mRNA expression of MMP-13 on the C28/I2 Chondrocytes was evaluated with qPCR *in vitro*. To study the effect of HF *in vivo*, a mice destabilization of the medial meniscus (DMM) osteoarthritis model was employed and untreated control group were compared with early treatment with HF (starting 48 hours post-surgery for 12 weeks) and late treatment (4 weeks post-surgery for 8 weeks). After sacrificing the animal, joint destruction in the knee tissue was assessed with Safranine O/Fast Green staining and MMP13 expression was evaluated by immunohistochemical staining.

**Results:** In chondrocytes, MMP-13 expression was significantly increased with IL-1β or TNF-α (13.77-fold, p-value<0.05 and 5.8-fold, p-value<0.05, respectively). Addition of HF Significantly reduced MMP13 expression close to baseline levels (1.73-fold, p-
value<0.05 when co-incubated with IL-1β and 1.6-fold p-value<0.05 when co-incubated with TNF-α). Injection of HF in the mice osteoarthritis model in vivo, significantly reduced Osteoarthritis progression according to OARSI scoring (3.8 vs. 1.16 vs. 1.07, p-value<0.05) and there was no difference between early vs. late administration of HF. While MMP-13 was overexpressed in the control (DMM surgery without HF treatment) groups in IHC staining, expression of MMP13 was suppressed by injection of HF in both groups.

**Conclusion:** Halofuginone inhibits MMP-13 expression and diminishes joint destruction. These preclinical findings provide supporting data for clinical investigation of HF as a therapeutic target for osteoarthritis.

**Keywords:** Osteoarthritis, Halofuginone, MMP-13, Knee Joint, TMJ
INTRODUCTION and REVIEW OF LITERATURE

I. Osteoarthritis:
   a. Overview

Osteoarthritis (OA) is the most common type of arthritis and musculoskeletal condition with a significant health and social burden and one of the main causes of disability worldwide. It is caused by destruction of articular components, which leads to the functional failure of synovial joints (1-3) (Figure 1). Over 70% of Americans above the age of 55 are affected by OA, which mainly influence knees, hands, hips, spine and Temporomandibular joint (TMJ) (4-6). It starts with joint pain accompanied by different degrees of limitation in the range of motion and reduced quality of life. Due to the increasing longevity of life and obesity in the world, the destruction caused by OA rapidly grows which influence life quality of the affected individuals, which has also enormous costs to the health care system.

**Figure 1:** Destruction of cartilage in an Osteoarthritic joint (Right image, pointed by Black arrow) compared to an intact cartilage in a healthy joint (Left image)
b. Pathogenesis

OA is a total joint disease that leads to loss of articular cartilage, peri-articular bone, synovial joint lining, the supporting connective tissue, and osteophyte formation (1, 2, 5, 7, 8). Adult articular cartilage is avascular, and its cellular components are chondrocytes, which has low turnover replacement of the extracellular matrix (ECM). Chondrocytes are specialized mesenchymal cells that are responsible for synthesis and repair of cartilage matrix (9). Articular chondrocytes can respond to direct biomechanical stress by increasing the synthetic activity and/or up-regulation of the inflammatory cytokines and they are believed to be the central players of osteoarthritis (10).

In a healthy joint, chondrocytes maintain the matrix components in a low turnover condition as opposed to an osteoarthritic joint where chondrocytes, alongside the other cells in the synovium, get activated due to exposure to abnormal insults, like high magnitude mechanical stress, inflammatory cytokines or different amount of matrix proteins and degradation products (11-13). After initiation of the disease, the pathological changes in OA follows a consistent pattern, which is chondrocyte clustering subsequent to up regulated cell proliferation, which in turn increase the general synthetic activities including ECM, expression of degradative proteinase genes, loss of proteoglycans and collagen type II degradation (14, 15).

OA is characterized by significant alterations in the composition, structure and function of the articular cartilage. Besides metabolic imbalance, up regulation of the whole
endochondral ossification process is an important determinant of OA progression, which starts with cell proliferation toward articular chondrocyte hypertrophy and apoptosis. To understand the pathophysiology of OA, it is critical to extend our knowledge of how abnormal biomechanics impact articular integrity and chondrocyte pathobiology (16) (Figure 2).

Figure 2: Biomechanical stress, leads to up-regulation of the synthetic activity and the inflammatory cytokines, chemokines, adipokines, Toll-like receptor (TLR) ligands, and other inflammatory mediators such as nitric oxide. The up regulation of cartilage-degrading proteinases by extracellular matrix (ECM) proteins and products can promote catabolic activation, phenotypic shift, and apoptosis.

Osteoarthritis. Current opinion in rheumatology. 2011;23(5):471-8. Figure used with permission.

Chondrocyte differentiation is an important event in the development and progression of
OA. Transforming growth factor-β (TGF-β) signaling pathway is a major regulator of chondrocyte differentiation and cartilage development, maintenance and repair and its alterations contribute to the pathogenesis of OA as well (10, 17-20). TGF-β initiates chondrogenesis and chondrogenic condensation via activation of fibronectin synthesis and N-CAM regulation (21, 22). It also stimulates chondroprogenitor cell proliferation and differentiation through Smad3-dependent activation of SOX9 transcription (23). Smads are intracellular effectors of membrane-bound serine/threonine kinase receptors which is the main route of TGF-β signaling. Modulation of receptor-Smads signaling is one important mechanism, through which TGF-β signaling exerts its biological functions.

TGF-β signaling can have a dual impact on chondrocyte differentiation. Chondrocyte terminal differentiation can be activated by TGF-β signaling via the Smad1/5/8 route while it can be inhibited via the Smad2/3 route (24).

The controversial role of TGF-β in regulating a chondrocyte’s terminal differentiation is a result of differential activation of various Smad routes, which can have opposite regulatory effects on the terminal differentiation of chondrocytes. One suggested mechanism for development of osteoarthritis is a switch in TGF-β signaling, from mainly Smad2/3 to dominant Smad1/5/8 signaling which alters articular chondrocytes and promotes progression of OA (10, 18, 25). It is anticipated that the role of TGF-β signaling on chondrocyte differentiation can be modified by factors like mechanical loading, inflammation and aging (10, 17, 26, 27) (Figure 3).
Chondrocytes have receptors for extracellular matrix (ECM) components which respond to mechanical overloading or trauma (28). Among these receptors, there are several
integrins, which are receptors for fibronectin and type II collagen fragments. Stimulation of these receptors activates the production of matrix degrading proteinases and inflammatory cytokines (29-33). Activation of inflammatory-induced and/or stress-induced signaling causes phenotypic shift, apoptosis and aberrant overexpression of inflammation-related genes and catabolic genes that play an important role in the pathogenesis of OA (12, 34-36). These signals stimulate the release of reactive oxygen species (ROS) that cause chondrocyte apoptosis and activation of stress induced kinases that promote production of several Metalloproteinases (MMP) especially MMP-13, nitric oxide synthase (NOS)-2, Cyclooxygenase (COX)-2 and a disintegrin and metalloproteinase (ADAM) (14, 31) (Figure 4).

**Figure 4:** Stimulation of receptors for type II collagen fragments activates the production of matrix degrading proteinases and inflammatory cytokines, which lead to release of ROS and production of MMP-13.

MMP-13 is the major enzyme that causes cartilage degradation and compared to other
MMPs, expression of MMP-13 is relatively restricted to the connective tissue (7, 37). Despite integrins, which bind to the collagen fragments, discoidin domain receptor 2 (DDR2) binds to collagen fibrils type II and X and subsequently stimulates its integral receptor tyrosine kinase. Activation of DDR2 in turn induces the expression of MMP-13 resulting in further cleavage of collagen type II (38-40). In other words, DDR2 is an additional receptor, which becomes activated by biomechanical triggers and subsequently leads to additional disruption of the peri-cellular matrix and activation of MMP-13 (41). MMP-13 not only causes irreversible joint damage in OA and promotes the progression of the disease, but more importantly also initiates the onset phase by causing the chondrocytes to leave their natural growth and differentiation arrested state (14).

Osteoarthritis have been considered as a non-inflammatory disease due to lack of neutrophil presence in the synovial fluid (42). On the other hand, some symptoms of the disease including pain, swelling, stiffness and loss of function of the joint show footprints of inflammation in the disease. Moreover, arthrocentesis from the osteoarthritic joints shows inflammatory cytokines, chemokines, and other inflammatory mediators traced in and can be measured in the synovial fluid of osteoarthritic patients (12),(43). (Table 1)
### Evidence of Inflammation in OA

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<th>Evidence</th>
<th>Observation</th>
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<tr>
<td>Clinical</td>
<td>Effusion, Joint swelling or palpable synovitis</td>
<td>Ayral et al 1999 (44)</td>
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<td>Local signs of inflammation</td>
<td>Krasnokutsky et al 2008 (45),</td>
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<td>Sudden increase in pain</td>
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<td>Night pain and morning stiffness</td>
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<td>Imaging</td>
<td>Association between ultrasound-detected synovitis and clinical symptoms of</td>
<td>Ostergaard et al 1997 (46),</td>
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<td>synovitis</td>
<td>Fernandez-Madrid et al 1995 (47),</td>
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<td>Macropscopic synovial changes detected by arthroscopy</td>
<td>Ayral et al 1999 (48),</td>
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<td>in about half of patients with knee OA</td>
<td>Loeuille et al 2005 (49),</td>
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<td>Gadolinium-enhanced synovium and increased synovial volume detected by MRI</td>
<td>D’Agostino et al 2005 (50),</td>
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<td>Correlation between MRI and histological observations</td>
<td>Ayral et al 2005 (51)</td>
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<td>Arthroscopic synovitis associated with progression of knee OA</td>
<td>Keen et al 2008 (52)</td>
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<td>Synovitis seen using ultrasonography of symptomatic joints</td>
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<td>Histological</td>
<td>Synovitis in the velocity of degenerative cartilage</td>
<td>Myers et al 1990 (53),</td>
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<td>Infiltration of mononuclear cells</td>
<td>Alsalamed et al 1990 (54),</td>
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<td>(monocytes/macrophages, activated B cells and T cells)</td>
<td>Nakamura et al 1999 (55),</td>
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<td>Synovial hypertrophy and hyperplasia</td>
<td>Shibakawa et al 2003 (56),</td>
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<td></td>
<td>Increased angiogenesis</td>
<td>Ayral et al 2005 (51),</td>
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<td></td>
<td>Adaptive immune T-cell and B-cell responses to fragments of extracellular</td>
<td>Walsh et al 2007 (57),</td>
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<td></td>
<td>matrix</td>
<td>Gobezie R et al 2007 (43),</td>
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<td>Sellam J et al 2010 (58),</td>
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<td>Maldonado M 2013 (59)</td>
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<tr>
<td>Molecular</td>
<td>Production and/or release of proinflammatory cytokines (TNF, IL-1β, IL-6, IL-8, IL-15, IL-17, IL-21)</td>
<td>Smith et al 1997 (60),</td>
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<td>Increased activity of MMPs</td>
<td>Furuwaza-Carballeda and Alcocer-Varela 1999 (61),</td>
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<td>Increased production of PGE2 and nitric oxide</td>
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<td>Release of EGF and VEGF</td>
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<td>Production of adipokines</td>
<td>Farahat et al 2003 (63),</td>
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<td></td>
<td>Release of proinflammatory and pain neurotransmitters (substance P, NGF)</td>
<td>Yuan et al 2004 (64),</td>
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<td>Involvement of macrophages in osteophyte formation via BMPs</td>
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<td>Presle et al 2006 (66),</td>
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<td>Raychaudhuri and Raychaudhuri 2009 (69)</td>
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<td>Biological Markers</td>
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<td>Increased levels of CRP (detected by ultrasensitive assay)</td>
<td>Masuhara et al 2002 (71),</td>
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<td>Pearle et al 2007 (72)</td>
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Table 1: Evidence of inflammation in OA
A growing list of studies now show that inflammation is the major factor in the progression of joint destruction, in addition to the signs and symptoms of the OA (58, 59, 73-78). Our current knowledge confirms that OA is initiated by both mechanical and inflammatory signals, activating similar signaling pathways and influences chondrocytes in a flawed attempt (31, 79-81).

Synovitis is common in the early stage and late stage of OA. This involves infiltration of mononuclear cells in the synovial membrane which accompanies with production of pro-inflammatory mediators like interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α) and various chemokines (58).

Non-traumatic cyclical loading of joints with appropriate magnitude can inhibit the induction of IL-1 that would initiate the cartilage matrix degradation (82). However, the impact of abnormal biomechanical stress on chondrocytes leads to expression of IL-1 at the concentrations which induce the expression of MMP-13, TNF-α, and other catabolic genes. IL-1β and TNF-α also activate other pro-inflammatory cytokines like IL-6, IL-17 and IL-18, and chemokines like IL-8, which many of these factors promote the catabolic responses in chondrocytes (12) (Figure 5).
Primitive CD4 T cells differentiate into different effectors and regulatory subsets to correlate immunity response, among them are T\textsubscript{H}1, T\textsubscript{H}2 effector subsets. These T cells, in turn, differentiate into pro-inflammatory T helper 17 (Th17) cells, which are also called tissue-protective induced T regulatory cells (83, 84). One important role for cytokines IL-1\textbeta, IL-23, IL-6 and TGF-\beta is their influence on differentiation and maintenance of
Th17 lineage (83-86) (Figure 6).

**Figure 6:** Th cells lineage (Th1, Th2 and Th17), T regulatory (Treg) cells and induced T regulatory (iTreg) cells (Th3 and Tr1) in Osteoarthritis. Osteoarthritis Cartilage. 2012 Dec;20(12):1484. Figure used with permission.

Th17 cells were initially introduced to contribute in the pathogenesis of autoimmune diseases, and later their significant role in other inflammatory processes, such those in infectious diseases was unravel (87-90) (Figure 7).
Th-17 cells produce pro-inflammatory cytokines, mainly IL-17, which activates other cytokines, chemokines and prostaglandins. Similar to rheumatoid arthritis (RA), the role of Th-17 in the pathogenesis of osteoarthritis has been recently suggested (Figure 8 and 9).

It has been shown that although there are quantitative differences in the number of Th17 cells between OA and RA, there is no qualitative difference between the expression of activation markers of Th17 cells between these two diseases (91).
**Figure 8:** The role of pro-inflammatory cytokines in the pathophysiology of OA.

Macrophages and T-cells have a central role.

**Figure 9:** The role of Th17 in the Osteoarthritis
c) Etiology and risk factors:

Various risk factors contribute to the development of osteoarthritis. One is mechanical factors such as joint injury, occupational/recreational usage, reduced muscle strength, joint laxity and joint misalignment, all through the same mechanism: abnormal biomechanics either from mechanical trauma on a normal joint or a normal loading on a mal-positioned joint (12). Other factors include genetic factors and constitutional factors such as ageing, female sex, obesity and high bone density.

The articular surface has a critical role in transferring the load in the joint. There is strong evidence that certain conditions in which a higher load transfer or altered patterns of load distribution are produced can initiate and accelerate the development of OA (92).

Studies from *in vitro* mechanical loading experiments show that traumatic static compression causes damage to the collagen network and down regulates the synthesis of cartilage matrix proteins, while dynamic compression up regulates the matrix synthetic activity (93). In response to trauma, the whole expression of inflammatory cytokines, stress response factors and cartilage-degrading proteinases are up regulated (36).


**d) Current treatment**

Several modalities are currently practiced for symptomatic treatment of OA. Non-Pharmacological management of Osteoarthritis includes exercise and manual therapy, weight loss, electrotherapy, acupuncture, aids and devices and invasive treatments such as debridement and total joint replacements. Pharmacological management of osteoarthritis includes analgesics and anti-inflammatory drugs such as NSAIDs and COX-2 inhibitors, opioids and intra-articular corticosteroids injections.

Currently, there is no effective treatment of OA that can alter the progression of the disease. Current therapeutic regimens only provide symptomatic pain relief and do not have a major effect on the inflammatory process that leads to the progression of the disease. Since these drugs are not targeting the molecular processes responsible for the initiation and progression of the disease, there is no proven structure modifying therapy available to date (94).

Studies have shown that the pain and severity of the disease are associated with the synovitis (65, 95) and synovitis can be considered as a potential target for therapy in OA (58, 96) (Figure 10).
**Figure 10:** Pharmacologic therapies and the site of their impact. Nat. Rev. Rheumatol. doi:10.1038/nrrheum.2010.178. Figure used with permission.


e) **Osteoarthritis in TMJ**

Most of the current understanding of cartilage degradation and OA is from large load bearing joints like the knee joint (97). In the field of prosthodontics, the temporomandibular joint (TMJ) is of large importance because reconstruction of the occlusion has a direct effect on the patient’s TMJ. Like the knee joint, the TMJ is a complex synovial joint that has an articular disk and it is believed that cartilage degeneration process of the TMJ in OA follows a very similar pattern to that of the knee joint (98, 99). Their main difference is the type of cartilage that lines the articular surfaces. In the knee joint, the articular lining is hyaline cartilage, while in TMJ the lining is fibrocartilage (100).

Temporomandibular disorder (TMD) affects a majority of the population as a myofacial dysfunction and degenerative joint disease, which is more common in women between the ages of 20-40 years old (101, 102). OA is the most common pathology of TMD (103).

Osteoarthritis of the TMJ is also a degenerative joint disease, which is age-related and is characterized by the continuous joint destruction of the articular tissues in the condyle of the mandible and glenoid fossa. Additionally, it has been shown that it is usually initiated with increased loading of the joint.

Signs of OA in TMJ include pain on palpation or pain during opening and lateral excursive movements with crepitation during mandibular movement (102, 104). Orthopantomogram or CT scans are usually used to evaluate the joint for any signs of
wear or osteophyte formation and to diagnose OA in TMJ (105) (Figure 11).

![Image: Osteoarthritic TMJs (B), CT-scan view, compared to the normal Joint (A).](image-url)

**Figure 11:** Osteoarthritic TMJs (B), CT-scan view, compared to the normal Joint (A). Dentomaxillofacial Radiology (2015) 44, 20140235. Pictures used with permission.

Interestingly, in a comparison between OA in the knee joint and OA of TMJ, it was demonstrated that production of inflammatory cytokines like PGE₂ is more significant in the TMJ compared to the knee joint, suggesting that inflammation may play a more significant role in pathogenesis of TMJ OA compared to the knee joint (100).
f) Osteoarthritis animal models

For better understanding of the disease, various animal models of OA have been established (106). The mice used for OA studies should have at least 10 weeks of age due to the fact that OA is a disease of adults and growing animals have a capacity to manage joint damage and at that age mice are mature skeletally. Generally, development of spontaneous OA is linked to a particular genetic background. For example, STR/ort mice spontaneously develop degenerative changes of knee joints with ageing, which is similar to human osteoarthritis.

However, spontaneous OA models develop over a much longer period of the animal’s life. Another method of creating an acute model for the study of OA is intra-articular injection of monosodium iodoacetate (MIA) or collagenase (107). However, it has some limitations. For example, due to the fact that MIA is a metabolic poison, there would be extensive death of chondrocytes in this model. Thus, application of this model is mainly limited to the induction of OA and it is mainly used for pain related Osteoarthritis research (108).

Mechanical instability-induced osteoarthritis mouse models have been established with a microsurgical technique to cause instability in the knee joints. This technique has been shown to be reproducible and the disease is very similar to human OA. Four slightly different methods have been developed which cause different grades of OA.
The partial medial meniscectomy (PMM) and medial collateral ligament transection (MCLT) models were the first surgical models of OA in the mouse (109). However, OA did not develop in the surgical knee and more severe OA was actually induced in the contralateral limb. This result could indicate that increased weight bearing was occurring in the non-operated limb (110). Visco et al (109) later hypothesized that mechanical instability created in the PMM and MCLT model could be too severe to overcome by the animal.

The two superior and more commonly used models of induced-OA in mice are the anterior cruciate ligament transection (ACLT) and destabilization of the medial meniscus (DMM). In the ACLT method (111) the first destructive changes in the mouse articular cartilage were a defect in the superficial zone evidenced by a decrease of Safranin-Orange (Safranin-O) staining, followed by a progressive cartilage destruction. These changes were very similar to human OA pathology recorded with arthroscopic and histological findings (111-113). Although instability was present in the whole knee joint, osteoarthritic changes were much more prominent in the tibial cartilage compared to the femoral condyle cartilage. This is due to the fact that tibial cartilage is much thinner and also osteophyte formation is very rare in the femoral condyle. For the same reason, current ACL models are more focused on OA changes in tibial cartilage (113).

In Figure 12 you can see an overview of the knee joint and the MMTL ligament which is transected to generate the generate destabilization of the medial meniscus (DMM) and the ACL is transected in the ACLT model (114).
**Figure 12:** (a) Outline of a right knee joint. The MMTL is transected to generate destabilization of the medial meniscus (DMM) and the ACL is transected in the ACLT model. (b) Overview of Safranin-O stained of a mouse knee joint 4 weeks after DMM induction. Grey arrow indicates articular cartilage, white arrow indicates growth plate. ×40 magnification. ACL: anterior cruciate ligament; F = femur; LCL = lateral collateral ligament; LFC: lateral femoral condyle; LM = lateral meniscus; LTP: lateral tibial plateau; MFC: medial femoral condyle; MM: medial meniscus; MMTL = medial meniscotibial ligament; MTP: medial tibial plateau; PCL: posterior cruciate ligament; T = tibia.

Taken from Mouse genetics Methods and Protocols ISSN 1064-3745, with permission.

DMM model has been used for induction of OA with great ease and reproducibility (114). Studies have been conducted to compare the severity of OA in both models of ACL and DMM surgery, aiming to identify a mild to moderate model of OA, in which the effects of disease modification in the knocked out mice would not be influenced by the severe biomechanical destruction associated with more severe models. Moreover, more severe models of OA have a great association with iatrogenic destruction, different biomechanical factors, or regenerative changes such as dramatic osteophyte formation or...
ankylosis, which could make a false impression in the assessment of cartilage degradation.

The DMM model in these studies was completely reproducible and showed a slower mild to moderate osteoarthritic progression, which is very similar to the osteoarthritic changes in human. DMM model has been applied for evaluation of knocked out mice models in order to evaluate the impact of specific genes in the development of OA, its progression and severity (114-116) (Figure 13).

**Figure 13:** Osteoarthritis progression after DMM in wild-type mice. Osteoarthritis progression is demonstrated on medial areas of Safranin-O stained sections of right hind knee joints at 2 (b), 4 (c), 6 (d), 8 (e), and 12 (f) weeks after DMM surgery. (a) is the contra lateral joint. *Arrows* indicate articular cartilage damage of femoral condyle and tibial plateau. (a–f) ×100 magnification

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II. Halofuginone

Halofuginone (HF) is a small molecule that is analogue of Febrifugine, an alkaloid derivative isolated from Dichroa febrifuga plant (117). This plant has been used for treatment of malaria for centuries (118). Halofuginone [7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone] (Figure 14) has been used in poultry centers (119, 120). It is also FDA approved additive for the animal’s food for prevention of coccidiosis (121) and against protozoan parasites in cattle (122).

![Chemical structures of Febrifugine and Halofuginone](image)

**Figure 14:** Chemical structures of Febrifugine and Halofuginone

Recently HF has attracted a lot of attention because of its broad beneficial biologic activities against a variety of diseases such as malaria, cancer and fibrosis related and autoimmune disorders (123-125).

HF exerts its biological functions through two distinct mechanisms: (1) inhibition of TGF-β signaling pathway (124, 126) and (2) Anti-inflammatory responses through inhibition of Th17, as a result of inhibition of prolyl-tRNA synthesis and activation of
Amino Acid Response (AAR) pathway (127-129).

The crosstalk between these two mechanisms may be the TGF-β pathway. Th17 differentiation in mice initiates with TGF-β, IL-1β and IL-6. In humans, combination of TGF-β and IL-21 initiates the differentiation of primitive T cells.

HF, \textit{in vitro}, was shown to down regulate Smad3 protein (130), reduce the amplitude of TGF-β dependent Smad3 phosphorylation and up regulate inhibitory Smad7 in fibroblasts, pancreatic and hepatic cells, myoblasts and tumor cells (131-135).

HF is an antifibrotic agent as well. Fibrosis is a result of chronic inflammation, which leads to destruction of organ’s architecture and function. Altering TGF-β, MMPs and the Tissue inhibitor of metalloproteinases (TIMPs) play an important role in the ECM regulation. The antifibrotic feature of HF was discovered by serendipity and later on explored further in animal models and in humans (117, 124, 126). \textit{In vitro}, HF reduces the amplitude of collagen α1 (I) gene expression in murine, avain and human fibroblasts derived from cornea (130) and \textit{in vivo} in scleroderma and graft versus host disease (GVHD) patients (136). In animal models, when excess collagen was a characteristic of the disease, HF can inhibit the collagen synthesis (131, 137) and resolve the established fibrosis (138, 139). The ability of HF to resolve the pre-existing fibrosis is because of its ability to down regulate the collagen synthesis and at the same time, up regulates collagenase activity by increasing synthesis of TIMPs, which that regulates MMPs activity (138, 140, 141).
HF inhibition of ProRS activity leads to intracellular accumulation of uncharged tRNA and mimics the reduction of available cellular proline. This pathway requires ATP, which binds the 2 parts of HF on the human ProRS in a way that one part mimics proline attachment and the other part mimics the 3’ end of tRNA (142).

Th17 cells regulate inflammation through its produced cytokines, mainly IL-17. IL-17 is a pro-inflammatory cytokine that stimulates other cytokines, chemokines and prostaglandins. HF down regulation of T-cell proliferation is correlated with up regulation of cell apoptosis and decrease in proline uptake, and shows the participation of amino acid starvation response (AAR) (128, 143, 144). This process takes place by activating the integrated stress response (IRS) which happens when cells experiencing metabolic, hypoxic or oxidative stress (145). HF activates the AAR by mimicking proline removal, and results in down regulation of inflammatory process (Figure 15).

**Figure 15:** AAR activation by HF inhibits prolyl tRNA charging enzyme EPRS results in uncharged tRNA accumulation. Uncharged tRNA activates the sensor kinase GCN2, leading to phosphorylation of eif2a and changes in translation and transcription of pro-inflammatory genes.
HF also has inhibitory effect on angiogenic cascade like prevention of endothelial cell MMP-2 expression and basement membrane invasion(146).

So far, HF has been studied in a number of clinical trials. It has been locally applied to the GVHD patients and has shown a significant decrease in collagen content without any systemic or local toxicity and no side effects (147). It has been used for AIDS related Kaposi sarcoma(148), in addition to a scleroderma trial, which revealed a statistically significant decrease in disease severity score (149).

HF has been also orally administered as a phase I clinical trial for patients with solid tumors and showed that therapeutically effective plasma levels can be reached without any toxicity. The minimum effective dose was 0.5 mg/kg and the maximum tolerant dose (MTD) was 3.5 mg/kg. The dose limiting toxicity (DLT) symptoms were vomiting, fatigue and nausea (150).

In a similar fashion to autoimmune and chronic inflammatory disease, therapeutic modulation of the tissue remodeling and invasive activities of cells could provide a powerful complementary approach to the inhibition of the pro-inflammatory activities of immune cells. Despite the previous dogma, we now believe that pathologic infusion of inflammation is a distinct characteristic of OA, which reflects failure of the immune system to restore tissue homeostasis. HF promotes restoration of immune homeostasis by inhibiting the differentiation and expansion of Th17 cells. Our research group at Whitman lab has also shown that HF can act directly on fibroblasts to prevent their
activation, inhibit chronic inflammation, and modulate pathologic tissue remodeling by activating the same target pathway (the AAR pathway) that is critical for immunomodulation (127, 145).

Inflammation plays an essential role in the pathogenesis of the osteoarthritis and preventing inflammation without inducing generalized immunosuppression would hypothetically be a great therapeutic strategy.

MMP-13 is interstitial collagenases that degrade type II collagen in cartilage and this is a committed step in the progression of OA (151). The expression of MMP-13 is regulated by inflammatory responses and is substantially increased in response to IL-1β and TNF-α. Elevated levels of these collagenases are observed in arthritic tissues including OA. Cytokine-mediated MMP-13 gene regulation plays a crucial role in the pathogenesis pathway and thus, it can serve as a potential therapeutic target for inflammatory processes of the joints i.e. osteoarthritis (152).

We hypothesize that HF can regulate the interplay between inflammatory activation and MMP13 expression, and subsequently through that, it can prevent MMP13 mediated joint destruction in OA.
HYPOTHESIS AND SPECIFIC AIMS

- **Hypothesis**: HF prevents OA associated joint destruction and inhibit the progression of OA by down regulation of MMP13 expression

- **Specific Aim 1**: To investigate the effect of HF on MMP-13 expression in chondrocytes during inflammation.

  CT-28I2 Human chondrocytes will be treated with HF both in the presence and absence of inflammatory cytokines and MMP-13 gene expression will be evaluated using q-PCR.

- **Specific Aim 2**: To investigate the effect of HF on the progression of OA in Mice model.

  DMM OA-induced model is employed and animals are treated with HF. Progression of OA and expression of MMP-13 will be evaluated using Safranin O/fast Green and Immunohistochemistry staining, respectively. We will address whether timing of administration of HF (early after injury vs. late administration, 4 weeks after the injury) affects potential therapeutic efficiency of HF.
SIGNIFICANCE AND INNOVATION

• **Significance:**

This is preclinical study that introduces a novel therapeutic approach for the treatment of OA and establishes a new paradigm for alleviating the destruction of joint that is driven by patho-physiologic inflammation.

• **Innovation:**

Currently, there is no treatment for OA, which can alter the progression of the disease. The existing pharmacological treatment for the mean time provides only symptomatic pain relief and have minor effect on the inflammation which leads to the progression of the disease since these drugs are not targeted to block the molecular processes responsible for the initiation and progression of the disease, so there is no proven structure modifying therapy available to date. Eventually, as the disease progresses, OA patients have to undergo total knee replacement as an end stage treatment. This will be the first preclinical study, which evaluates the effect of HF in the osteoarthritis, and can provide supporting data for further clinical investigations to develop a drug to prevent the progression of Osteoarthritis.
METHODS AND MATERIALS

In vitro:

✓ Cell Culture:

Immortalized C-28/I2 chondrocytes (153), were used for the in vitro studies. Ten percent pure HF was kindly provided as a gift by Hangpoon Chemical Co. (Seoul, South Korea). Then it was further purified via HPLC to >99% purity and used for the experiments.

Cells were seeded at 3.5 x 10^5 cells per well and cultured at 37°C in 5% CO in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD) and 1% Penicillin-Streptomycin (Gibco). Upon confluence, cells were split using 0.03% trypsin-EDTA and seeded at the same density. The second day after seeding, the media was changed to DMEM/F-12 with 0.2% FBS and 1% PS. In order to assess the effects of Halofuginone on MMP-13 expression in vitro, we added Halofuginone (200nM per well), IL-1β (10ng/ml) or combination of them and an equal volume of the vehicle DMSO to the 4th group as a control. On the third day, we added 200nM of Halofuginone and on the fourth day we added the IL-1β 10ng/ml per well and 6 hours later we harvested the RNA for quantitative polymerase chain reaction (qPCR) assay.
To validate the findings with another inflammatory marker, we repeated this experiment and used TNF-α (10ng/ml) instead of IL-1β (Table 2).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day1</th>
<th>Day2</th>
<th>Day3</th>
<th>Day4</th>
</tr>
</thead>
<tbody>
<tr>
<td>- HF - IL-1β/TNF-α</td>
<td>10% FBS</td>
<td>1% FBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ HF - IL-1β/TNF-α</td>
<td>10% FBS</td>
<td>1% FBS</td>
<td>+ 200 nM HF</td>
<td></td>
</tr>
<tr>
<td>- HF +IL-1β/TNF-α</td>
<td>10% FBS</td>
<td>1% FBS</td>
<td></td>
<td>+10ng/ml IL-1β/TNF-α</td>
</tr>
<tr>
<td>+HF +IL-1β/TNF-α</td>
<td>10% FBS</td>
<td>1% FBS</td>
<td>+ 200 nM HF</td>
<td>+10ng/ml IL-1β/TNF-α</td>
</tr>
</tbody>
</table>

Table 2: Experimental conditions for qPCR experiments. Medium and supplements were added to the chondrocytes culture medium at the following concentrations: Halofuginone (200nM per well), IL-1β (10ng/ml)/ TNF-α (10ng/ml)

✓ Quantitative real-time PCR:

C-28/I2 chondrocytes were activated as described above, collected at the indicated times and cell pellets were flash-frozen in liquid nitrogen. Total RNA was isolated from cultured cells using RNeasy mini kits (Quiagen, Hilden, Germany) according to the
manufacturer’s instructions. Afterwards the RNA samples were treated with DNase I (Sigma).

Sybrgreen quantitative real-time PCR was performed on RNA samples following reverse transcription via SuperScript II first-strand cDNA synthesis kit (Invitrogen). All qPCR data was collected on an iCycler thermal cycler (Bio-Rad). Quantitative real-time PCR was performed using primers for MMP-13. Primer sequences are listed below.

(5’-CCAGTCTCC- GAGGAGAAACA-3’ [forward] and 5’-AAAAACAGCTCC-GCATCAAC-3’ [reverse]) and GAPDH (5’-GGTGAAGGT- CCGAGTCAACGGA-3’ [forward] and 5’-GAGGGATCTC- GCTCCTGGAAGA-3’ [reverse])

PCR reaction was performed at 95 °C for 3 min followed by 50 cycles of 95 °C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 4 min. A melting curve (temperature range between 55 and 95 °C with +0.5 °C intervals) was generated to test the specificity of the PCR product, at the end of the PCR cycles. A cDNA sample in each experiment was tested in triplicate and each experiment was performed two times. As an internal control, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. We tested the efficiency of PCR (standard curve) by plotting the amount of PCR product versus the known amount of a template, 0.001, 0.01, 0.1, 1, and 10ng. In theory, when the slope of the standard curve is -3.322, the efficiency of PCR is considered 100%. In our experiments, the efficiency reached 90% or higher.
In vivo:

✓ DMM OA model:

DMM injury-induced osteoarthritis model was used for the in vivo analyses of this study. Mice at the age of two months were anesthetized with Ketamine (100 mg/kg BW) and Xylazine (10 mg/kg BW) intra-peritoneally, and knees were prepared for aseptic surgery. Buprenorphine was provided peri-operatively at 0.09 mg/kg subcutaneously. After sedation, a bland ophthalmic ointment was placed in both eyes to prevent desiccation of the cornea. The criteria used to assess the level of anesthesia during the surgical procedure was respiration rate, muscular relaxation and toe or tail pinch. The level of consciousness was assessed every five minutes. Mice were clipped around the site of incision with an animal clipper. The clipped site was scrubbed with surgical disinfectants 10% Povidone-iodine three times and wiped with 70% alcohol. The joint capsule immediately medial to the patellar tendon was incised and opened with a #15 blade. To expose either the intercondylar region and providing visualization the meniscotibial ligament of the medial meniscus, a blunt dissection of the fat pad over the intercondylar area was performed. Then, The medial meniscotibial ligament (MMTL) was identified running from the cranial horn of the medial meniscus laterally into the anterior tibial plateau. Sectioning of MMTL with a #11 blade results in the destabilization of the medial meniscus (DMM). The MMTL anchors the medial meniscus (MM) to the tibial plateau, while the anterior cruciate ligament restricts the tibia from moving anteriorly, relative to
the femur. When MM is intact, there is more balance and area of contact between the articulating surfaces, providing a larger area to transfer the weight-bearing forces. Upon transection of the MMTL, the MM displaces medially, and weight bearing area will concentrate on a smaller area, leading to increased mechanical stress. Upon transection of the MMTL, the mechanical load on the knee joint is altered, which leads to increased mechanical stress so approximately 12 weeks post operation there is extensive articular cartilage degeneration in the surgical knee.

The joint’s capsule was closed using a continuous 8-0 tapered Vicryl suture and for the subcutaneous layer we used 7-0 cutting Vicryl. The skin was closed by one or more sutures and triple antibiotic ointment was applied on the incision. For the post-surgery recovery period, the mice were placed on an electric blanket to prevent hypothermia. Mice were stimulated every 10 minutes. After they return to sternal recumbency and have regained the ability to control their airways, the mice were returned to their room and monitored continuously until they have recovered from anesthesia. Buprenorphine at 0.05 mg/kg was provided subcutaneously every 12 hours in first 48 hours post-surgery. We monitored post-operative animals within 3 hrs post-operation including respiration rate, muscular relaxation and toe pinch. Animals were monitored twice daily for four days following surgery. We recorded the care (including administration of analgesics) and monitoring in animals post-operatively with the Rodents Surgery Report (RSR). After 4 days post-operation, we used the Experimental Illness Report (EIR) to record the care and monitoring of experimental illness in animals.
There are no reports indicating that such surgery affects the health of the mice, their eating, drinking and joint movement and the mice undergone surgery develop early histological signs of OA by the time when they are euthanized at 12 weeks post-surgery (154).

However, we were aware that animals may develop some complications/impairment such as lameness, irritation and sluggish immediately post surgery. Additional care, such as food and hydragel on a Petri dish placed on the bottom of the cage, were arranged to ensure that animals are able to reach food and water. In this case, the vet staff would contacted or animals were humanely euthanized.

In addition, if the animals show any sign of infection in the surgical area, we would have treat them with antibiotics, such as Vetropolycin (bacitracin-neomycin-polymyxin ointment) applied on surgical area.

The animals used for this study didn’t develop any complications or infection during the study.

Mice were kept in a virus-free animal facility at Harvard Medical School under a 12-hour lighting schedule (12 hours with light and 12 hours without light).
After the DMM surgery, we divided the animals into 3 groups, 6-7 mice per group and for Test 1 group we started the Halofuginone treatment with subcutaneous injections 2 days post DMM surgery (400 µg/kg diluted in 1ml PBS) every other day for 12 weeks. In the Test 2 group we started the Halofuginone treatment with subcutaneous injections 4 weeks post DMM surgery (400 µg/kg diluted in 1ml PBS) every other day for 8 weeks. The 3rd group served as the control group which they received 1ml PBS subcutaneous injections with the same intervals and pattern as their peer test groups.

Joint collection, processing and sectioning:

12 weeks post DMM surgery all the mice sacrificed by CO2 asphyxiation. We displace the air in a suitable airtight container with CO2 fed in from a compressed gas cylinder. We placed the animals gently in the container. We verified that the regulator has the correct psi (pounds per square inch), which is no higher than 5 psi. The flow rate should displace no more than 30% of chamber/cage volume per minute. Based on the guidelines, for a typical mouse cage this would be ~2 liters/minute. Unconsciousness occurs within 30 seconds, but animals were left in the container for several minutes to ensure death. We confirmed death by lack of cardiac pulse. We patiently waited approximately 3-5 minutes for the animal to stop moving or breathing. The Eyes should be fixed and dilated then we turned off CO2 tank and the regulator valve to stop the flow of CO2.
All protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Harvard University.

Then both left and right knee joints were harvested and prepared for sectioning and analysis. We dissected the knee joints and removed the skin and muscles. Sample tissues were fixed in 10% neutral buffered paraformaldehyde solution (VWR, Radnor, PA, USA) at the room temperature for 6 hours. Then they have kept in 4°C overnight, then we washed the samples with tap water every 30 min for 5 hours. Afterwards we decalcified them with 20% EDTA solution for two weeks and changed the decalcifying solution every two days. After 2 weeks we washed the joints with PBS for 30 mins. To check the efficacy of decalcification we did the neutralizing test with Cal-arrest (Decal Chemical Corp.). Then we dehydrate the specimens with ascending alcohol row (2–3 h EtOH 50 %, 2–3 h EtOH 70 %, overnight EtOH 96 %, 4 h EtOH 96 %).

We removed the alcohol and solvent with 2-propanol (twice 2–6 h) afterwards. The specimens were then ready for embedding.

The samples were processed and embedded in paraffin at 60°C and the blocks cooled down at least for 24 hours when they were ready for sectioning. We cooled the paraffin blocks at 0°C for at least 30 min before sectioning.

Six-µm-thick mid-saggital sections at different levels were cut from the medial compartment of the joints. The sections were mounted on the slide and dried for at least 3 days at 37°C, then they were stained with safranin O/Fast Green (SO/FG).
Safranin O/Fast Green staining:

All samples were deparaffinized in two 6 and 8-min serial washes of xylene. After deparaffinizing, the sections were rehydrated using different concentrations of Ethanol from 100% to 70% then the slides were stained with hematoxylin for 3 min, and rinsed and placed in Scott’s buffer for 2 min. After rinsing the slides into the tap water, slides were stained in 0.2% aqueous Fast green for 4 min and counterstaining with 0.5% Safranin O for 5 min. Lastly, the slides were dehydrated using ethanol serial dilution, then cleared in xylene, and mounted onto glass slides using Pre-mount. Each of the stained sections were evaluated under light microscope and photographed using a light microscope equipped with a CCD video camera.

Histomorphometric measurements were recorded by two blinded observers based on the semi-quantitative Modified Mankin scoring system recommended from the OARSI (155)(Table 3).
<table>
<thead>
<tr>
<th>Grade</th>
<th>Osteoarthritic damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>0.5</td>
<td>Loss of Safranin-O without structural changes</td>
</tr>
<tr>
<td>1</td>
<td>Small fibrillation without loss of Cartilage</td>
</tr>
<tr>
<td>2</td>
<td>Vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina</td>
</tr>
<tr>
<td>3</td>
<td>Vertical clefts/erosion to the calcified cartilage extending to &lt;25% of the articular surface</td>
</tr>
<tr>
<td>4</td>
<td>Vertical clefts/erosion to the calcified cartilage extending to &lt;25-50% of the articular surface</td>
</tr>
<tr>
<td>5</td>
<td>Vertical clefts/erosion to the calcified cartilage extending to &lt;50-75% of the articular surface</td>
</tr>
<tr>
<td>6</td>
<td>Vertical clefts/erosion to the calcified cartilage extending to &gt;75% of the articular surface</td>
</tr>
</tbody>
</table>

**Table 3:** Semi-quantitative Modified Mankin scoring system recommended

We evaluated all the specimens through all the weight bearing area of the joint with 50µm intervals. We scored each section according to the scoring system in the Table 2. We recorded the maximum number for each animal through the joint (figure 16).
Figure 16: Here is a representative image for morphometrical evaluation of osteoarthritis using a Safranin-O stained section. The histology image has taken from a right knee joint 4 weeks after DMM surgery. Black arrow shows area of acellular/unstained cartilage, grey arrow is the whole cartilage area and white arrow refers to subchondral bone plate (SBP). At ×100 magnification.
**Immunohistochemistry staining:**

The samples were processed for paraffin embedding. For each knee joint, a series of sectioning each 6-µm in thickness was taken. Approximately 120-150 sections represent the entire mouse knee joint from anterior to posterior side for the surgically induced OA mice. Every 20th section was collected for immunohistochemistry staining.

One slide from each mouse from all 3 groups has been selected for double immunohistostaining (Total of 6 or 7 per groups). The selection of the slide was based on the evaluation of safranin O/Fast Green stainings. We chose the slide right after or before the highest scored slide so in this case that one slide from each animal was representative of the worst condition of joint in each animal. Each slide contains up to 10 knee joint sections.

The sections were deparaffinized and quenched for endogenous peroxidase activity. The slides were incubated with primary polyclonal antibodies [rabbit polyclonal antibody against mouse MMP-13 (1:400 dilution, cat. AB8120; Chemicon, Temecula, CA)], at 4°C overnight. After washing with PBS, the slides were treated with secondary antibodies, goat anti-rabbit IgG Biotinylated at room temperature for 30 minutes. Color development was performed using a peroxidase substrate (Vector Nova-RED Substrate, cat. no. SK-4800; Vector Laboratories, Burlingame, CA) with avidin and biotinylated horseradish peroxidase (Vectastain ABC Kit, cat. no. PK-4000; Vector Laboratories).
Sections were counterstained with 0.2% Fast Green solution. Staining without primary antibody also were performed as negative controls.

✓ **Statistical analysis**

For experiments comparing two groups (e.g. relative mRNA expression), two-tailed unpaired student’s t-test was applied. Results of all quantitative assays involving multiple time points (e.g. OARSI score of joint destruction) were analyzed using analysis of variance (ANOVA) followed by post-hoc tukey test. P < 0.05 was considered to be significant difference.
RESULTS

To evaluate the anti-inflammatory effects of HF and their possible therapeutic effects in OA, we initially evaluated the effects of HF on expression of MMP13, the important mediator of joint destruction, in vitro. To confirm the in vitro findings, we then evaluated the effect of administration of HF in an OA animal model in vivo.


MMP-13 the central mediator in pathogenesis of OA and its overexpression is associated to joint destruction. The new paradigm of OA pathogenesis emphasizes on the role of inflammation in the progression of osteoarthritis. To evaluate the potential role of HF as a treatment that inhibit the progression of OA, we studied the effects of inflammation and addition of HF on MMP-13 mRNA expression. We used IL-1β and TNF-α, which are the main inflammatory cytokines in OA pathogenesis to produce the inflammatory environment, in vitro. We co-incubated chondrocytes with HF, IL-1β, or the combination of both, and compared the mRNA expression of MMP-13 to that of the untreated vehicle group. Inflammatory cytokine (addition of IL-1β) significantly increased expression of MMP-13 (relative expression was 13.77 ±1.3, p value= 0.006). When HF was also added
in addition to IL-1β, expression of MMP-13 was significantly reduced, close to the level of the untreated group (relative expression was 1.73 ±0.9, p value= 0.01).

Compared to the vehicle group, adding HF alone did not significantly alter the expression of MMP-13 (relative expression was 0.76 ±0.13, p value= 0.4). These results indicate that Inflammation induces overexpression of MMP-13 and HF inhibits inflammation-induced production of MMP-13. (Figure 17)

Figure 17: HF inhibits the expression of MMP-13 when it is co-incubated with the IL-1β in the CT28/I2 cells
To validate these findings, we also used TNF-α as another inflammatory marker and we observed similar results. Addition of TNF-α significantly increased the expression of MMP-13 while co-incubation of HF and TNF-α did not significantly alter MMP-13 compared to vehicle group, which indicates that addition of HF blocks TNF-α induced overexpression of MMP-13. (Figure 18)

Figure 18: HF inhibits the expression of MMP-13 when it is co-incubated with the TNF-α in the CT28/12 cells
Then to prove our findings, we used animal model of OA to see if HF will inhibit joint
destruction and expression of MMP-13 and if timing of administration of HF would have
any effect on the progression of OA.

*HF inhibits the joint destruction of OA in DMM-surgery osteoarthritic model.*

None of the animals had any health related issues after 12 weeks post DMM-surgery.
Neither surgery, nor the HF treatment caused any complication for the animals. Injection
of HF in the mice osteoarthritis model significantly reduced Osteoarthritis progression
according to OARSI scoring. The mean OARSI score for the DMM-surgery non-treated
group was 3.8±0.33 vs. the scoring for the two treated groups 1.16±0.38 (in the group
that had HF treatment 4 weeks post DMM surgery) and 1.07±0.20 (in the group that had
HF treatment started right after the surgery) with the p-value<0.05. Post hoc turkey test
showed there was no difference between early vs. late administration of HF (Figure 19).
Figure 20 illustrates representative Safranin O/Fast green staining of histologic sections
of osteoarthritis induced by DMM surgery.
Figure 19: HF treatment reduced the progression of OA in DMM surgery osteoarthritis model.
Figure 20: Safranin O/Fast green staining at 12 weeks after DMM surgery (10x magnification) (a) Control group (PBS injected, untreated animals) shows destruction of cartilage, wider erosion, irregular surface and demonstrates grade 5 OARSI semi-quantitative scoring. (b) Early treatment (HF injections 2 days post DMM surgery every other day for 12 weeks) demonstrates grade 0.5 OARSI semi-quantitative scoring (c) Late treatment (received Halofuginone injections 4 weeks post DMM surgery every other day for 8 weeks, and demonstrates grade 1 OARSI semi-quantitative scoring. In the treated animals (b and c) less cartilage destruction is observed compared to untreated animals (a).
In vivo: HF reduces the expression of MMP-13 in DMM-surgery osteoarthritic model

While MMP-13 was overexpressed in the control (DMM surgery without HF treatment) groups in IHC staining, expression of MMP13 was suppressed by injection of HF in both test groups and MMP13 was hardly detected in the both treated groups (figure 21).

**Figure 21:**
Immunohistochemistry staining for MMP-13, in histological sections of osteoarthritic knee induced by DMM surgery in mice model
(a) Control group (PBS injected, untreated animals) prominent expression of MMP-13 *(Brown spots pointed by blue arrows)*
(b) Early treatment (received HF injections 2 days post DMM surgery every other day for 12 weeks) and MMP-13 is hardly detected
(c) Late Treatment (received Halofuginone injections 4 weeks post DMM surgery every other day for 8 weeks, and MMP-13 expression is rare. In (b) and (c) there is significantly less MMP-13 signal observed.
DISCUSSION

Current treatments for OA mainly focus on symptomatic management, such as medications to relieve the pain and maintaining quality of life and everyday activities of patients. The current therapeutic approach includes pharmacological therapy and surgery. The patient may start physical therapy (PT) or occupational therapy (OT) but when pain is severe and daily activities become difficult, surgery is recommended which is total joint replacement. As an example, knee OA patients wait, on average, 4.4 years from diagnosis to joint replacement (156). However, there is a tremendous need for non-surgical therapy and intervention to improve the quality of life of OA patients and currently, there are no proven therapies capable of preventing or slowing down the process of the disease. In this study, we investigated HF as a potential therapeutic drug for preventing the progression of OA. We hypothesized that HF inhibits OA associated joint destruction and inhibit the progression of OA by down regulation of MMP-13.

It has been proven that IL-1β and TNF-α up regulate the catabolic processes in OA and are important mediator cytokines in the development of the disease (68, 157-160). These inflammatory cytokines were shown to stimulate and significantly increase the expression of MMP-13 in chondrocytes (161-168). On the other hand, it is well established that the expression of MMP-13 is increased in OA and it plays a critical role in the destruction of cartilage and it is considered to be the main factor in the development and progression of
OA (169-171). Thus, hypothetically, suppressing IL-1β and TNF-α and subsequent down regulation of MMP-13 should inhibit the destructive processes induced by overexpression of MMP-13.

In line with the previous published studies, we showed in our in vitro studies that, expression of MMP-13 was significantly increased in the presence of the important inflammatory cytokines IL-1β and TNF-α. When HF was added to the chondrocytes that were co-incubated with IL-1β and TNF-α, the expression of MMP-13 was down regulated close to the baseline expression when there was no inflammatory cytokine added.

Suppressing effect of HF on MMP-13 was also confirmed in the in vivo model as well, where expression of MMP-13 was detected by IHC in control animals, while in those who were treated with HF, MMP-13 expression was suppressed.

To prove this hypothesis that administration of HF and subsequent suppression of MMP-13 will lead to prevention of joint destruction, we applied Safranin O/fast Green staining and OARSI scoring system which is the standard way to assess joint destruction. We observed that the destruction of joint was significantly decreased by administration of HF. In our animal model, in untreated animals there was successful induce of OA with joint destruction.

We also studied the timing of starting HF treatment on our preclinical model. We considered two time points for starting the treatment and administration of HF (early vs.
late) and observed that both of them effectively decreased joint destruction with no substantial difference. In other words, we showed that there is no difference if HF is administered during the acute phase of inflammation (right after DMM surgery), compared to delayed treatment (4 weeks after DMM surgery) when the acute inflammation has been resolved. Thus, according to these preclinical data, HF does not need to be immediately started after injury, to prevent injury induced osteoarthritis.

A therapeutic tool which can suppress inflammation induced MMP-13 can potentially be useful to inhibit joint destruction in OA. We showed that HF down regulates the production of MMP-13, thus, decreases the joint destruction associated with OA. In line with our results, there is one published study by Pines et al that demonstrates the role of HF in suppressing MMP-13 in pancreatic tumors where HF decreased MMP-13 levels significantly (172).

The inhibitory effect of HF on pro-inflammatory cytokines has been previously shown in various studies (128, 144, 145). Liang et al have demonstrated that HF significantly decreases the levels of TNF-α and IL-1β and it suppresses the secretion of inflammatory cytokines (173); these are the cytokines that play a critical role in pathogenesis of osteoarthritis and we have shown their impact on stimulation and upregulation of MMP-13.

Two different mechanisms of action have been described for HF. The first is inhibition of Th17 differentiation, which leads to inhibition of the inflammation by activation the AAR response by binding to prolyl-tRNA synthetase (127-129, 145). Th-17 cells produce pro-
inflammatory cytokines and have been shown to be active in OA as well (87-91). Therefore, inhibition of their differentiation can lead to diminished production of inflammatory cytokines and consequently, lower production of MMP-13. The second known mechanism of HF is inhibition of Smad3 phosphorylation and down regulation of the TGF-β signaling pathway (124, 126). Morales et al has shown that TGF-β is stored in cartilage in high amounts (174). Homandberg et al have demonstrated that following trauma and injury, TGF-β is released from the ECM in the cartilage (175). There is also evidence that during OA, TGF-β is increased in cartilage (176). Higher levels of TGF-β have been reported in serum of OA patients and Kapetanakis et al claimed that there is a strong correlation between TGF-β levels and severe pain and dysfunction according to a higher grade of Kellgren Lawrence and WOMAC scale (177). On the other hand, Xavier et al have reported that HF down regulates the Smad7 and TGF-β, which leads to inhibiting the activation of Smad2 and Smad3 (178). All together, with HF down regulating the TGF-β signaling, we would expect less cartilage damage in OA.

HF has been previously studied on different diseases like cancer (179-183), scleroderma (149) and demonstrated therapeutic effects as anti-malaria (184), anti-fibrotic (117, 126), inhibiting angiogenesis in the tumors (146) and anti-inflammatory effects (128, 144, 185). However, there was no published study on the effects of HF on OA to this date. This is the first study that evaluates the efficacy of HF in a preclinical OA model and these results introduce HF as an inhibitor for the progression of OA in DMM surgery in mice. HF is an FDA approved medication and it has been administered orally in a phase I
clinical trial for patients with solid tumors and has been proven to reach effective therapeutic plasma levels without any toxicity.

The result of this preclinical study shows that HF is a very potent inhibitor of MMP-13 expression and pathologic joint destruction in OA and p ave the ground for further clinical studies that are required to provide better insight into long-term outcomes relative to other treatment modalities and clarify whether inhibiting the pro-inflammatory cytokines and blocking the MMP-13 production by HF may replace current techniques as the gold standard.
CONCLUSIONS

This is the first study that investigates Halofuginone (HF) for the prevention of progression of Osteoarthritis (OA). We demonstrate that HF inhibits inflammatory induced MMP-13 expression and diminishes joint destruction. There is no difference between administration of HF in acute early inflammatory phase verses late phase when acute inflammation has subsided. These preclinical findings provide supporting data for future clinical investigations for HF as a therapeutic target for osteoarthritis.
REFERENCES


Gene profiling in experimental OA models has provided additional targets for consideration [Appleton et al. 2007; Bernardo et al. 2011; Lodewyckx et al. 2012; Loeser et al. 2012; Yasuhara et al. 2011]. Furthermore, certain mediators that determine initiation and progression of cartilage damage are common to all of these models. The importance of proteoglycan depletion in cartilage erosion was demonstrated in Adamts5 knockout mice, which are protected against progression of cartilage destruction [Glasson et al. 2005; Stanton et al. 2005]. However, aggrecan depletion, by itself, does not drive OA progression, as suggested by recent studies in Mmp13 knockout mice showing that MMP-13 deficiency inhibits cartilage erosion, but notaggrecan depletion [Little et al. 2009]. The importance of the stability of the extracellular matrix to cartilage health is also documented in studies of Timp3–/– mice, which each show age-dependent cartilage degeneration similar to that of patients with OA because of loss of this key MMP and ADAMTS inhibitor [Sahebjam et al. 2007]. Similarly, Fgf2–/– mice exhibit accelerated spontaneous and surgically induced OA due to loss of the intrinsic capacity of FGF-2 to inhibit ADAMTS5 [Chia et al. 2009].

Mouse models have also taught us about the patterns of receptors on chondrocytes that sense changes in the pericellular matrix. The receptors on the resting chondrocyte are protected from interacting with certain matrix components by the unique structure of the pericellular matrix. But their expression and activation change in response to mechanical or inflammatory stimuli. In Col9a1 knockout mice and Col11a1 haplosufficient mice, the development of OA-like changes can be observed with aging, owing to decreased amounts of the minor collagens that contribute to type II collagen fibril formation [Hu et al. 2006; Xu et al. 2005]. In these mice, there is little pericellular matrix and the fibrillar collagen bundles can be observed closer to the chondrocytes. This results in exposure of the receptor tyrosine kinase, discoidin domain receptor 2 (DDR2) to its ligand, native type II collagen, and preferential induction and activation of MMP-13. The association of DDR2, MMP-13,

**Figure 1:** Destruction of cartilage in an Osteoarthritic joint (Right image, pointed by Black arrow) compared to an intact cartilage in a healthy joint (Left image)
Figure 2: Biomechanical stress leads to up-regulation of the synthetic activity and the inflammatory cytokines, chemokines, adipokines, Toll-like receptor (TLR) ligands, and other inflammatory mediators such as nitric oxide. The upregulation of cartilage-degrading proteinases by extracellular matrix (ECM) proteins and products can promote catabolic activation, phenotypic shift, and apoptosis.

Osteoarthritis. Current opinion in rheumatology. 2011;23(5):471-8. Figure used with permission.
Figure-3: Effects of TGF-β signaling on healthy and osteoarthritic cartilage
Activation of canonical NFκB (p65/p50) and stress-induced and mitogen-activated protein kinase (MAPK) signaling is required for the chondrocytes to express MMPs, ADAMTSs, and inflammatory cytokines themselves [Goldring et al. 2011; Marcu et al. 2010; Pulai et al. 2005]. NFκB signaling strongly induces the expression of transcription factors such as HIF2α [Yang et al. 2010] and Elf3 [Otero et al. 2012], which in turn bind to and activate MMP13 and other gene promoters (Figure 1). The stress-induced MAPK pathways, including the ERK, c-Jun N-terminal kinase (JNK) and p38 MAPK cascades, coordinate the induction and activation of gene expression through transcription factors such as activator protein 1 (cFos/cJun), ETS, C/EBPε, and Runx2 [Goldring and Sandell, 2007; Liu et al. 2010; Long and Loeser, 2010; Tetsunaga et al. 2011; Tsuchimochi et al. 2010]. Induction of both ADAMTS4 and 5 requires Runx2 [Tetsunaga et al. 2011], and NFκB and HIF2α [Yang et al. 2010] mediate ADAMTS4 upregulation, whereas MMP-13 induction requires all three transcription factors. Recent studies indicate that epigenetic mechanisms also play a role through modulation of the DNA methylation status on promoters driving expression of, for example, IL1B and MMP13 genes [Hashimoto et al. 2009] or through dysregulation of the microRNAs that are important for maintenance of homeostasis [Dudek et al. 2010; Miyaki et al. 2010].

As articular cartilage matrix proteins are degraded, activation of certain receptors stimulates the production of matrix-degrading proteinases and inflammatory cytokines and chemokines, either

Figure 4: Stimulation of receptors for type II collagen fragments, activates the production of matrix-degrading proteinases and inflammatory cytokines which leads to release of ROS and production of MMP-13.
Figure-5: Molecular pathogenesis of osteoarthritis under mechanical stress. Nat Clin Pract Rheumatol. 2006 Jun;2(6):304-12. Figure used with permission.
Th cells (Th1, Th2, and Th17), T regulatory (Treg) cells and induced T regulatory (iTreg) cells (Th3 and Tr1).


**Figure-6:** Th cells lineage (Th1, Th2 and Th17), T regulatory (Treg) cells and induced T regulatory (iTreg) cells (Th3 and Tr1) in Osteoarthritis
**Figure-7:** Involvement of the synovium in OA pathophysiology


Figure used with permission.
Figure-8: The role of proinflammatory cytokines in the pathophysiology of OA.

Macrophages and T-cells have a central role in the joint space.

**JOINT SPACE**
- Release of cytokines & degenerative enzymes into the synovial fluid
- Degenerative enzymes (ADAMTS, MMPs)
- Inflammatory cytokines & growth factors (e.g. IL-1, IL-6)
- Cartilage degradation
- Increase of ADAMTS, MMP
- Cartilage antigens
- Inhibiton of Collagen II Proteoglycan TIMP
- Osteophyte formation
- Subchondral bone sclerosis

**SYNOVIAL**
- Synoviocyte
- Synovitis
- IL-1
- IL-6
- IL-8
- TNFα
- INFγ
- IL-12
- IL-10
- IL-17
- TGF
- M1
- M0
- M2a
- M2c
- Th0
- Th1
- Th2
- Th17
- Synoviocyte
- Fibrosis
- TGF

**Figure-9:** The role of Th17 in the Osteoarthritis
**Figure-10:** Pharmacologic therapies and the site of their impact. Nat. Rev. Rheumatol. doi:10.1038/nrrheum.2010.178. Figure used with permission.
bony changes. Flattening, which is a typical change of Type F, may classi

compared with other classi

convenient for the evaluation of bone changes in TMD patients

CBCT was the imaging method used in this study.

lateral slices (analyzing lateral slices in isolation, and combining coronal and

superior to CT for visualizing bony changes in TMJ patients, ef

cost, better access to equipment, lower radiation, and diagnostic

advent of CBCT. CBCT has several advantages over CT, such as lower

ef

The above-mentioned barriers have been overcome with the

accurate as high as CT, but superior to those of panoramic radiog-

comparison, types F and E are comparatively the initial stages of

quantities of degraded products that could not be ef

the surface of the articular soft tissue. When there were large

occurred. The pain may be caused by the soft tissues around the

diagnosis of OA was dif

diagnosis was already introduced into the eval-

Koyama et al., 2007; Honey et al., 2007

Kordass et al. (2012)

Barrera-Mora et al., 2012; Perillo et al., 2011; Rauhala et al., 1999

Bentzen et al., 2011; Larsson and Rönnerman, 1981

Shahidi et al. (2013)

failed to

investigated the correlation between computer-assisted measurements of mandib-

Samad et al., 2007; Parnham et al., 2007

Kazemi et al., 2008

The results in this study showed that the score and degree of

s Di was 7, and the degree was 2. (b) Male aged 48 years with positive glenoid fossa bony change; the score of

s Di was 11, and the degree was 3. (c) Male aged 49 years with maximum bony change of condyle was Type E; the score of

s Di was 2, and the degree was 1. (b) Female aged 21 years with maximum bony change of condyle was Type E;

s Di has been

investigated the correlation between articular

maximum intercuspation

D or type S because of the progression of condylar resorption, while

and that the bone surface will change. Moreover, some clinical

deviation and frequent popping

s Di was 7, and the degree was 2. (d) Female aged 39 years with maximum bony change of condyle was Type S; the score of

Katzberg, 1989

Kryger and Helkimo, 1980

Katzberg et al., 1988

Figure-11: Osteoarthritic TMJs (B), CT scan view, compared to the normal Joint (A).

Figure-12: (a) Outline of a right knee joint. The MMTL is transected to generate destabilization of the medial meniscus (DMM) and the ACL is transected in the ACLT model. (b) Overview of Safranin-O stained of a mouse knee joint 4 weeks after DMM induction. Grey arrow indicates articular cartilage, white arrow indicates growth plate. ×40 magnification. ACL: anterior cruciate ligament; F = femur; LCL = lateral collateral ligament; LFC: lateral femoral condyle; LM = lateral meniscus; LTP: lateral tibial plateau; MFC: medial femoral condyle; MM: medial meniscus; MMTL = medial meniscotibial ligament; MTP: medial tibial plateau; PCL: posterior cruciate ligament; T = tibia.

Taken from Mouse genetics Methods and Protocols ISSN 1064-3745, with permission.
**Figure-13:** Osteoarthritis progression after DMM in wild-type mice. Osteoarthritis progression is demonstrated on medial areas of Safranin-O stained sections of right hind knee joints at 2 (b), 4 (c), 6 (d), 8 (e), and 12 (f) weeks after DMM surgery. (a) is the contra lateral joint. *Arrows* indicate articular cartilage damage of femoral condyle and tibial plateau. (a–f) ×100 magnification

Taken from Mouse genetics Methods and Protocols ISSN 1064-3745, with permission.
Figure-14: Chemical structures of Febrifugine and Halofuginone

Figure-15: AAR activation by HF inhibits prolyl tRNA charging enzyme EPRS by HF results in uncharged tRNA accumulation. Uncharged tRNA activates the sensor kinase GCN2, leading to phosphorylation of eif2a and changes in translation and transcription of pro-inflammatory genes.
**Figure-16:** Here is a representative image for morphometrical evaluation of osteoarthritis using a Safranin-O stained section. The histology image has taken from a right knee joint 4 weeks after DMM surgery. Black arrow shows area of acellular/unstained cartilage, grey arrow is the whole cartilage area and white arrow refers to subchondral bone plate (SBP). At ×100 magnification.
Figure 17: HF inhibits the expression of MMP-13 when it is co-incubated with the IL-1β in the CT28/I2 cells
Figure 18: HF inhibits the expression of MMP-13 when it is co-incubated with the TNF-α in the CT28/I2 cells.
Figure 19: HF treatment reduced the progression of OA in DMM surgery osteoarthritis model
Figure 20: Safranin O/Fast green staining at 12 weeks after DMM surgery (10x magnification) (a) Control group (PBS injected, untreated animals) shows destruction of cartilage, wider erosion, irregular surface and demonstrates grade 5 OARSI semi-quantitative scoring. (b) Early treatment (HF injections 2 days post DMM surgery every other day for 12 weeks) demonstrates grade 0.5 OARSI semi-quantitative scoring (c) Late treatment (received Halofuginone injections 4 weeks post DMM surgery every other day for 8 weeks, and demonstrates grade 1 OARSI semi-quantitative scoring. In the treated animals (b and c) less cartilage destruction is observed compared to untreated animals (a).
Figure 21: Immunohistochemistry staining for MMP-13, in histological sections of osteoarthritic knee induced by DMM surgery in mice model (a) Control group (PBS injected, untreated animals) prominent expression of MMP-13 (*Brown spots pointed by blue arrows*) (b) Early treatment (received HF injections 2 days post DMM surgery every other day for 12 weeks) and MMP-13 is hardly detected (c) Late Treatment (received Halofuginone injections 4 weeks post DMM surgery every other day for 8 weeks, and MMP-13 expression is rare. In (b) and (c) there is significantly less MMP-13 signal observed compared to (a) 10x magnification
<table>
<thead>
<tr>
<th>Level of evidence</th>
<th>Observation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical</strong></td>
<td>Effusion, Joint swelling or palpable synovitis</td>
<td>Krasnokutsky et al (2008), Ayral(1999)</td>
</tr>
<tr>
<td></td>
<td>Local signs of inflammation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sudden increase in pain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Night pain and morning stiffness</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Correlation between MRI and histological observations</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synovitis seen using ultrasonography of symptomatic joints</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Association between ultrasound-detected synovitis and clinical symptoms of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>synovitis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Macroscopic synovial changes detected by arthroscopy in about half of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>patients with knee OA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arthroscopic synovitis associated with progression of knee OA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infiltration of mononuclear cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(monocytes/macrophages, activated B cells and T cells)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adaptive immune T-cell and B-cell responses to fragments of extracellular</td>
<td></td>
</tr>
<tr>
<td></td>
<td>matrix</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased angiogenesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synovitis in the velocity of degenerative cartilage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-8, IL-15, IL-17, IL-21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased production of PGE2 and nitric oxide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased activity of MMPs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Production of adipokines</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Release of EGF and VEGF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Involvement of macrophages in osteophyte formation via BMPs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Release of proinflammatory and pain neurotransmitters (substance P, NGF)</td>
<td></td>
</tr>
<tr>
<td><strong>Markers</strong></td>
<td>Increased levels of MMP-13, MMP-3 and MMP-9</td>
<td></td>
</tr>
</tbody>
</table>

Table-1: Evidence of inflammation in OA
<table>
<thead>
<tr>
<th>Groups</th>
<th>Day1</th>
<th>Day2</th>
<th>Day3</th>
<th>Day4</th>
</tr>
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<tbody>
<tr>
<td>- HF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- IL-1β/TNF-α</td>
<td>10% FBS</td>
<td>1% FBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ HF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- IL-1β/TNF-α</td>
<td>10% FBS</td>
<td>1% FBS</td>
<td>+ 200 nM HF</td>
<td></td>
</tr>
<tr>
<td>- HF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ IL-1β/TNF-α</td>
<td>10% FBS</td>
<td>1% FBS</td>
<td></td>
<td>+10ng/ml IL-1β/TNF-α</td>
</tr>
<tr>
<td>+HF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+IL-1β/TNF-α</td>
<td>10% FBS</td>
<td>1% FBS</td>
<td>+ 200 nM HF</td>
<td>+10ng/ml IL-1β/TNF-α</td>
</tr>
</tbody>
</table>

**Table-2:** Experimental conditions for qPCR experiments. Medium and supplements were added to the chondrocytes culture medium at the following concentrations: Halofuginone (200nM per well), IL-1β (10ng/ml)/ TNF-α (10ng/ml)
<table>
<thead>
<tr>
<th>Grade</th>
<th>Osteoarthritic damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>0.5</td>
<td>Loss of Safranin-O without structural changes</td>
</tr>
<tr>
<td>1</td>
<td>Small fibrillation without loss of Cartilage</td>
</tr>
<tr>
<td>2</td>
<td>Vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina</td>
</tr>
<tr>
<td>3</td>
<td>Vertical clefts/erosion to the calcified cartilage extending to &lt;25% of the articular surface</td>
</tr>
<tr>
<td>4</td>
<td>Vertical clefts/erosion to the calcified cartilage extending to &lt;25-50% of the articular surface</td>
</tr>
<tr>
<td>5</td>
<td>Vertical clefts/erosion to the calcified cartilage extending to &lt;50-75% of the articular surface</td>
</tr>
<tr>
<td>6</td>
<td>Vertical clefts/erosion to the calcified cartilage extending to &gt;75% of the articular surface</td>
</tr>
</tbody>
</table>

**Table-3:** Semi-quantitative Modified Mankin scoring system recommended from OARSI.