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The role of transcription factor Nrf2 in osteoarthritis

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Résumé

Introduction: L'arthrose est caractérisée par une destruction progressive du cartilage, une inflammation synoviale, et un remodelage de l'os sous-chondral avec une production excessive des médiateurs inflammatoires et cataboliques. Nous avons démontré que le niveau du 4-hydroxynonénal (4-HNE), un produit de la peroxydation lipidique, est augmenté dans le cartilage humain arthrosique sans qu'on sache le mécanisme exacte impliqué dans l'augmentation de cette molécule. Des données de la littérature indiquent que l'accumulation du HNE est contrôlée par l'action de la glutathione S-transférase A4-4 (GSTA4-4), une enzyme impliquée dans la détoxification du HNE. Au niveau transcriptionel, l'expression de cette enzyme est régulée par la transactivation du facteur de transcription Nrf2. **Objectif:** L'objectif de cette étude vise à démontrer que l'augmentation du HNE dans le cartilage arthrosique est attribuée, en partie, à l'altération de l'expression de la GSTA4-4 et de Nrf2. **Méthode**: Le niveau d'expression de la GSTA4-4 et de Nrf2 a été mesurée par Western blot et par PCR en temps réel dans le cartilage humain arthrosique et dans le cartilage provenant des souris atteintes d'arthrose. Pour démontrer le rôle du Nrf2 dans l'arthrose, les chondrocytes humains arthrosiques ont été traités par l'interleukine 1beta (IL-1β) ou par le H_2O_2 en présence ou en absence des activateurs du Nrf2 tels que le Protandim®, AI, et du 6-Gingérol. Par ailleurs, les chondrocytes ont été transfectés par un vecteur d'expression de Nrf2 puis traités par l'IL-β. En utilisant le modèle d'arthrose chez la souris, les animaux ont été traités par voie orale de 10 mg/kg/jour de Protandim® pendant 8 semaines. **Résultats**: Nous avons observé une diminution significative de l'expression de la GSTA4-4 et de Nrf2 dans le

cartilage humain et murin arthrosique. L'activation de Nrf2 bloque la stimulation de la métalloprotéinase-13 (MMP-13), la prostaglandine E2 (PGE_2) et de l'oxyde nitrique (NO) par l'IL-1β. En outre, nous avons montré que l'activation Nrf2 protège les cellules contre la mort cellulaire induite par H_2O_2. Fait intéressant, l'administration orale de Protandim® réduit la production du HNE par l'intermédiaire de l'activation de la GSTA4. Nous avons démontré que le niveau d'expression de la GSTA4-4 et de Nrf2 diminue dans le cartilage provenant des patients et des souris atteints d'arthrose. De plus, la surexpression de ce facteur nucléaire Nrf2 empêche la production du HNE et la MMP-13 et l'inactivation de la GSTA4-4. Dans notre modèle expérimental d'arthrose induite par déstabilisation du ménisque médial chez la souris, nous avons trouvé que l'administration orale de Protandim® à 10 mg / kg / jour réduit les lésions du cartilage. **Conclusion**: Cette étude est de la première pour démontrer le rôle physiopathologique du Nrf2 in vitro et in vivo. Nos résultats démontrent que l'activation du Nrf2 est essentielle afin de maintenir l'expression de la GSTA4-4 et de réduire le niveau du HNE. Le fait que les activateurs du Nrf2 abolissent la production de la HNE et aussi un certain nombre de facteurs connus pour être impliqués dans la pathogenèse de l'arthrose les rend des agents cliniquement utiles pour la prévention de la maladie.

Mots-clés : Arthrose, cartilage, hydroxynonénal, glutathione S-transferase, Nrf2, catabolisme, inflammation.

Summary

Background: Osteoarthritis (OA) is characterized by progressive cartilage destruction, synovial inflammation, and subchondral bone remodelling with increased inflammatory and catabolic responses. Elevated levels of oxidative stress lead to the accumulation of reactive oxygen species and subsequently the production of lipid-peroxidation products (LPO). The toxic aldehyde 4-hydroxynonenal (HNE) is a LPO product that was found, at pathological concentrations, strongly related to the release of different catabolic and inflammatory mediators in OA. Transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) acts as a key modulator for the expression of multiple cellular stress-response genes such as glutathione S-transferase A4-4 (GSTA4-4), an important HNE detoxifying enzyme. Protandim®, a commercial product composed of a mixture of natural antioxidant products is reported to activate and increase the levels of Nrf2 in different tissues. **Objective**: In this study we are evaluating the biological effects of Nrf2 activators such as, Protandim®, A-I, and 6-Gingerol. **Results**: The activation of Nrf2 can lead to indirect blockage of inflammatory and catabolic responses as well as oxidative stress. Using human OA chondrocytes, we demonstrated that Nrf2 activation by Protandim®, A-I, and 6-Gingerol abolished interleukin-1beta (IL-1β)-induced metalloproteinase-13 (MMP-13), prostaglandin E2 (PGE_2), and nitric oxide (NO). Furthermore, we showed that Nrf2 activation protects cells against H_2O_2-induced cell death. Interestingly, the oral administration of Protandim® reduces HNE production via GSTA4-4 activation. We found that Nrf2 protein and mRNA levels decrease in OA cartilage from human and mice and match GSTA4-4 changes. Moreover, the overexpression of this

nuclear factor abrogates IL-1β-induced GSTA4-4 inhibition as well as HNE and MMP-13 production. In our experimental mouse model of OA induced by surgical destabilization of the medial meniscus (DMM), we found that oral administration of Protandim® at 10 mg/kg/day reduces cartilage damage. **Conclusion**: This is the first *in vitro* and *in vivo* study to demonstrate the pathophysiological role of HNE in OA. In addition, this study indicates that Nrf2 activators abolish HNE production and number of factors known to be involved in OA pathogenesis. These findings render such activators clinically-valuable agents in the prevention of OA.

Keywords: Osteoarthritis, cartilage, hydroxynonenal, glutathione S-transferase, Nrf2, catabolism, inflammation.

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LIST OF ABBREVIATIONS

4-hydroxy-2-hexenal	4-HHE
4-Hydroxynonenal	HNE
Activator protein 1	AP-1
Advanced LPO-end products	ALEs
Alkoxyl radical	LO
Anterior cruciate ligament transection	ACLT
Antioxidant response element	ARE
Arachidonic acid	AA
Carnosine	CAR
Chemokine (C-X-C motif) ligand 1	CXCL-1
c-Jun N-terminal kinase	JNK
Collagen	Col
Cyclooxygenase	COX
Cyclooxygenase-2	COX-2
Destabilization of the medial meniscus	DMM
Extracellular matrix	ECM
Fibroblast-like synoviocytes	FLS
Glutathione	GSH
Glutathione peroxidase	GPx
Glutathione S-transferase	GST
Glutathione S-transferase A4-4	GSTA4-4

Heme oxygenase-1	HO-1
Hydrogen peroxide	H_2O_2
Hydroxyl radicals	$OH^{\bullet}$
IL-1 receptor antagonist	IL-1Ra
Inducible nitric oxide synthase	iNOS
Intercellular Adhesion Molecule 1	ICAM-1
Interferon-gamma	INF-ɣ
Interleukin-1beta	IL-1β
Kelch ECH associating protein 1	Keap1
Lipid hydroperoxide	LOOH
Lipid peroxidation	LPO
Lipid peroxyl radical	$LOO^{\bullet}$
Malondialdehyde	MDA
Matrix metalloproteinase-13	MMP-13
Matrix metalloproteinases	MMPs
microsomal-PGES	m-PGES
Mitogen-activated protein kinase	MAPK
Myeloperoxidase	MPO
N-acetyl-cysteine	NAC
NG-monomethyl-t-arginine	NMMA
N-iminoethyl-L-lysine	L-NIL

Nitric oxide	NO
Non-steroidal anti-inflammatory drugs	NSAIDs
Nrf2 Activator II	AI-1
Nuclear factor erythroid 2-related factor 2	Nrf2
Nuclear factor-kappa B	NF-κB
OA Research Society International	OARSI
Osteoarthritis	OA
Peroxisome proliferator-activated receptor gamma	PPARɣ
Peroxynitrite	$ONOO^-$
Polyunsaturated fatty acids	PUFAs
Post-translational modification	PTM
Prostaglandin	PG
Prostaglandin E2	PGE_2
Prostaglandin-E synthase	PGES
Protein kinase C	PKC
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Rheumatoid arthritis	RA
small Maf	sMaf
Superoxide dismutase	SOD
Tissue inhibitors of metalloproteinases	TIMPs
Tumor necrosis factor-alpha	TNFα

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CHAPTER I: INTRODUCTION

1. THE DIARTHRODAL JOINT

The diarthroidal or synovial joint is the most abundant movable type of joint in the body of a mammal. Its encapsulated system consists of bone, articular cartilage, synovial membrane and synovial fluid [1]. **Fig.1** shows a simplified synovial joint.

1.1 Articular Cartilage

Cartilage is a non-innervated anaerobic connective tissue found in many areas around the human body. There are three main types of cartilage classified according to the relative amounts of main components; elastic cartilage, hyaline cartilage and fibrocartilage. Cartilage constitutes mainly of sole type of cells named chondrocytes distributed within a mesh of an extracellular matrix (ECM). It can be divided into four zones according to the morphological changes in chondrocytes and matrix from the articular surface to the subchondral bone [1]. **Fig.2** illustrates the structure and different zones within hyaline cartilage. A synovial joint contains hyaline cartilage which forms a protective layer covering the two bone ends of the joint to reduce contact stresses there and facilitate bone movement without pain. Moreover, articular cartilage protects bone surface from impact stresses, and minimizes friction and wear in the joint [1, 2]. Therefore, loss or destruction of cartilage can be associated with extreme pain especially during movement, which is often the case in advanced OA patients.

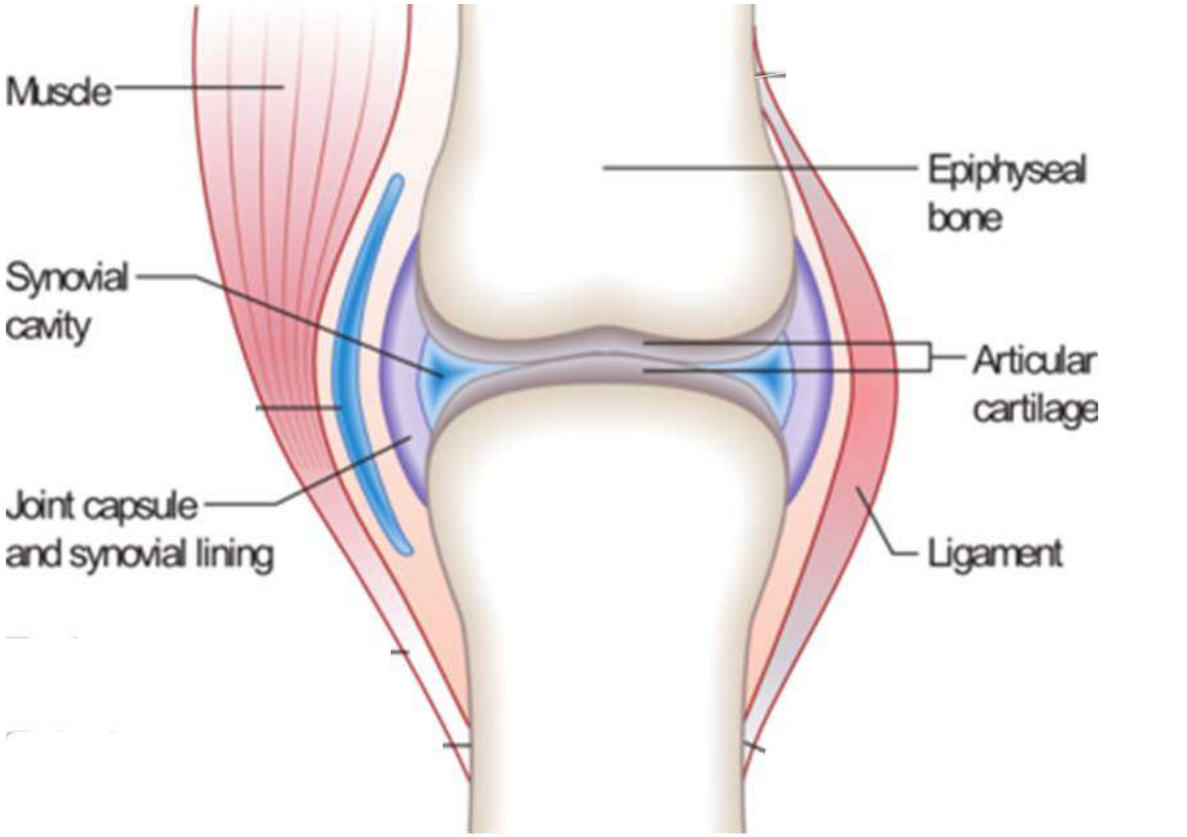

Figure 1 : The structure of a healthy typical Synovial joint.

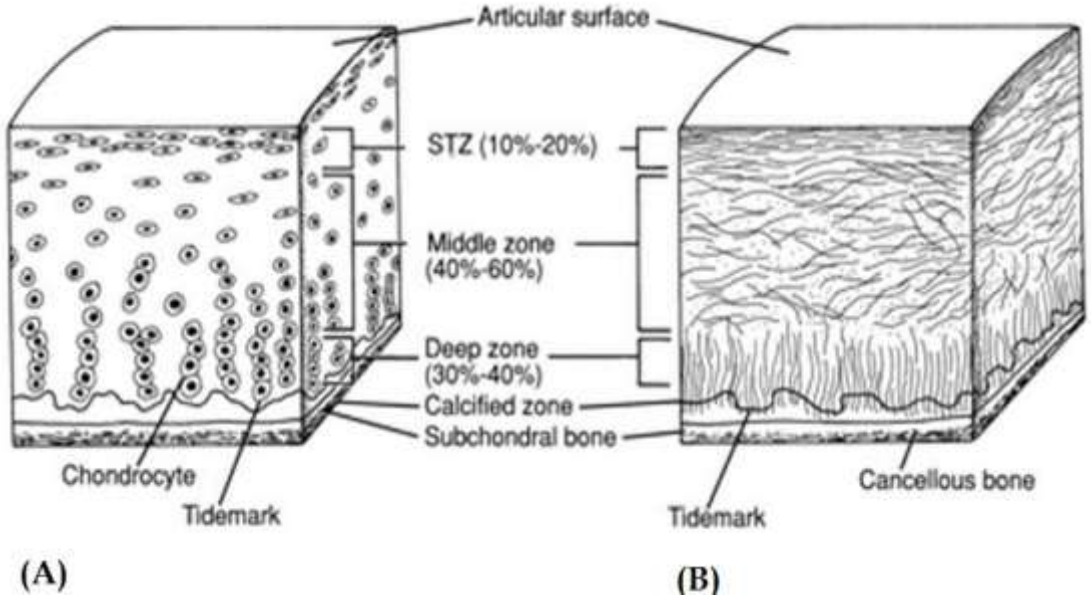

Figure 2: **Schematic diagram of the cellular organization in the zones of articular cartilage (A) and of the collagen fiber architecture (B)** (from Buckwalter et.al [3]).

The composition, structure and functions of chondrocytes vary depending on their zone or location within the cartilage [4, 5]. In the superficial zone chondrocytes are in contact with the synovial fluid, cells are closely spaced and aligned with the cartilage surface. This zone, serves mainly to support the tensile stresses generated when compressive loads are applied to the cartilage. The transitional intermediate zone holds lower density of randomly dispersed chondrocytes and high concentration of proteoglycan and collagen. In the deep radial zone chondrocytes forms rows of cells parallel to the collagen fibers. The calcified zone is the innermost zone that connects the cartilage to the subchondral bone. This zone consists of lower count of chondrocytes uniquely capable of synthesizing type V collagen (Col V) which was presented as an important factor in the structural integrity to the less elastic subchondral bone [6].

1.1.1 Chondrocytes

Chondrocytes, the sole cellular component, form only 1-5% of cartilage volume, yet the highly specialized cells are responsible for producing other macromolecular components of the ECM. Depending on their location within the matrix the highly metabolically active chondrocytes can produce multiple collagen types including type II Collagen (Col II), Col VI, Col IX, Col X, Col XI, and proteoglycan among other mediators [2]. Chondrocytes are responsible for maintaining ECM integrity by controlling both the degradation and synthesis of matrix macromolecules. Though the exact process in still under study, it is believed to be under careful balance between multiple catabolic and anabolic mediators produced by the cells. Interleukin-1 beta (IL-1β) presents an example as it triggers a cascade of signals that can induce the expression of catabolic enzyme matrix metalloprotease-13 (MMP-13), up-regulate the expression of pro-inflammatory enzymes like cyclooxygenase-2 (COX-2) and molecules like prostaglandin E_2 (PGE_2), and affect the transcription of genes needed for the synthesis of proteoglycans [7]. On the other hand, cytokines, such as insulin-dependent growth factor-I, oppose these catabolic activities by stimulating matrix synthesis and cell proliferation. Chondrocytes can also produce tissue inhibitors of metalloproteinases (TIMPs) that antagonize the catabolic effect of MMPs. Furthermore, the interaction between chondrocytes and matrix in an interdependent pattern is essential for tissue maintenance and has huge effect on the metabolic activity of chondrocytes. [2, 7].

1.1.2 Extracellular Matrix (ECM)

Articular cartilage derives its form and mechanical properties from its matrix [2].Water, the main component contributing up to 80% of the matrix, collagen and proteoglycans interact to gives tensile strength to the cartilage. The matrix also functions as an environment to protect chondrocytes from mechanical stress, store cytokines and growth factors needed to maintain cell function and activity, act as a media for nutrient diffusion to chondrocytes and as signal transductor for the cells [8]. Col II is the principal component of ECM accounts for 90-95% of collagen. Deformations in the matrix due to the of loss of balance between catabolic and anabolic factors in the cartilage can further alter the response of chondrocytes, and lead to overall abnormal cartilage structure and function such as the case in advanced OA [2] . **Fig.3** shows main differences between normal and OA joint.

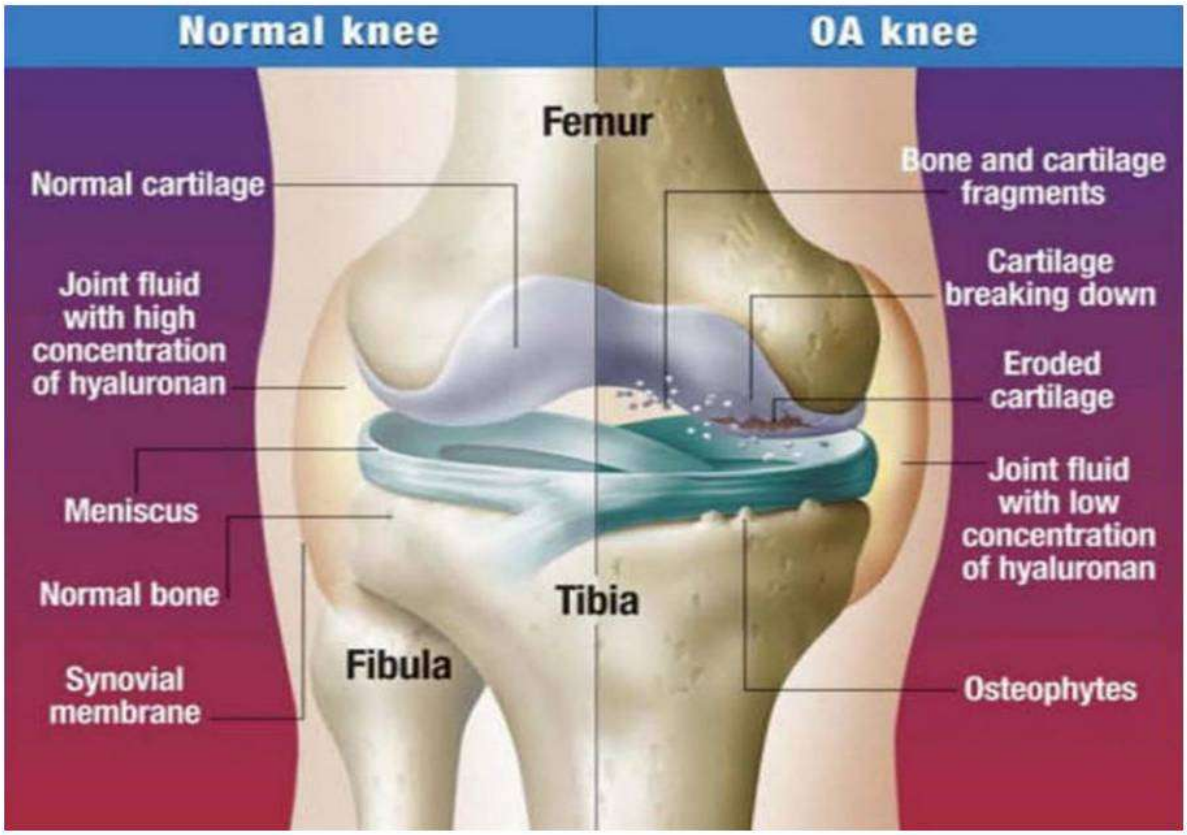

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Figure 3: Normal joint vs. OA joint shows changes such as loss of cartilage integrity and erosion, loss of joint space and the formation of osteophytes.

1.2 Bone

The calcified zone of the cartilage is bordered by the subchondral bone plate, a very thin cortical bone structure to which the articular cartilage is anchored. Arterial terminal branches in the subchondral bone plate it can supply approximately 50% of the glucose, oxygen, and water required by articular cartilage [9, 10]. Bone cells include osteoblasts and osteoclasts secret factors that can affect the activity and differentiation of chondrocytes through crosstalk between subchondral bone and cartilage. Though the relationship is still

under investigation it is well reported that, changes affecting metabolic activity of bone cells, especially in the subchondral bone, may alter local signaling factors and trigger changes affecting near chondrocytes and articular cartilage in general [11].

1.3 Synovial Membrane

The Synovial membrane is bi-layer membrane of connective tissue that lines the cavities of joints. There are two types of cells forming the innermost or internal layer of the membrane; fibroblast-like synoviocytes (FLS) and macrophages. While FLS are responsible for producing hyaluronan long chain sugar polymers [12], macrophages act to clear unwanted substances and debris [13]. This membrane seals off the synovial fluid from the surrounding tissue and plays essential role in supporting joint lubrication by secreting synovial fluid and other important macromolecules like hyaluronan. Moreover, the membrane acts to regulate the amount and content in it by controlling the passage or filtration of nutrients into the synovial capsule and the clearance of waste products from it [1, 11] .

1.4 Synovial Fluid

The synovial fluid is a viscose plasma dialysate secreted by the joint tissues especially synovial membrane characterized by high content of hyaluronic acid. The synovial fluid holds three main advantages; the viscous fluid lubricates the articulating surfaces, provides a media for carrying nutrients to chondrocytes, and clearing waste products from the cartilage while acting as a shock absorber [1]. Whereas cell counts of synovial fluid are normally low, healthy samples contain a detectable level of mononuclear cells. It has been suggested that the main

function of the mononuclear cells of synovial fluid is the removal of debris that normally appear in joints. However, the fact that vascular and synovial membrane permeability are altered by inflammation, accounts for changes in protein and cellular content in diseased synovial fluid [14]. Moreover, a direct correlation between high levels of leukocytes and synovial fluid pH was described in samples from patients with various forms of acute and chronic arthritis [15]. In addition, the level of different enzymes present in the synovial fluid can vary in the presence of articular disease. These enzymes could be either produced locally by the synovial membrane or released by synovial fluid macrophages or entering from plasma. Such changes can have strong implication on disease progression and symptoms development [1, 16].

2. OSTEOARTHRITIS (OA)

2.1 Definition and classification

OA is one of the most prominent chronic degenerative disease of the joints; it is the most common joint disorder in the world and the second most common diagnosis among ageing population [17]. Disease diagnosis and stage determination can be achieved through analyzing clinical and idiographic outcomes. Clinical examination evaluates symptom such as pain, local inflammation and change or limitation in joint function, while radiographic imaging can aid in viewing articular changes like deformations and joint space narrowing. The most commonly affected joints are the distal interphalangeal joints of the hands and weight baring joints such as the hips and the knees [18].

OA is a multifactorial disease that is mainly characterised pathologically by progressive loss of articular cartilage in synovial joints. Moreover, disease development and progression also involves synovial inflammation, bone remodeling and increased inflammatory and catabolic responses. Therefore, OA is being considered a disease that affects the joint as an organ including cartilage, bone, synovium, muscles and ligaments [19]. The structural integrity of the matrix of human articular cartilage is maintained by a dynamic equilibrium between synthesis and degradation. In OA disruption of the differentiation and function of chondrocytes influences the composition and structure of the cartilage matrix. This disruption causes catabolic factors to overcome the anabolic capacity of chondrocytes leading to overall loss of normal ECM composition and cartilage properties.

2.2 Epidemiology of OA

Advanced OA is considered a major socio-economical problem not only in Canada but in the Western countries and the world. The disease significantly reduces the productivity, activity and quality of life of patients as well as exerting high stress on the health system. Moreover, an anticipated increase in OA prevalence within the next two decades is expected to make it the fourth leading cause of discordance, this increase is mainly attributed to ageing population, sedentary lifestyle and obesity epidemic [20]. According to a report published by the Arthritis Alliance of Canada in 2011 titled "The impact of arthritis in Canada: today and the next 30 years", the prevalence of OA is expected to further increase in Canada to reach 71% by 2040 among seniors above the age of 70 years old.

OA can be generally classified into two forms either as primary or secondary OA. While the primary form of the disease is the most common type, it is considered as an idiopathic phenomenon that is usually related to aging process, typically occurring in older individuals. Though primary OA has no specific apparent trigger or initiating factor, multiple risk factors can play a role in disease progression. However, secondary OA refers to damage affecting the synovial joint as a result of predisposing condition like trauma or congenital deformity and can happen earlier in life [21]. Risk factors; OA can be considered as the product of an interaction between different factors; these factors can be categorized as systemic risk factors affecting the biological activity of the body or local risk factors that exert an influence on specific joint or joints. Systemic risk factors; age is considered as the strongest factor for OA development and progression where changes in biological functions are associated with cumulative exposure to different risk factors [21]. Women have higher prevalence not only for developing OA but for having more severe form of the disease especially during and after menopause [22, 23]. Therefore, both gender and hormonal factors are considered to be important risk factors. Race and ethnicity can also play a role where studies on different location revealed variation in OA prevalence and joints affected. For example, African Americans reported more joint and hip symptoms than whites in general [24]. Further study is needed to comprehend the origin of this variation. Moreover, factors such as genetics and the presence of congenital or developmental conditions also have an influence on disease progression [25]. Local risk factors; obesity is highly targeted as a potent risk factor for developing OA particularly in weight-bearing joints especially the knee. However, weight loss and exercise were found to reduce pain and risk of developing symptomatic OA. Knee surgery or injury are considered among the strongest risk factors

especially for early OA of the knee, also having an occupation or playing certain sports that exerts strong impact or strain on certain joints can increase the prevalence for developing OA [21, 26].

3. PATHOPHYSIOLOGY OF OA

OA is described as a disease affecting the whole joint. However, articular cartilage is considered a major target of tissue injury that is most related to symptoms development and a hallmark of disease progression regardless of triggering factors. Alterations in ECM and cartilage are associated with changes in chondrocyte and disruption of homeostasis leading to loss of the balance between anabolic and catabolic pathways necessary for maintaining the properties of cartilage tissue. Studies on OA indicate that cytokines and growth factors are implicated in the disease. Degradation of cartilage ECM and the suppression of its biosynthesis by chondrocyte are regulated by the release of several cytokines in the joint. According to the triggered response these factors can be classified as proinflammatory, which are generally also catabolic (e.g. IL-1, IL-6, IL-8, and tumor necrosis factor-alpha (TNFα)), or anti-inflammatory (e.g. IL-4, IL-10, IL-13). Other factors like IL-1 receptor antagonist (IL-1Ra) and interferon-gamma (IFN-ɣ) are also classified as inhibitory cytokines, since they may block the actions of catabolic cytokines. Though many of these factors are necessary at low levels for normal homeostasis, in OA disruption in their level can indicate an important role in the disease. Despite the absence of clear or specific initiating factor, the progression of the disease is mostly regulated by pro-inflammatory cytokines mainly IL-1β and TNFα [27, 28]

3.1 Pro-inflammatory mediators

In OA, upregulation of cytokines production such as IL-1β and TNFα in synovial membrane leads to increase in their level in cartilage triggering the activation of chondrocytes. Upon activation by IL-1β, cells show a pronounced upregulation in gene expression of MMPs, inducible nitric oxide synthase (iNOS) and COX-2, as well as in the synthesis of inflammatory mediators such as PGE_2 and nitric oxide (NO). Current research on OA suggests a central role of IL-1β in joint destruction and other pathological features of the disease. While IL-1β is largely involved in the induction of catabolic effect on cartilage in OA, TNFα is presented more as an inducer of the inflammatory effect [29]. Further investigating its effect indicated cytokine ability to induce deleterious changes in chondrocyte function and in the integrity of ECM. In addition, understanding cytokine-induced signal transduction pathways involved the OA and/or normal cartilage homeostasis can provide new potential targets for intervention in OA [30, 31]. In fact, several clinical trials have tried to modulate the effect of major cytokines in order to control their role in OA pathogenesis. This approach had several successes in rheumatoid arthritis (RA). For example, the administration of TNF antagonists have been effective in treating RA, yet TNF antagonist therapy has less dramatic effects in patients with OA. Clinical trials on the use of several inhibitors of biological targets propose certain factors as part of the disappointing outcome such as side effects of medication, time of intervention and pharmacokinetics of the used agents [32].

3.1.1 Pro-inflammatory cytokine IL-1β

IL-1β protein is primarily produced as a precursor (pro-IL-1β) that needs to be processed by caspase-1 to generate its mature and active cytokine. Upon binding its type 1 receptor (IL-1RI), IL-1β triggers several pathways leading to activation of several signal transduction kinases as well as inducing nuclear translocation of number of transcription factors. These effects can be inhibited by naturally present IL-1Ra and type2 IL-1 decoy receptor. Several studies have presented strong indications of the significant role played by IL-1ß in OA [30]. In normal articular tissue the cytokine is produced in limited amount. However, its level markedly increased in chondrocytes as well as synovial cells from patients with OA [33]. The reported increase in IL-1β production and also in cellular response to the cytokine might be explained by several observations; while the level of IL-1 antagonists in OA synovium is downregulated, there is a concomitant upregulation of IL-1β-converting enzyme and IL-1RI in human chondrocytes and synovial fibroblasts. Therefore, these observations may provide a molecular explanation for the enhanced catabolic effects of IL-1β in OA joint tissues.

The destructive effects of IL-1β in OA include both elevation of cartilage catabolism and suppression of cartilage anabolism. IL-1β can trigger multiple signal transduction pathways that upregulates gene expression and subsequently the levels of major extracellular proteolytic enzymes involved in cartilage degradation such as MMPs [34]. In addition, the cytokine has the capacity to induce several proinflammatory mediators involved in the increase of local hematopoietic cells during OA. An increase in articular hematopoietic cells

can cause increases in oxidative burst activity and decreases in O_2 concentration, and generation of additional inflammatory and proteolytic enzymes that can lead to progression of OA [30]. Among proinflammatory mediators induced by IL-1β the upregulation in gene transcription of iNOS and COX-2 that causes a subsequent increase in levels of NO and PGE_2 respectively hold significant importance in the pathology of OA. The detrimental effects on articular tissue associated with significantly high levels of these proinflammatory mediators have been closely investigated in the literature [30, 31]. Moreover, the cytokine can also induce downregulation in biosynthesis of Col II, major articular joint protein as well as through stimulating chondrocyte apoptosis [35]. As a result, IL-1β can decrease ECM synthesis by affecting the anabolic activities of chondrocytes and/or the cell densities of articular cartilage. Consequently, the outcomes of triggering of IL-1β pathway in OA can be related to deleterious effects that include both elevation of cartilage catabolism and suppression of cartilage anabolism [30].

Several strategies aiming to inhibit or modify the activity of IL-1 have been investigated as possible treatments in OA such as the administration of IL-1Ra and soluble IL-1 receptors. The results of these investigations revealed potential agents that may be used in the treatment of OA. However, further research is needed to establish sufficient data about its safety and effectiveness. The role of different IL-1 inhibitors in OA has been discussed in an evidence based review [36].

3.1.2 COX-2 and PGE_2

Cyclooxygenase (COX) also named prostaglandin H synthase is the main enzyme in the metabolism arachidonic acid (AA) and subsequent production of prostaglandins (PG). Membrane-bound phospholipids are converted into AA through phospholipase A2 activity. COX enzymes convert AA first to PGG_2 and then to an unstable metabolite PGH_2 that will be rapidly converted into three types of prostanoids: prostaglandins, thromboxanes, prostacyclins in addition to leukotrienes [37]. Currently, three isoform of COX enzyme have been identified each encoded by a different gene. The inducible nature of the isoforme COX-2 expression by several mediators such as pro-inflammatory mediators like IL-1β and TNFα and lipid peroxidation (LPO) product 4-hydroxynonenal (HNE) made it a target for closer investigation [38, 39]. COX-2, but not other isoforms, synthesis was significantly increased in different diseases known to be of inflammatory nature is well reported in disease-related pattern. The increase in both mRNA and protein levels of COX-2 in arthritic joint tissues of animal models of joint inflammation indicate an important role played by the enzyme in arthritic articular tissues [40]. PGE_2, the most abundant PG produced in the articular tissue, is involved in many physiological events, such as cell growth, immune regulation, and inflammation. Three prostaglandin-E synthase (PGES) isozymes, each encoded by a separate gene, are responsible for PGH_2 isomerization to produce PGE_2. Further studying the activity of microsomal-PGES (mPGES) isoforme showed inducible nature in the presence of inflammatory stimuli providing an interesting target in the development of inflammatory arthropathies. Both COX-2 and mPGES are key enzymes for PGE_2 biosynthesis especially under inflammatory conditions [39].

The study of OA cartilage specimens indicated not only the expression of COX-2 but also showed spontaneous increase in PGE_2 levels that are at least 50-fold higher than normal cartilage [41]. Therefore, the noticed increase in local PGE_2 production, suggests an attribution through COX-2 activity. However, recent evidence was provided that PGE_2 in human synovial fibroblasts can also play a role in controlling COX-2 expression through a positive feedback mechanism [42]. In OA, PGE_2 is well reported to induce a catabolic effect where it can inhibit collagen expression, increase the production of MMPs, and modulate bone resorption by stimulating osteoclasts activity [43, 44]. PGE_2 has also apoptotic effect on chondrocytes by enhancing NO-induced apoptosis. In addition, other researchers suggested a direct apoptotic role for PGE_2 perhaps through cAMP activity [45].

The expression of COX genes is highly regulated. The inducible nature of COX-2 gene expression is regulated through its gene promoter which contains several inducible enhancer elements [46]. Several stimulators can interact with these elements to play a role in COX-2 gene expression through different pathways. Interestingly, 15d-PGJ2, the endpoint metabolite of PGD_2 is found to prevent IL-1β induced COX-2 expression and PGE_2 production [47]. Yet, it also induced COX-2 expression but not PGE_2 production in the absence of IL-1β. This observation suggests the presence of another signaling pathway involved in PGE_2 production with IL-1β stimulation on which 15d-PGJ2 inhibitory effect takes place [39].

The use of COX-2 specific inhibitor caused marked suppression in COX-2 expression, PGE_2 production, along with swelling, and cellular infiltration in the joints [48]. It was also shown that pre-treating rat models with neutralizing anti-PGE_2 monoclonal antibodies prevented signs of tissue inflammation, presenting PGE_2 as a valuable target for preventing symptoms of inflammation [49]. Non-steroidal anti-inflammatory drugs (NSAIDs) are among

the most widely prescribed drugs. They work by inhibiting COX enzymes in a non-specific way. Majority of the reported undesirable effects of NSAIDS are attributed to COX-1 isoform inhibition. Therefore, developing agents to specifically block COX-2 while simultaneously keeping COX-1 activity unaffected is considered a rational approach to obtain efficacy without deleterious side effects. COX-2 specific agents, generally called coxibs, are the most common clinically prescribed drugs as analgesics and for the management of several conditions including OA. Coxibs proved to be effective with significant reduction in known side effects usually associated with conventional NSAIDs usage [50]. However, recently concerns are rising regarding the permanence of some gastrointestinal and renal side effects, also regarding potential cardiovascular adverse effects. Up to date results released are considered inconclusive triggering the need for developing new agents or targets that help better control the expression and activity of COX-2 and PGE_2 [51].

Amongst COX-2 and mPGES-1 gene expression inducers HNE is also reported to act as inflammatory mediator that enhances protein and mRNA levels by affecting gene promoter activity in dose-time dependant pattern [45, 52]. This induction is also associated with increases in PGE_2 production. The expression of COX genes is highly regulated. The inducible nature of COX-2 gene expression is regulated through its gene promoter which contains several inducible enhancer elements. Several stimulators can interact with these elements to play a role in COX-2 gene expression through different pathways. For example, the cytokine IL-1β induces gene expression via mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways while HNE appears to modulate different pathways that affect COX-2 and mPGES-1 expression such as p38MAPK signaling pathway [45, 52].

Interestingly, 15d-PGJ2, a potent activator of peroxisome proliferator-activated receptor gamma (PPARγ), is found to prevent IL-1β induced COX-2 expression and PGE_2 production. Yet, this endpoint metabolite of PGD_2 could induce COX-2 expression but not PGE_2 production in the absence of IL-1β suggesting the presence of another signaling pathway involved in PGE_2 production with IL-1β stimulation on which 15d-PGJ2 inhibitory effect takes place [39, 47].

3.2 Catabolic molecules

The maintenance of healthy cartilage requires a system of ongoing balance between building and breaking mechanisms. However, when a shift in the endogenous system causes increases in the catabolic activity it leads to increase in cartilage ECM degradation. In OA, the destruction of cartilage components is considered a central character of disease development especially the degradation of Col II by collagenases activity of MMPs such as MMP-13 [53]. Moreover, other molecules can also contribute to the catabolic process like the free radical NO which is considered among the main molecules that mediate catabolic changes [54] .

3.2.1. MMPs

MMPs are a group of endopeptidase that share similar structure. Since their discovery the enzymes were studied for their function within different tissue not only for connective tissue remodeling, but also in wound healing, angiogenesis, and metastasis as well [55]. Among the group four enzymes MMP-1, -8, -13 and -14 have collagenolytic activity and are capable of breaking the highly stable fibrillar collagen. Samples from synovium and

cartilage from OA and RA patients showed an increase in the activities of several MMPs. However, an upregulation in cartilage expression and activity of collagenase-3 (MMP-13) in OA cartilage present the enzyme as the primary collagenase in OA [53, 56]. In arthritis endogenous inhibitors of MMPs such as TIMPS are produced; however their activities are not sufficient to compensate for the increase in catabolic activity of MMPs [57].

In an effort to reduce/manage the catabolic activity of MMPs, a number of MMP inhibitors have been developed and tested in various animal models of arthritis. An inhibitor of the collagenases (MMP-1, -8, and -13) prevents cartilage degradation in animal models of both RA and OA [58]. Other MMP inhibitors were tested in arthritis clinical trials, but serious side effects mainly due to lack of specificity have largely hindered their further development [59]. MMP-13 is known to be activated by a number of mediators, such IL-1β. In OA, the increase in MMP-13 expression and activity might therefore be attributed, in part, to the increase in IL-1β release in the synovial fluid [60, 61].

Moreover, locally produced inorganic oxidants were found capable of degrading cartilage mainly by inducing the oxidation of ECM components and/or posttranslational modification of MMPs [62, 63]. Moreover, reactive oxygen species (ROS) might shift the balance of proteolytic potential by decreasing the production and/or the activity of TIMPs [64]. In addition, HNE can also induce MMP-13 expression at protein and mRNA levels. [65-68]. Interestingly, an earlier study revealed that the pathway needed for activating the transcription and protein synthesis of MMP-13 by HNE is different from the pathways triggered by proinflammatory mediators like IL-1β and TNF-α. Whereas IL-1 induction of MMP-13 gene expression requires the activity of p38 and, c-Jun N-terminal kinase (JNK) with the translocation of NF-κB translocation [66], TNF-α triggered MMP-13 gene

expression involves MAPK, activator protein 1 (AP-1) and NF-κB [65]. Nevertheless, HNE induction of MMP-13 transcription requires p38 MAPK activity with possible role of AP-1 and c-Jun but not NF-κB [67].

3.2.2 Inducible nitric oxide synthase (iNOS) and NO in OA

NOS is the enzyme responsible for the production the highly reactive molecule NO through l-arginine metabolism. The enzyme is present within different tissues in three isoforms; neuronal (nNOS) and endothelial (eNOS) and inducible (iNOS). The constitutive nNOS and eNOS produced low level of NO play an important role in intercellular signaling and the homeostasis in neurons and endothelial cells [69, 70] . However, in the presence of certain stimuli like inflammation the release of certain cytokines and mediators such as IL-1β and TNFα can trigger the transcription and activity of iNOS; as a result large quantities of NO are produced [71]. In fact, the inducible production of NO is significant not only for inflammatory, and immunological host defense responses, where NO can exert cytotoxic effects against microbes, but for tumor cells and tissue repair as well [72]

Earlier studies indicated the presence of higher levels of NO in human synovial fluids from RA and OA patients when compared with normal samples [73]. The observation suggests an important role for the free radical as a mediator of inflammation in both diseases. In OA, sustained high levels of inflammatory cytokines especially IL-1β leads to prolonged activation of iNOS usually in the presence of highly oxidative environment. The release of high amount of NO in the presence of superoxide anion leads to the formation of the highly reactive peroxynitrite which in turn induces proapototic and proinflammatory responses in cells [74-77]. In chondrocytes, NO is not only cytotoxic; in fact, the molecule has an

additional predominant catabolic effect where it inhibits the synthesis of ECM components; Col II [78] and proteoglycan [79], as well as activating MMPs [80]. These observations indicate essential role of induced NO release and its derivatives as mediators in OA pathogenesis through different mechanisms [81].

The production of NO by chondrocytes and synoviocytes in OA is regulated by the level of NOS transcription and translation. Therefore, several studies tested the effect of different inhibitors of NOS in OA and RA. Such studies helped also elucidate the role of iNOS and NO as mediators in disease pathogenesis. Studies on animal models of OA using different inhibitors such as NG-monomethyl-t-arginine (NMMA) and specific iNOS inhibitor N-iminoethyl-L-lysine (L-NIL) showed interesting and promising findings in RA and OA respectively. The data showed that the inhibition of iNOS results in marked suppression in tissue destruction associated with chronic inflammation [82, 83]. In a study on an animal model of RA, the administration of NMMA greatly reduced leukocytes effusion, joint erosion and other signs of inflammation in the area. Interestingly, the beneficial effect was not limited to earlier stages of the arthritis, but it was also present at the chronic phase of the disease [84]. Similar results were also obtained by selectively inhibiting iNOS in experimental OA model using L-NIL. The inhibition of iNOS was associated with reduced production of NO in tissue which also resulted in a significant decrease in the production of catabolic factors such as MMPs and peroxynitrite [83]. In a more recent study in OA the use of L-NIL could also prevent NO-induced lipid peroxidation and subsequent production of highly reactive toxic compounds like HNE; a key mediator in OA [85]. Moreover, L-NIL significantly reduced the cytotoxic, proinflammatory and catabolic effects induced at different pathological concentrations of HNE. L-NIL also blocked the inhibitory effect of IL-1β on glutathione S-

transferase (GST) and the stimulation of ROS in a dose dependent pattern [85]. The reported beneficial effects of NOS inhibitors indicated a potential promising protective effect on chondrocytes in OA.

4. THE OXIDATIVE STRESS IN OA

Closer study of the chronic degenerative nature of OA led to significant shift in the search for therapeutic solutions for the disease. In fact, most available pharmacological agents targeting the formation of inflammatory mediators have not provided a real solution for OA. Therefore, there is an essential need for other secondary therapies designed to prevent the progression of this chronic disease. Current attempts based on recent data aim to develop effective chondroprotective disease-modifying agents to control different molecular pathways involved in the initiation and progression of OA. Oxidative stress within articular tissue has been pointed out as an aspect with a significant role in the pathogenesis of OA, an observation supporting the predicted therapeutic potential of targeting pathways involved in joint metabolism controlled or affected by oxidative stress [86, 87].

Though physical exercise is important for maintaining normal cartilage function, excessive repetitive mechanical load and sheer stress can trigger apoptotic and inflammatory responses in chondrocytes. The increase in ROS production within the articular tissue due to excessive mechanical load and stimulated pro-inflammatory mediators can lead to diffusion of free radicals into cartilage. Interestingly, studies also show a concomitant decrease in the protective antioxidant response within the joint leaving chondrocytes highly prone to cell

death through caspase activation mediated by the oxidative radicals [88]. Moreover, research on the effects of ageing show that cartilage homeostasis is highly affected by ageing process where chondrocytes are less responsive to mechanical anabolic stimuli. This observation can be correlated with our knowledge about OA to explain much higher incidence of the disease among older patients. Another condition affecting cartilage termed cellular senescence may lead to an irreversible arrest of chondrocytes growth causing detrimental outcomes on cartilage homeostasis relevant to cartilage ageing. A study by Yudoh et al. [89] investigated the role of oxidative stress and reactive radicals in cartilage senescence and in the development of OA. The study demonstrates that high level of oxidative stress affects chondrocytes in the presence of profound decrease in antioxidative capacity of articular tissue. Consequently, the oxidative stress induces a mechanism triggering the acceleration of chondrocyte senescence by causing abnormal erosion of cellular telomere end. Therefore, oxidative stress is considered closely involved in cartilage senescence and the development of OA. In addition, both mechanical and chemical stressors may alter cellular adaptation to hypoxia during disease progression, causing oxidative damage and changes in the microenvironment ultimately negatively affecting the synthesis of chondrocytes [89]. The same study reported an important reduction in the antioxidative potential in degenerating regions in comparison with the intact regions from the same OA articular cartilage sample. Obtained results suggested that oxidative damage can induce catabolic changes to cartilage matrix in articular cartilage. Furthermore, the treatment of cultured cartilage with an antioxidative agent showed a protective effect against the observed oxidative stress-induced chondrocyte dysfunction and cartilage damage. Collectively these findings support the theory focusing on oxidative stress as an inducer of catabolic changes in cartilage matrix [89].

4.1. Oxidative stress and antioxidant molecules

Reactive molecules are normally present within a biological system. Different reactive species can be produced as byproducts of normal activities such as aerobic metabolism, during pathological conditions or result from external resources such as pollutants and cigarette smoke. At certain concentration reactive radicals do play a role in regulatory functions, cell signaling and in immunological response [90]. However, when present in large volumes, reactive molecules can interact with and damage major cellular components like proteins, lipids and DNA [90]. The induced damage can be attributed to the production of peroxides and free radicals like ROS and reactive nitrogen species (RNS). To avoid excessive cellular damage the body contains number of defense and protective mechanisms including continuous repair machinery and the antioxidant system. Disturbances in the balance between produced oxidative molecules and available reducing agents due to uncontrolled production of free radicals with or without a decline in reduction capacity of the cells can cause tissue damage in several pathophysiological conditions [91]. Under such conditions the cell is considered under oxidative stress. The effect of this stress on the cell and the body overall depends on the extent of damage caused by the reactive species and on cell's ability to constantly repair any damage. At different levels oxidative stress can cause several changes in cellular response. The induced effect may range from inducing altered cellular activity by affecting cell signaling pathways; either directly as chemical messengers or indirectly via modifying cellular components involved in the pathway such as lipids, to induce apoptosis. Moreover, under severe levels of oxidative stress the accumulating damage can prevent the cell from undergoing programmed cell death and cause necrosis [92, 93].

The antioxidant system contains different enzymes which protect against oxidative stress. Moreover, the role of antioxidants in controlling the production of inflammatory cytokines, LPO and the activation of transcription factors which trigger other inflammatory components is of major importance in several pathological conditions. The enzymes glutathione peroxidase (GPx), heme oxygenase-1 (HO-1) and superoxide dismutase (SOD) are among the best studied cellular antioxidants. In OA, studies indicated the importance of GSX, HO-1, SOD2 and SOD3 in the oxidative defense in cartilage [86, 94]. In addition, there is a well reported change in the expression of these genes in OA chondrocytes and in the presence of inflammatory cytokines such as IL-1β [86, 90].

Antioxidant molecules such as ascorbic acid, flavonoids, N-acetyl-cysteine (NAC) and glutathione (GSH) have been shown to play an important role in different inflammatory diseases such as atherosclerosis, arthritis and OA [95, 96]. In particular GSH, the substrate for GST and GPx, have shown a promising protective role in OA [97].

4.2. Reactive oxygen species (ROS)

Aerobic cellular activities such as cellular respiration can produce oxygen containing reactive molecules named ROS. The highly reactive molecules and the less reactive of these species are important for redox signaling pathways [98]. The immune system also benefits from the destructive activity of ROS in killing pathogens [99]. However, due to their high reactivity, ROS are widely linked to the induction or aggravation of different pathological conditions such as atherosclerosis, cancer, neurodegenerative disease and OA [90]. The production of ROS is not solely influenced by internal mechanisms but external sources like environmental toxins can also induce their generation within the body. Therefore, the role of

antioxidants within the biological system is considered primordial to maintain an optimal level of these molecules at all times [100].

In OA, several ROS have been closely studied as main factors in the pathology of the disease. While articular cartilage is an avascular tissue that depends on nutrient supply needed for basic cellular functions from the synovial fluid, chondrocytes are adapted to work within low oxygen pressure to anaerobic environment [101]. In pathological conditions associated with OA, fluctuations in partial oxygen pressure, mechanical stress and inflammatory mediators such as IL-1β can put chondrocytes under the influence of oxidative stress. Subsequently, significant increase in the production of ROS such as NO and O_2^- takes place. Different ROS molecules can further interact to generate reactive radicals, including peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2). Moreover, recent studies reported increase in the production of myeloperoxidase (MPO) in OA as well [102]. In the presence of H_2O_2 and Fe^{+2}, chondrocytes produce hydroxyl radicals ($OH^•$). The highly reactive radical can initiate further series of reactions with unsaturated fatty acids within the lipids of cell's membrane. Chain reactions can then result in the formation of other lipid radicals with much longer half life ($RO^•$,$ROO^•$) such as LPO end product HNE [103]. The role of oxidative stress along with produced ROS and LPO end products in OA is a subject for deep investigation and research.

4.3 Lipids within the cell

Lipids are one of the major building blocks of the eukaryotic cell. Different forms of lipids such as; fats and fatty acids, phospholipids, certain vitamins, and steroidal

hormones are essential for the structure and function of living cells. Earlier opinions focused on lipids as a source of storage and provision of energy through their oxidation however, a much complicated and diverse role in the regulation of biological processes have been described later on [104]. For example, lipids can act as chemical messengers in cellular signaling; they are involved in hormone regulation, membrane lipid layer formation, and cholesterol synthesis (cholesterol is also the precursor of bile acids, vitamin D and steroidal hormones). The hydrophobic properties of lipids make them excellent candidates for signaling as they can diffuse through different membranes to carry signals by binding to either membrane-bound or intracellular receptors [105]. Lipids can act as potent chemical messengers for paracrine, autocrine, and endocrine signaling forming a complex signaling network. In fact, imbalances in this network can be associated with different pathological conditions [106]. Interestingly, the essential fatty acid linoleic acid has an important role as precursor for the formation of prostaglandins which was first described by Samuelsson et al in 1964 [107, 108]. Prostaglandins in turn play a significant role in inflammation and other diseases including OA [39, 107].

4.4. Lipid peroxidation (LPO)

The lipids within the cell can undergo non-enzymatic oxidative degradation by ROS, especially when the cell is under oxidative stress. Polyunsaturated fatty acids (PUFAs) within the cellular membrane are main target for different ROS due to the presence of multiple double bonds within their structures. The breakdown of PUFAs within cellular membrane can lead to further oxidative reactions and the production of potentially toxic LPO-end products. In the absence of protective antioxidant response, the self-propagating nature of the chain

reaction can result in significant tissue damage starting from the oxidation of only a few lipid molecules [109].

The production of advanced LPO-end products (ALEs), such as aldehydes, within the cell is of special interest because of their unique biological properties and potential role in different pathological conditions. ALEs are more stable and have longer half life than ROS, making their ability to form protein adducts causing protein dysfunction and affecting cellular response of major importance. Moreover, the rates of ALEs production and protein adduct formation increase along with reduction in antioxidant capacity, such as during ageing, causing the accumulation of ALEs and modified proteins [110]. Depending on their target protein, different ALEs can be associated with different pathological conditions, such as atherosclerosis, asthma and OA [67, 111-113]. Whether studied LPO-products are the cause of a certain disease or they are merely produced as a result of the pathological condition, is still under investigation. However, the role of LPO-products in disease progression is predominant [109]. The process of LPO can affect the cell on different levels; disturb the structure of the cellular membrane, causing changes in its fluidity and permeability, and affect metabolic processes and ion transportation through the membrane [114].

During LPO three main steps take place; initiation, propagation, and termination. The initiation phase generally results in the abstraction of a hydrogen atom. In the case of PUFA the initial reaction with radicals produces lipid radical (L•), which then reacts with oxygen molecules to form a lipid peroxyl radical (LOO•). In the next step, the LOO• can become lipid hydroperoxide (LOOH) by abstracting hydrogen atom from another fatty acid molecule which in turn becomes a second lipid radical [115]. In the presence of reduced metals, such as Fe^{+2}, a reductive cleavage of LOOH can occur producing lipid alkoxyl radical

(LO•). Both alkoxyl and peroxyl radicals can abstract additional hydrogen atoms from other fatty acid molecules thereby triggering lipid peroxidation chain reaction [116]. Additionally, in the presence of reducing agents like ascorbate, LOOH can break to produce reactive aldehydes, such as malondialdehyde (MDA), HNE, and 4-hydroxy-2-hexenal (4-HHE) [117]. LPO products such as HNE can then interact with proteins, peptides, phospholipids, and nucleic acids within the cell. As a result, HNE can act as second toxic messenger affecting cellular pathways and causing cytotoxic, mutagenic and genotoxic changes [118, 119].

4.5. HNE

4.5.1. Synthesis and characteristics of HNE

The α,β-unsaturated hydroxyalkenal is the primary LPO product in cells from ω−6 PUFA such as linoleic acid and arachidonic acid [118]. HNE is normally found within different biological tissues at basal concentration where it acts as second messenger [120]. However, the increase in its level especially during oxidative stress is being linked to different pathological conditions. The electrophilic nature of HNE due to its α,β-unsaturated carbonyl makes it highly reactive to cellular nucleophiles such as proteins especially via 1, 2- and 1, 4-Michael addition. These properties make HNE at level beyond its basal concentration to possible toxicity to the cell [118, 121]

4.5.2. Metabolism of HNE

The role of HNE in the pathology of several diseases made intracellular HNE degrading pathways an important part of the antioxidative defense system. By preventing the

accumulation of aldehydic LPO-products, these pathways can protect proteins from modification by the reactive compounds. HNE is metabolized mainly through intracellular metabolism by different mechanisms including Michael additions, oxidation and reduction [122]. Several enzymes such as alcohol dehydrogenases, aldehyde dehydrogenases, and GST in addition to cofactors like NAD+ and NADH are needed for these reactions to take place [123]. The cellular capacity for HNE enzymatic metabolism depends on the level and availability of the metabolizing enzyme and/or cofactor. For example, though GST is present in almost all cell types the availability of this pathway is controlled by the level of GSH within the cell. Therefore, when a rapid or prolonged increase in HNE production leads to a fast decrease in intracellular level of GSH, the role of this antioxidant in HNE detoxification is greatly compromised [121].

4.5.3. HNE trapping molecules (Carnosine, N-acetyl-cysteine, Glutathione)

The high bioactivity of HNE can be attributed to mainly to the electrophilic nature of its α,β-unsaturation. This character makes cellular nucleophiles such as GSH amino acids cysteine, lysine and histidine within side chains of proteins main target for the aldehyde. The interaction between HNE and a nucleophile results in the formation of adducts especially Michael adducts formation, a stable and strong bond [121]. The same reaction is also a method for HNE detoxification where forming similar adducts with reducing molecules like GSH hinders free HNE molecules preventing their interaction with other cellular components. However, benefiting of this pathway as reliable detoxification method depends on the presence of elevated levels of the reducing agent GSH. Exogenous neucleophilic compounds such as Carnosine (CAR) and NAC can interact with and trap free HNE molecules by forming

stable covalent adducts. Therefore, such compounds referred to as HNE-trapping molecules are being studied for possible protective properties to prevent deleterious effect of free and protein-bound HNE molecules on cellular components [124]. Additionally, these compounds can be used to investigate the effect of free HNE molecules and HNE modified proteins on the cell. The natural antioxidant GSH (Glu–Cys–Gly) is essential for maintaining antioxidants in an active state, and exerts high detoxification reactivity against HNE. CAR, a natural antioxidant composed of β-alanyl-L-histidine dipeptide has been found to inhibit HNE-induced protein modification in cartilage and other tissues by directly binding to free HNE molecule or displacing bound HNE off protein surface. Therefore, CAR is being presented as a quencher of highly cytotoxic aldehydes [125]. Another amino acid derivative NAC has highly protective scavenging properties against HNE and MDA. Studies reported that the pretreatment with NAC protects chondrocytes against HNE-induced apoptosis in chondrocytes and several cell lines [97].

4.5.4. HNE and GSTA4-4

GSTs are important enzymes for cellular metabolism and detoxification. GST facilitates the conjugation between GSH molecule and an electrophilic center like HNE. In fact GST is more than 600 times more active than the non-enzymatic Michael addition reaction. Under oxidative stress or in case of pathological condition, high LPO rates accelerate HNE formation. The increase in GST activity to neutralize the produced HNE can lead to a concomitant fast drop in intracellular GSH concentration [122]. GSTs could also aid in reducing hydroperoxide formation during LPO process leading to less HNE generation [126]. Among different isoforms, GSTA4-4 plays an important role in the detoxification of HNE

within articular tissue due to its high catalytic efficiency with the toxic aldehyde [127]. On one hand, studies on OA showed that the loss of GSTA4-4 enhanced the cytotoxic effect of HNE on chondrocytes. On the other hand, enzyme's overexpression succeeded in protecting the cells from HNE-induced cell death [97].

4.5.3. HNE in OA

Research on OA showed increased production of LPO-products especially MDA and HNE in articular tissue. Moreover, synovial fluid samples from OA patients revealed significant increase in the levels of HNE adducts in OA samples in comparison with control subjects There is also evidence that the influence of HNE follows dose dependant pattern, where at lower concentration (≤10 μM) HNE acted as an important mediator of catabolic and inflammatory processes in OA. At higher concentration ≥20 μM, HNE had pronounced cytotoxic effect on chondrocytes [97, 125] . This important influence of HNE on the cell is caused mainly by its interaction with cellular proteins especially through the formation of adducts. HNE-modified proteins may show various changes in their function and/or activity [125, 128]. HNE is capable of affecting a wide array of biological activities, such as signal transduction, gene expression, and modulation of cell proliferation [129] mainly by modulating the expression of different genes [130]. Such HNE-induced effects including apoptosis are well reported in several cell types of different origins including chondrocytes [131]. However, HNE cytotoxicity was preventable in the presence of antioxidant agent NAC or by the overexpression of HNE-detoxifying enzyme GSTA4-4 [97].

In chondrocytes isolated from OA cartilage HNE, directly and indirectly, triggered several changes affecting the production and activity of collagen network component Col II.

Whereas HNE inhibited the expression of Col II, the HNE-modified collagen was not only more susceptible for degradation by MMPs especially MMP-13, but it could also interact with chondrocytes to induce inflammatory and catabolic responses. Furthermore, HNE binding to Col II can cause multiple abnormalities in both phenotype and function of interacting chondrocytes by modulating the expression of adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and α1β1 integrins indicating a role for the aldehyde in the alteration of cell-ECM interaction in OA [125]. HNE can also increase in the activity, protein synthesis, and mRNA levels of MMP-13 mainly through the activation of p38 MAPK [67]. Furthermore, it was also suggested that a post transcriptional HNE-induced conformational change on MMP-13 leads to protein activation without proteolytic cleavage of its pro-domain [68]. In addition, HNE is well reported to stimulate the oxidation and subsequent fragmentation of collagen. These changes can affect the material properties of collagen fibrils, making them less resilient hence more prone to mechanical pressure. Consequently, the produced brittle cartilage might induce the initiation of OA [128].

4.5.4. HNE and signaling pathways

The role of HNE has long been a subject of debate whether the aldehyde has a "second toxic messenger" role as first hypothesized by Esterbauer and colleagues [118], or if it merely represents a marker of extensive tissue damage. This controversy changed recently when HNE was found to be a normal constituent of mammalian tissue membranes. In addition, growing evidence supported a role for HNE as a modulator of signal transduction and posttranslational modification in both physiological and pathophysiological responses. This role of HNE is highly regulated in a time-dose-dependent pattern [128, 132-134].

In OA, it was reported that free HNE can induce catabolic and inflammatory responses in isolated OA chondrocytes and alter the cellular phenotype of OA osteoblasts possibly by the modulation of several signaling pathways both directly and indirectly by modifying pathway components [52, 67, 113]. HNE binding to Col II modulates the expression of adhesion molecules such as ICAM-1 and α1β1 integrins. Adhesion molecules are expressed on cell surface and their interactions with the surrounding environment is important for a vast array of biological processes including cell viability, inflammation and catabolism. HNE-modified Col II affected the expression of both ICAM-1 and α1β1 integrin at the protein and mRNA level [125]. Additionally, HNE-modified Col II could affect multiple OA related catabolic and inflammatory responses via the activation of MAPKs and nuclear factor-kappa B (NF-κB) [52, 113]. Data obtained suggest that NF-κB/ ERK1/2 and p38 kinase oppositely regulated cell viability and adhesion in the presence of HNE-Col II adducts and this regulation could be linked to the level of Col II alkylation [125]. A concomitant increase in the activity, protein synthesis, and mRNA levels of MMP-13 by HNE occurred at the transcriptional level and required the activation of p38 MAPK, but not p54/46 and p44/42 MAPKs, which is in contrast to the cytokine signaling pathways involved in MMP-13 synthesis [65-67]. Nevertheless, p38 MAPK is not the sole pathway for HNE signaling [135, 136]. HNE can also trigger modifications in markers of apoptosis, redox status, and energy metabolism in chondrocytes. In HNE-triggered apoptosis, the activity of several classical apoptosis markers was highly recognized during HNE-induced chondrocyte death [52]. **Fig**.**4** illustrates possible scheme for HNE role as modulator of cellular transduction pathways in both its free and protein bound forms. Furthermore, different types of cells showed a well documented upregulation and activation of pro-apoptotic factors, followed by translocation of

certain factors to the nuclease to undergo apoptosis in response to HNE stimulation [137, 138]. Focusing on apoptosis signaling pathways in chondrocyte revealed that HNE suppressed pro-survival Akt kinase activity but, in contrast, induced Fas/CD95 and p53 expression [45, 97]. Akt activation is known to lead to the phosphorylation of numerous other proteins involved in the regulation of glucose metabolism, cell proliferation, apoptosis, and gene expression [139]. The analysis of cellular energy and redox status indicated that HNE induced ATP, NADPH, and GSH depletion and inhibited glucose uptake and citric acid cycle activity. Such HNE induced changes in factors known to be essential for normal cell activities can exert deleterious effects on chondrocytes ultimately inducing apoptosis. Interestingly, while the complete loss of HNE-detoxifying enzyme GSTA4-4 in chondrocytes augmented HNE cytotoxicity, significant protection against HNE-induced cell cytotoxicity was achieved by enzyme overexpression. Therefore, change in GSTA4-4 expression is expected to have an impact in HNE-induced apoptosis. [52, 97].

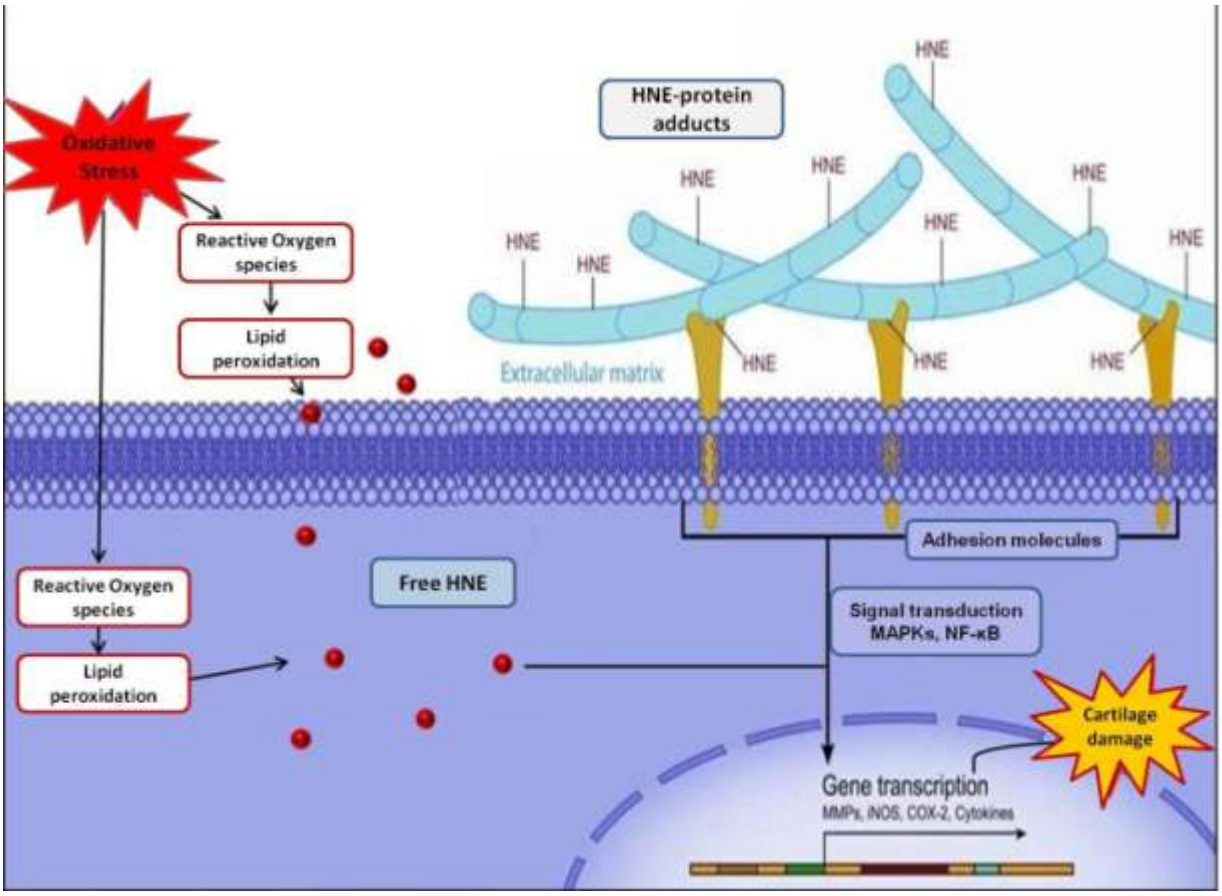


Figure 4: Possible scheme for HNE role as modulator of cellular signaling pathways in its free and protein bound forms.

HNE can modulate the transcription of pro-inflammatory and pro-catabolic mediators leading to cartilage damage in OA. Adapted from Bentz et al. 2012[85]

5- TRANSCRIPTION FACTOR Nrf2

5.1 Definition and function.

Since its identification in 1994 [140] the transcription factor named nuclear factor-erythroid-2-related factor 2 (Nrf2) has been gaining a lot of attention as a master positive regulator of the antioxidant response [141]. Nrf2 is a cap'n'collar basic leucine zipper

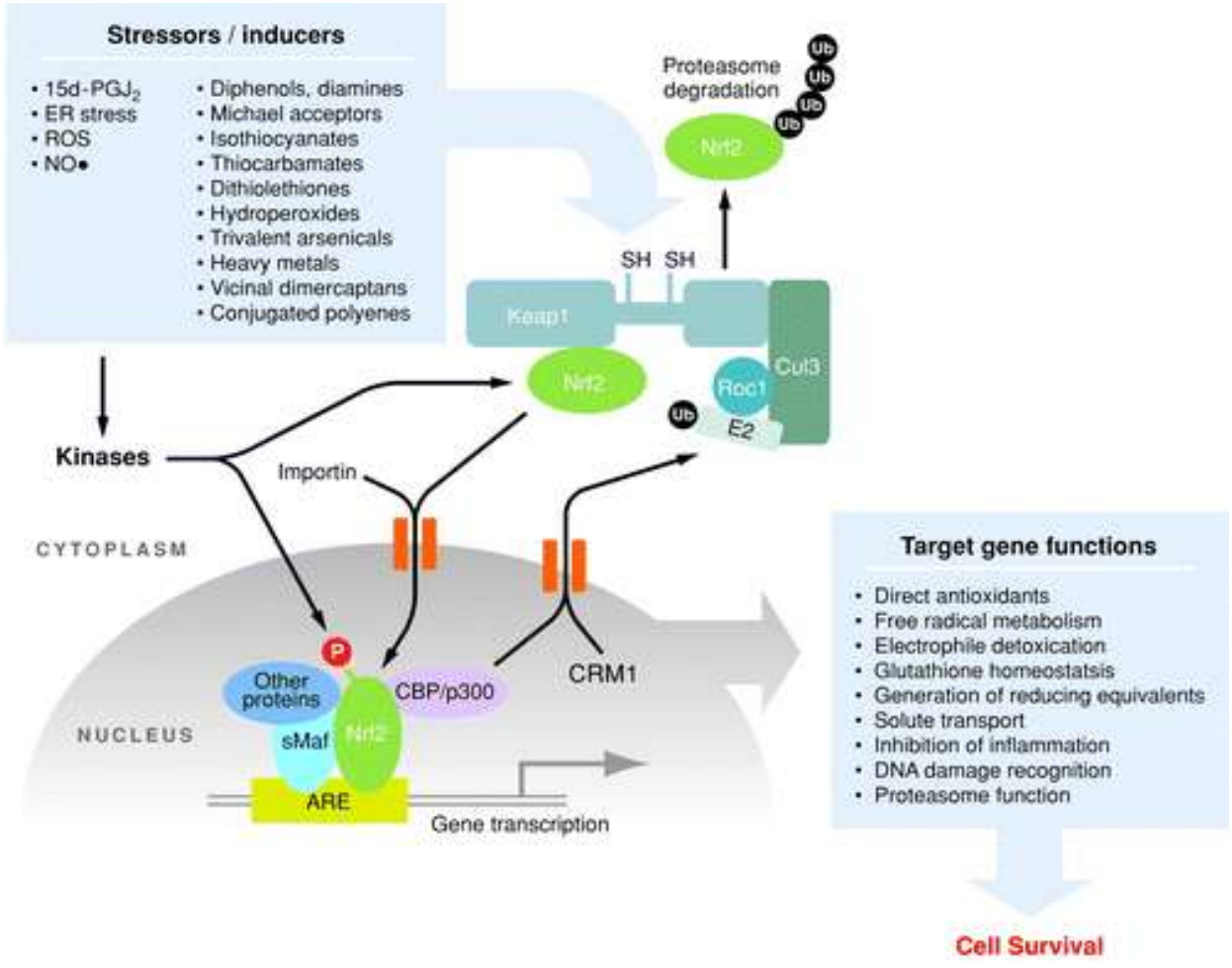
transcription factor that controls the expression of several enzymes by modulating human antioxidant response element (ARE). In response to endogenous and exogenous stressors Nrf2 can bind to and positively regulate the transcription of genes having ARE regulatory sequences [142]. Nrf2 transcriptional regulation mainly involves ARE-regulated genes essential for homeostasis, detoxification, immune, inflammatory responses, and tissue remodeling [143, 144].

5.1.1. Signaling pathway of Nrf2

Regulation of gene transcription through Nrf2 is achieved through signaling pathway that involves interaction with Kelch ECH associating protein 1 (Keap1) and ARE. Keap1 is a repressor protein in the cytoplasm that binds to Nrf2 to prevent its translocation to the nucleus and promotes its proteasomal degradation. The presence of stress signals triggers Nrf2 phosphorylation and dissociation from Keap1 followed by Nrf2 translocation to the nucleus. In the nucleus Nrf2 stimulates the transcription of several genes involved in stress response such as GST and inhibits cytokine-mediated inflammation [145]. Interestingly, Nrf2 itself contains an ARE-like sequence in its promoter, hence the transcription factor can autoregulate its own expression [146]. **Fig.5** illustrates the regulation of gene expression through Keap1-Nrf2-ARE pathway. The discovery of Keap1-Nrf2-ARE pathway and insights on it at molecular level can be found in detail in dedicated review [142].

In fact, the Keap1-Nrf2-ARE pathway is highly regulated and plays a cardinal role in cellular homeostasis. Therefore, Nrf2 is being studied for its relevance to disease prevention especially with the discovery of natural and synthetic Nrf2 activators such as, 6-Gingerol and

Nrf2 activator II (AI-1), respectively. The use of specific Nrf2 activators provides a precious path for enhancing the expression of cytoptotective genes [147, 148].



Kensler TW, et al. 2007.
Annu. Rev. Pharmacol. Toxicol. 47:89–116

Figure 5: General scheme depicts the regulation of gene expression through Keap1-Nrf2-ARE signaling pathway [142].

5.1.2. The pathophysiological role of Nrf2

Oxidative stress and loss of balance between oxidants and antioxidant protection within the cell are proposed as important factors associated with ageing and many pathological

conditions [149]. The increased production of pro-inflammatory cytokines such as IL-1β, chemokines, MMPs, COX-2, iNOS is a hallmark in the pathology of inflammatory diseases. However, the continuous cycle of oxidative stress and overproduction of inflammatory mediators can be stopped by the activation of Nrf2/ARE system. The activation of Nrf2 induces the expression of antioxidant molecules thus negatively regulates the production of pro-inflammatory mediators and enzymes [92, 149]. The role Nrf2 and the effect of losing its effect in various inflammation-associated disorders such as OA has been reported by several studies and discussed in literature review [92]. Recent studies have indicated that constitutive Nrf2 activation improves cell survival by enhancing resistance to oxidative stress possibly by enhancing the expression of cytoprotective genes such as GSTA4-4 [142]. However, loss or disturbance in Nrf2 level and/or activity can strongly affect cellular defense against ROS and LPO-products such as HNE [147]. Accordingly, the use of Nrf2 activators to enhance the transcription factor activity are considered a promising approach to help the cell eliminate the effect of oxidative stress, ROS and their products such as HNE [150] .

In OA, studies have demonstrated the role of HNE in disease pathogenesis. Ageing, an important risk factor for OA, is also associated with decline in Nrf2 expression leading to impairment in the expression of cytoprotective molecules [151].

5.1.3 Activators of Nrf2

Several natural and synthetic compounds are being investigated and/or developed in an attempt to regulate and enhance the transcriptional activity of Nrf2. Though its activation occurs mainly by triggering the release of Nrf2 from Keap1, several scenarios have been described. While the phosphorylation of Nrf2 at serine 40 appears to be an important event in

both release from Keap1 and translocation of Nrf2 to the nucleus, kinase signaling pathways may be involved, and adduct formation with Keap1 can also take place [152]. The anticipated subsequent increase in the expression of cytoprotective molecules makes such activators of great benefit especially in inflammatory disorders. Nevertheless, finding a specific Nrf2 activator that is pharmacologically effective for a specific disorder is still a great struggle [92]. Protandim® is a commercial dietary supplement composed of five phytochemicals with antioxidant properties. The mixture of these natural Nrf2 activators enhances the expression of endogenous antioxidant enzymes through multiple kinase pathways [153]. Moreover, dietary administration of Protandim® showed chemopreventive effect on the cell and reduced markers of oxidative stress, LPO and subsequently production of HNE in-vitro and in-vivo animal models [92, 154]. 6-Gingerol was also reported to be a potent activator of Nrf2. Lee et al. [155] have reported that 6-Gingerol exhibits preventive and/or therapeutic potential for the management of Alzheimer's disease via augmentation of antioxidant capacity and Nrf2 transactivation. **Fig.6** lists some of natural Nrf2 activators.

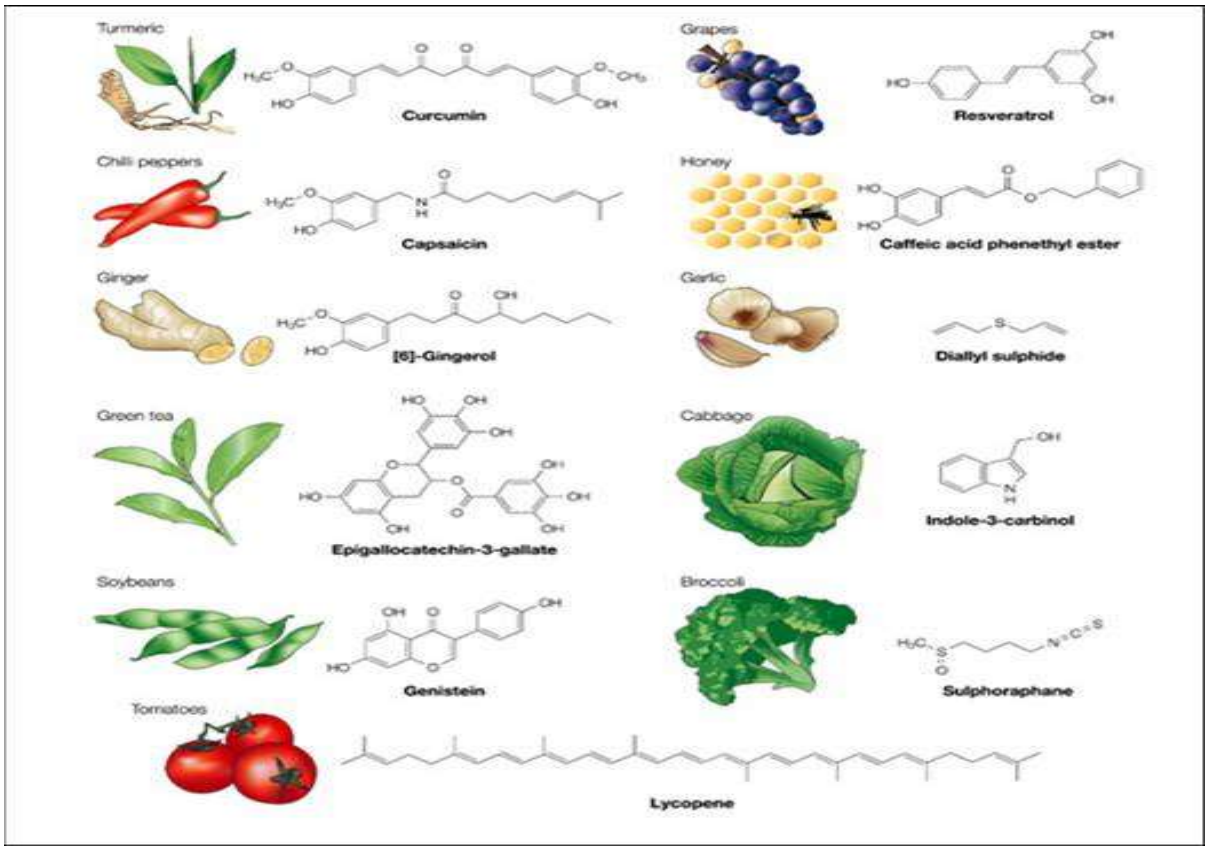


Figure 6: Some reported Nrf2 activators in natural dietary supplements.

5.2. Transcription regulation of HNE-detoxifying GSTA4-4 by Nrf2

GSTs are part of an important cellular response against intrinsic and extrinsic cellular stressors. The enzyme facilitates the addition of the antioxidant GSH to electrophiles such as HNE for detoxification and to prevent their interaction with cellular components. The expression of both inducible and constitutive forms of GSTs is controlled by Nrf2 activity [145]. Loss or disturbance in Nrf2 activity greatly affects the inducible expression of GSTs and leaves the cell more vulnerable to oxidative stress [142]. GSTA4-4 isoform plays an important role in HNE detoxification and in protecting the cell from HNE-induced apoptosis [97, 127]. Interestingly, HNE can trigger Nrf2 release from Keap1 ultimately inducing the

expression of GSTA4-4 through Nrf2-ARE activity. This cellular adaptive mechanism is programmed to counteract the accumulation of HNE. Upon activation, transcription factor Nrf2 can indirectly inhibit HNE production and subsequently the effect of free HNE on signal transduction pathways in OA chondrocytes through stimulating GSTA4-4 gene transcription. However, the protective antioxidant system within the cell can be overwhelmed by increased HNE production in the presence of oxidative stress leading to detrimental effects on the cell [156]. **Fig.7** represents the proposed role of Nrf2 in the transcriptional regulation of GSTA4-4 and subsequently in regulating HNE level.

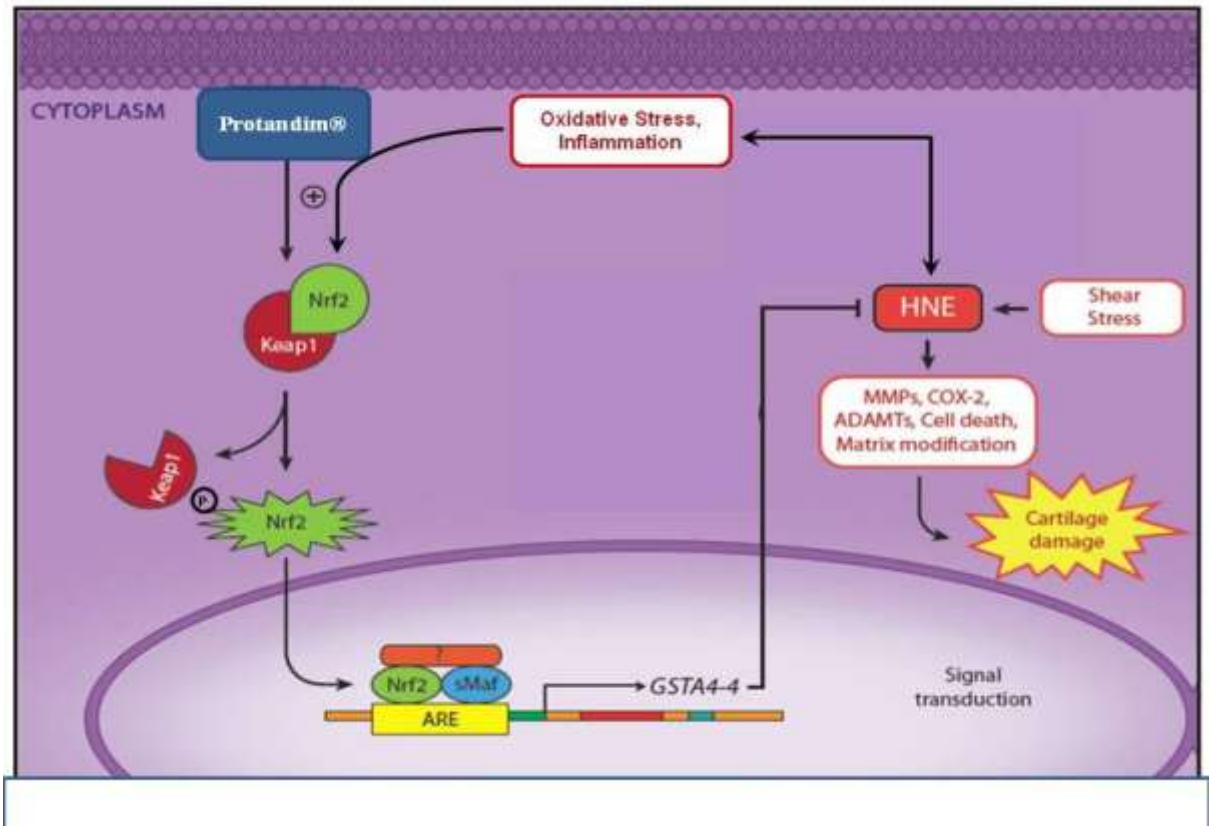


Figure 7: A scheme represents the proposed role of Nrf2 activation in regulating HNE level. (Adapted from Abusarah et al. 2012) [157]

6. OBJECTIVES AND HYPOTHESIS

It is well demonstrated in the literature that oxidative stress contributes in the pathogenesis of OA by causing the accumulation of ROS. Within different tissues the effect of ROS might be attributed in part to the production of toxic aldehydes, such as HNE, through LPO. Therefore, mechanisms targeting the role of HNE in the disease would be a beneficial approach in the treatment of OA. Previously obtained preliminary data indicated interesting findings that the expression of both transcription factor Nrf2 and GSTA4-4, is dramatically decreased in human OA cartilage as compared with control. This observation prompted further investigation regarding the significance of decreased GSTA4-4 and Nrf2 in cartilage. The current study focuses on the role of Nrf2 as a key transcription factor for multiple cellular stress-response genes such as phase-2 detoxifying enzyme GSTA4-4 which plays an important role as HNE detoxifying enzyme. Protandim®, a commercial product composed of a mixture of natural antioxidants is reported to activate and increase the levels of Nrf2 in different tissues. The activation of Nrf2 by Protandim® can prevent the production of HNE along with the pro-inflammatory and catabolic factors implicated in cartilage degradation in OA, mainly by stimulating the expression of GSTA4-4. The concept of activating cellular protection against the effect of HNE using Nrf2 as endogenous cellular defence switch can provide a promising technique in preventing further HNE-associated cartilage degradation and disease progression.

CHAPTER II: MATERIALS AND METHODS

2.1 Specimen Selection

Human OA articular cartilage was obtained from OA patients aged (67 ± 9 years, mean ± SD) who underwent total knee arthroplasty. All specimens were collected within the first 24 hours post-surgery. Informed consent had been obtained from OA patients for the use of their tissues for research purposes. All patients were evaluated by rheumatologists according to the criteria set by the American College of Rheumatology [158]. The experimental protocol and use of human tissues were approved by the Research Ethics Board of Hôpital du Sacré-Coeur de Montréal.

2.2 Sample Preparation and Isolation of Chondrocytes

OA knee cartilage was aseptically obtained from each specimen, spliced and rinsed to prepare the cartilage for the extraction of chondrocytes by sequential enzymatic digestion as described previously [159]. OA cartilage was digested with 1 mg/ml of pronase (Sigma, Oakville, Ontario, Canada) for 1 hour at 37ºC , followed by 0.5 mg/ml of type IV collagenase (Sigma) for 6 hours in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technology, Inc., Grand Island, NY, USA), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen Life Technology, Inc.). The cells were seeded at high density in culture flasks at 37ºC in a humidified atmosphere of 5% CO_2/95% air until they were confluent.

2.3 Cell Culture

When cells were confluent they were seeded (at 10^5 cells/cm^2) in tissue culture plates and incubated for 48 hours before treatment. To preserve the phenotype of the cells only chondrocytes from first-passage are used. The experiments were performed in DMEM containing 1% FBS and antibiotics with the factors under study. Chondrocytes were pre-treated with different concentrations of, Protandim® (LifeVantage Canada LTD), 6-Gingerol (Sigma-Aldrich), or AI-1 (Calbiochem) for one hour. Then cells were treated either with IL-1β (1 ng/ml) or with H_2O_2 (500 mM/ml) for 24 hours.

2.4 Protein Detection by Western Blotting

20 micrograms of total proteins from chondrocyte lysates under the indicated conditions were loaded for discontinuous 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). They were then transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON, Canada) for protein immunodetection, and semi-quantitative measurement as described previously [159].

The primary antibodies were: rabbit anti-human Nrf2 (R&D Systems); rabbit anti-human Gsta4-4 (Abcam Inc); mouse anti-b-actin (Sigma-Aldrich). After serial washes, primary antibodies were detected by anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Immunoreactive proteins were detected with SuperSignal blotting substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) and exposed to half-blue x-ray film (Bioflex, Clonex, Interscience)

2.5 MMP-13 Assay

Active MMP-13 enzyme was quantified in conditioned medium using Human pro-MMP-13 Immunoassay enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The sensitivity of the kit is 8 pg/ml. Assay was performed in duplicates. Absorbance was measured using the micro-ELISA Vmax photometer (Bio-Tek Instruments, Winooski, VT, USA).

2.6 PGE_2 Assay

PGE_2 level was assessed in conditioned medium by enzyme immunoassay (Cayman Chemical Company) according to the manufacturer's instructions. Detection sensitivity of the kit is 9 pg/ml. The assay was performed in duplicates. Absorbance was measured with the micro-ELISA Vmax photometer (Bio-Tek Instruments, Winooski, VT, USA).

2.7 NO Assay

Nitrite, a stable end-product of NO, was quantified in the supernatant according to a spectrophotometric method based on Griess reaction [160]. Assay was performed in duplicates. Absorbance was measured with the micro-ELISA Vmax photometer (Bio-Tek Instruments, Winooski, VT, USA).

2.8 Viability test

Chondrocyte viability was evaluated using 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) assay in 96-well plates (Fisher Scientific Company, Ottawa, ON, Canada) as described previously [161]. Cells were incubated with 0.5 mg/ml MTT reagent (Sigma-Aldrich) for 15 minutes at 37ºC, and then 100 µL of solubilization solution (0.04 M HCl-isopropanol) was added to dissolve the formed formazan salt. Absorbance was measured at 570 nm with the micro-ELISA Vmax photometer (Bio-Tek Instruments, Winooski, VT, USA).

2.9 Cartilage homogenization

Cartilage explants (~50 mg) were homogenized on ice in 1 ml of radioimmunoprecipitation assay (RIPA) buffer (150 m*M* NaCl, 1 m*M* EDTA, 40 m*M* Tris, pH 7.6, 1% Triton X-100), supplemented with protease inhibitors and 2 M guanidine hydrochloride. Extracts were dialyzed overnight against RIPA buffer at 4°C and conserved at -80°C.

2.10 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol reagent according to the manufacturer's instructions (Invitrogen). RNA was quantitated with RiboGreen RNA quantitation kits (Molecular Probes, Eugene, OR, USA), dissolved in diethylpyrocarbonate-treated H_2O, and stored at -80°C until used. 1 µg of total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Fermentas, Burlington, Ontario, Canada), as detailed in

the manufacturer's guidelines. One-fiftieth of the reverse transcriptase reaction product was analyzed by real-time quantitative PCR. The following specific sense and antisense primers were purchased from Bio-Corp Inc. (Montreal, QC, Canada): mouse Gsta4-4, 5′-GGA AGA ACT CAG TGC CCC TGT AC-3' (forward) and 5′- TGT AGG AAT GTT GCT GAT TCT TGT C-3' (reverse); Nrf2, 5′- AGC AGG ACA TGG AGC AAG TT-3' (forward) and 5′- TTC TTT TTC CAG CGA GG AGA-3' (reverse); mouse GAPDH, 5′- TGG AAT CCT GTG GCA TCC ATG-3′ (forward) and 5′- TAA AAC GCA GCT CAG TAA CAG TC-3′ (reverse).

Quantitative PCR analysis was performed in a total volume of 50 µl containing template DNA, 200 nM sense and antisense primers, 25 µl of SYBR Green Master Mix (Qiagen, Mississauga, Ontario, Canada), and 0.5 units of uracil-N-glycosylase (UNG, Epicentre Technologies, Madison, WI, USA). After incubation at 50°C for 2 minutes (UNG reaction) and at 95°C for 10 minutes (UNG inactivation and activation of AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 minute at 60°C for annealing and extension). Incorporation of SYBR Green dye into the PCR products was monitored in real-time with a Mx3000 real-time PCR system (Stratagen, La Jolla, CA, USA), allowing determination of the threshold cycle (Ct) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with 1 peak, indicating amplification specificity. A Ct value was obtained from each amplification curve with software provided by the manufacturer (Stratagen).

Relative mRNA expression in chondrocytes was quantified according to the ΔΔCt method, as detailed in the manufacturer's (Stratagen's) guidelines. A ΔCt value was calculated, first by subtracting the Ct value for the housekeeping gene GAPDH from the Ct value for each sample. A ΔΔCt value was then calculated by subtracting the ΔCt value for the control

(unstimulated cells) from the ΔCt value for each treatment. Fold changes compared to the controls were then determined by 2-ΔΔCt. Each PCR generated only the expected specific amplicon, as shown by melting temperature profiles of the final product and by gel electrophoresis of the test PCRs. Each PCR was run in triplicate on 2 separate occasions for each independent experiment (n=3-4).

2.11 Surgically induced OA mouse model.

OA was surgically induced in 10-week-old male 129S6/SvEv mice by destabilization of the medial meniscus (DMM) in the right knee, as previously described [162]. The mice were anesthetized with isoflurane and O_2, and the right knee joint was destabilized by transection of the anterior attachment of the medial meniscus to the tibial plateau. A sham operation, which involved a similar incision to the knee without compromising the joint capsule, was also performed on the right knee of 10-week-old 129S6/SvEv mice. Animals with DMM received intraarticular injection (10 mL) of saline solution (0.9 g/L NaCl) or Protandim® at 1 mg/ml. Mice were observed daily to verify healing and to ensure that they were using their right limb. The description of animal groups were reported in table

Groups	10-weeks old
Group 1: Sham	N=3
Group 2: OA	N=4
Group 3: OA + Protandim	N=4

Table 1: Description of different groups of Sham, OA, and OA + Protandim® mice.

2.12 Cartilage histology

Operated mice (sham and OA mice) were euthanized at 12 weeks after surgery. To assess OA severity, we evaluated isolated knee joints histomorphometrically. The right knee joints were dissected free of skin tissue, fixed in TissuFix (Chaptec), decalcified in RDO Rapid Decalcifier (Apex Engineering), and embedded in paraffin, as previously described [163]. Briefly, frontal histological sections (5 μM) were obtained throughout the knee joint at 80 μM intervals, deparaffinised in xylene, followed by a graded series of alcohol washes, stained with Safranin O–fast green (Sigma-Aldrich), and scored by 2 blinded observers according to the OARSI mouse scoring system [164], which grades cartilage structure and PG content on a scale of 0-6 (where 0 = normal cartilage, 0.5 = loss of PGs without structural changes, 1 = superficial fibrillation without structural changes, 2 = vertical clefts and loss of surface lamina, 3 = vertical clefts/erosion of the calcified layer lesion for 1-25% of quadrant width, 4 = lesion reaches calcified cartilage for 25-50% of quadrant width, 5 = lesion reaches calcified cartilage for 50-75% of articular surface, and 6 = lesion reaches calcified cartilage for >75% of quadrant width). All joint quadrants (medial tibial plateau, medial femoral condyle, lateral tibial plateau, and lateral femoral condyle) were scored separately, and the scores were added to obtain summed OA scores for the whole joint.

2.13 Plasmids and transient Nrf2 transfection

Wild type and mutant GSTA4-4 expression plasmids were generously gifted by Dr. S. Awasthi [126] (University of North Texas Health Science Center, Fort Worth, TX, USA). The

expression vector of Nrf2 is gifted by Dr. Yue Xiong (University of North Carolina, Lineburger Cancer Center, NC, USA). Subconfluent chondrocytes were transiently transfected by Lipofectamine 2000TM reagent (Invitrogen Life Technology) according to the manufacturer's protocol. Briefly, transfections were conducted for 6 hours with DNA lipofectamine complexes containing 10 µl of lipofectamine reagent, 2 µg empty DNA plasmid, 2 mg Nrf2 expression plasmid, and 0.5 µg of pCMV-β-gal (as a control of transfection efficiency). After washing, experiments were performed in 2% FBS fresh medium supplemented or not with 1 ng/ml IL-1β. Then, Nrf2 expression was analyzed by Western blotting, as described previously. β-gal level was measured with ELISA kits from Roche Diagnostics Canada, Laval, QC, Canada).

2.14 Cellular level of HNE-protein adducts

Total cellular levels of HNE-protein adducts were assessed by enzyme-linked immunosorbent assay, as described by Benderdour et al. 2003 [128] with some modifications. To prepare the HNE-BSA standard, BSA (Sigma) was dissolved in 50 mM phosphate-buffered saline (PBS; 5 mg BSA/ml), pH 7.2, and incubated at 37°C for 1 hour in the presence of 0.1 mM HNE. The HNE-BSA solution was then filtered and washed four times with PBS using 10,000 MWCO centrifugal filters (Millipore). Immulon II microtiter plates (VWR International) were precoated overnight at 4 °C with 100 µl of HNE-BSA standard or sample (tissue protein extracts concentrated 5-fold), washed four times with PBS, and then incubated with 200 µl of blocking buffer (5% BSA and 5% fetal bovine serum) for 4 hours at room temperature. The blocking buffer was discarded, and the plates were incubated overnight at

4°C with 200 µl of primary antibody rabbit anti-HNE (1:1,000 dilution in 0.05% Tween-PBS). After washing four times with Tween-PBS, 200 µl of the goat anti-rabbit IgG-horseradish peroxidase conjugate (1:50,000; The Jackson Laboratory) were added, and the plates were incubated for 2 hours at room temperature. Detection was carried out with a Sigma Fast™ POD kit (Sigma). After a 10 to 20 min incubation at room temperature, the reaction was stopped with 50 µl of 2N H_2SO_4, and absorbance at 450 nm was measured with a microplate reader. A linear response in the range up to 5 µg/ml of HNE-modified BSA standard was observed (slope = 0.30 ± 0.03; coefficient of correlation = 0.99; $p < 0.0001$).

2.15 Statistical Analyses

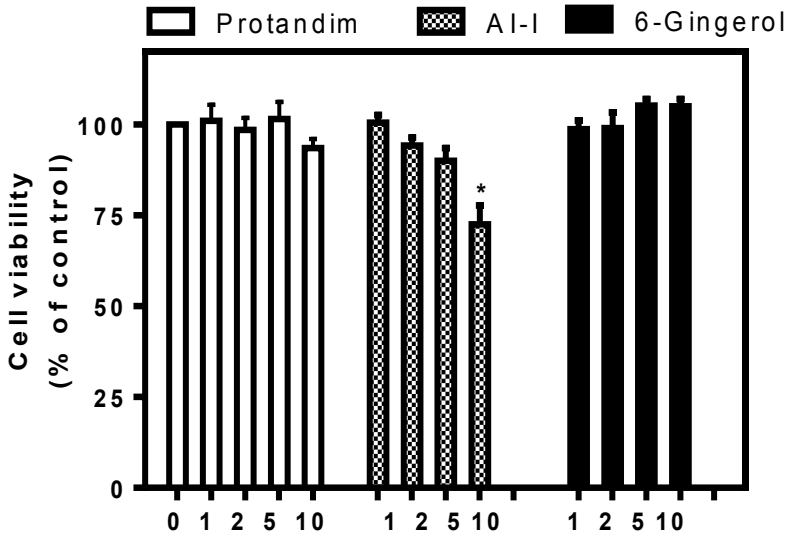
Results were expressed as the mean ± standard error of the mean of eight specimens, and assays were performed in 3 independent experiments. Statistical analysis was performed using the two-tailed paired Student t test, and a difference of less than or equal to 0.05 was considered significant.

CHAPTER III: RESULTS

1- Natural and synthetic Nrf2 activators safely increase Nrf2 protein level

To evaluate the effect of Nrf2 activation samples were treated with two natural and one synthetic activators of Nrf2; Protandim®, 6-Gingerol and Nrf2 activator II **(**AI-1), respectively. Chondrocytes were first treated with three different Nrf2 activators at increasing concentrations for 24 hours. Cell viability is then determined using MTT test and calculated as percentage of control. Obtained results depicted in figure 8A show no change in cell viability with Protandim® and 6-Gingerol at tested concentrations. Synthetic activator AI-1 however, shows some toxicity at concentration ≥10 µM. The level of total Nrf2 protein was then assessed in cellular extracts using Western blot. Figures 8B shows visible dose-dependent increase in total Nrf2 with Protandim®. However, it is well reported in the literature that Protandim® can activate Nrf2 in different cell lines by inducing the latter's nuclear localization. This activation is proposed to take place via the activation of various kinases, subsequently prompting the phosphorylation of Nrf2 [147, 153]. Therefore, we are anticipating that the reported increase in Nrf2 activity is associated with simultaneous increase in the level of Nrf2 phosphorylation. Collectively, our obtained findings confirmed both safety and Nrf2 activation capability of Protandim®, AI-1 or 6-Gingerol at concentrations (0-10 µg, 0-5 µM and 0-10 µM respectively).

A



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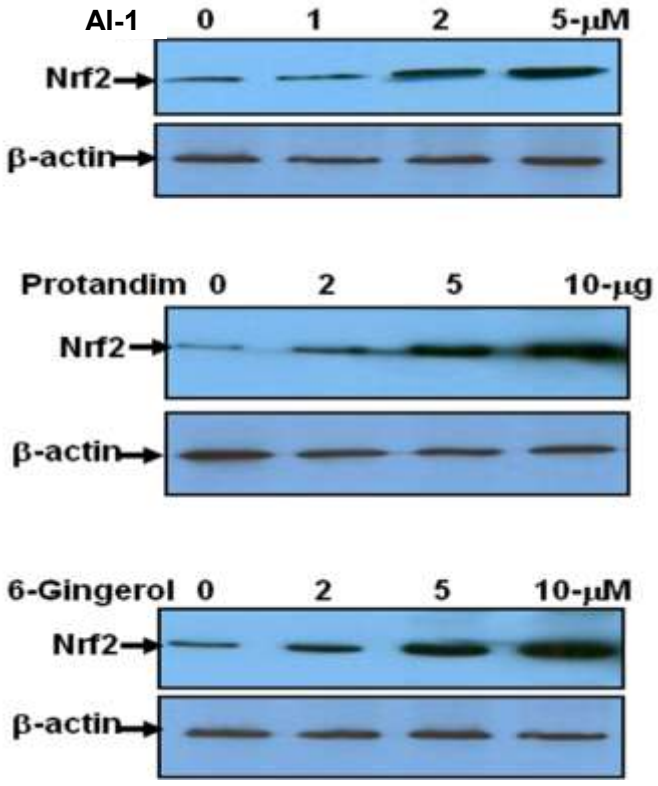

Figure 8: Effect of Nrf2 activators on cell viability and Nrf2 expression.

Human OA chondrocytes were incubated with natural (Protandim® and 6-Gingerol) or synthetic activator of Nrf2 (AI-1) at different concentrations for 24 hours. A) Cell viability was evaluated using 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) assay in 96-well plates by incubating them with 0.5 mg/ml MTT reagent for 15 min at 37^0C. Then, 100 μL of solubilization solution (0.04 M HCl-isopropanol) was added, formazan salt was dissolved, and absorbance was read at 570 nm with the micro-ELISA Vmax photometer. (B) Nrf2 total protein expression was determined by Western blot using monoclonal anti-Nrf2 antibody. Data are the means ± SEM of 4 independent experiments. *$P < 0.05$, compared to untreated cells (1% FBS).

2- Treatment with Nrf2 activators protects chondrocytes against H_2O_2-induced cell death

It is well reported that H_2O_2 can induce cell death mainly by increasing oxidative stress. However, overexpression of antioxidant molecules and enzymes can protect the cell from such outcome [165]. To examine the protective effect of Nrf2 transcription factor against oxidative stress-induced cell death, three different Nrf2 activators were tested. First, isolated human OA chondrocytes were pre-incubated with increasing concentrations of Nrf2 activators; Protandim®, 6-Gingerol or AI-1 for one hour. Cells were then treated with 0.5 mM H_2O_2 for 24 hours. Cell viability assay presented in Figure 9 reveals dose-dependent protection with all three activators. The maximum activation was obtained with 6-Gingerol at 10 µM with 105% when compared to untreated cells. These readings suggest that Nrf2 activation can protect the cells from the cytotoxicity of H_2O_2.

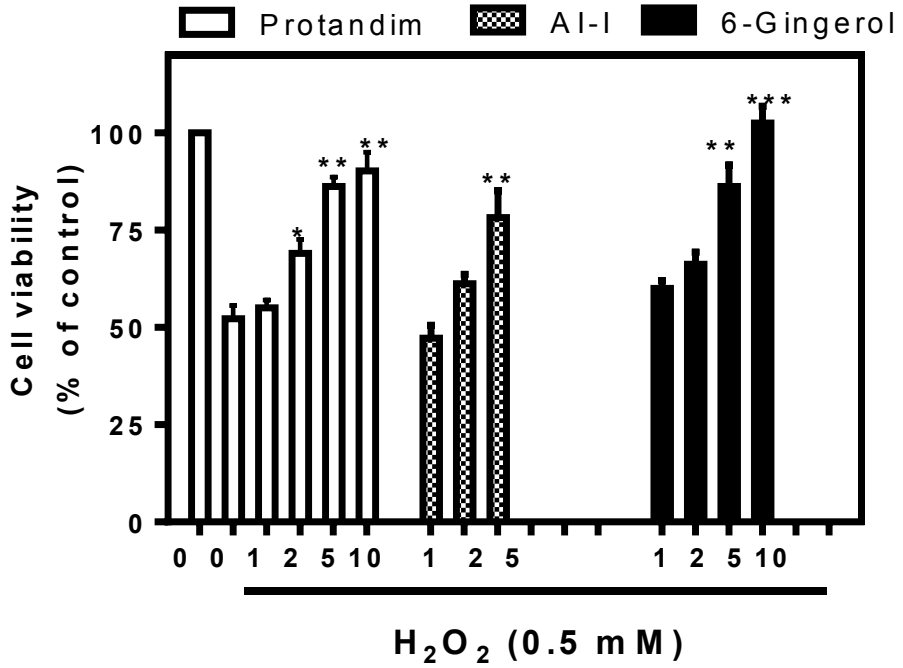

Figure 9: The activation of Nrf2 protects the cell against H_2O_2-induced cell death.

Human OA chondrocytes were pre-incubated with Protandim®, AI-1 or 6-Gingerol (concentrations 0-10 µg, 0-5 µM and 0-10 µM respectively) and then incubated with 0.5mM H_2O_2 for 24 hours. Cell viability was assessed by MTT assay as described in the legend of the figure 8.

3- Nrf2 activation inhibits the production of catabolic and inflammatory mediators

There are number of inflammatory and catabolic mediators implicated in the pathogenesis of OA. Increase in the level of PGE_2, MMP-13 and NO have been associated with disease progression and cartilage damage. Pro-inflammatory cytokine IL-1β has an important role in the release of PGE_2, MMP-13 and NO by triggering inflammatory and catabolic signaling pathways. In OA, samples from articular tissue show persistent increase in

the level of IL-1β [29]. In this study we tested the influence of Nrf2 activation on the level of IL-1β-induced release of PGE_2, MMP-13 and NO in isolated human OA chondrocytes. To do so, cells were pre-incubated with increasing concentrations of Protandim®, AI-1 or 6-Gingerol for one hour followed by stimulation with 1 ng of IL-1β for 24 hours. The concentration of PGE_2, MMP-13 and NO was then calculated in collected cell culture medium using commercial assays. Interestingly, as shown in Figure 10, Nrf2 activators have an inhibitory effect on the level of three mediators. The reported effect shows dose dependant response as well. At 10 μM, 6-Gingerol showed to be the most potent inhibitor of IL-1β-induced MMP-13, NO, and PGE_2.

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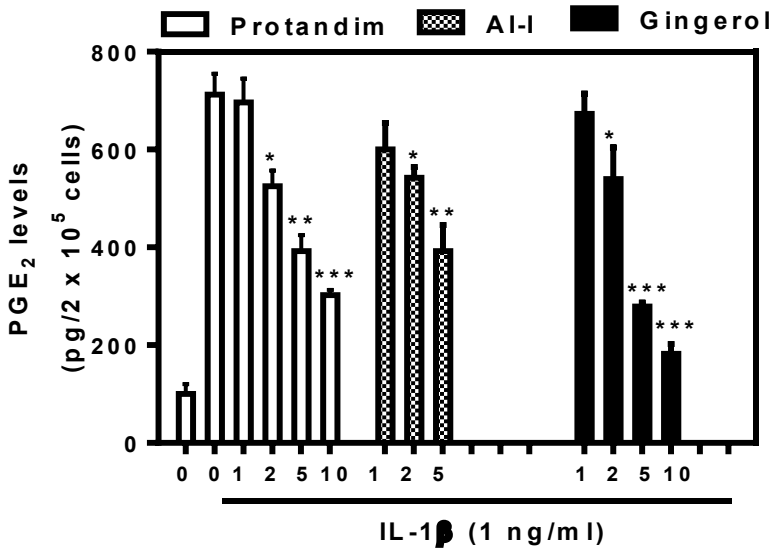

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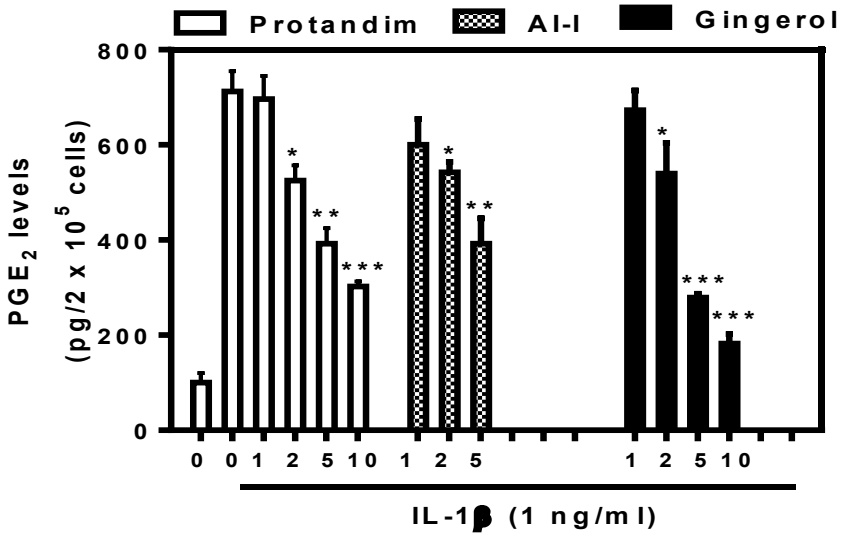

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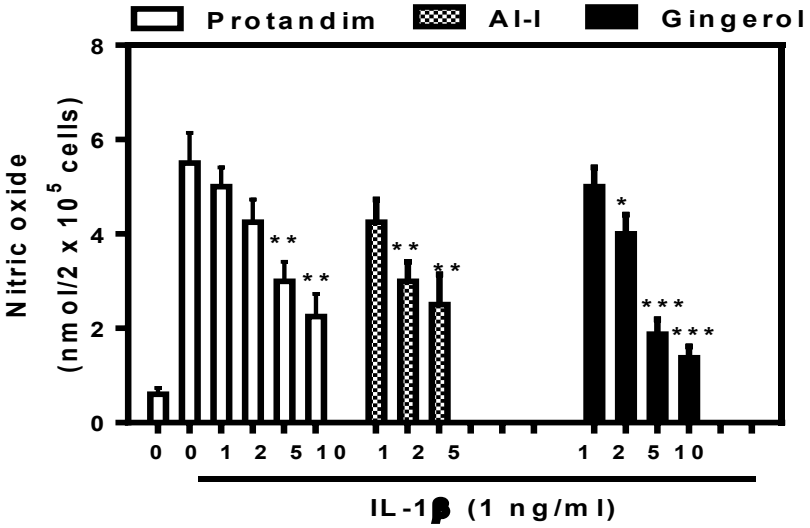


Figure 10: Isolated human OA chondrocytes were pre-treated with increasing concentrations of three different Nrf2 activators; Protandim®, AI-1 or 6-Gingerol (0-10 µg, 0-5 µM and 0-10 µM respectively) for one hour. The cells were then treated with 1 ng/ml of IL-1β for 24 hours. Cell culture medium was collected and assayed against A) PGE_2, B) MMP-13 and C) NO.

Values represent the means ± SEM of 4 separate experiments performed in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001 compared to IL-1β-treated cells.

4- The dietary use of Protandim® in DMM mouse model of OA shows reduced cartilage degradation

To further evaluate the overall protective capability of Nrf2 activation, we tested the influence of the dietary consumption of Protandim® in the surgical DMM model of OA. Mice were given either vehicle or Protandim® for 8 weeks post-surgery. After sacrifice, histological cartilage samples from the knee were evaluated for signs of cartilage degradation such as fibrillation with loss of chondrocytes and loss of aggrecan. Each sample was given a score according to OA Research Society International (OARSI) score system. Figure 11 presents the obtained findings which indicate that oral administration of Protandim® is associated with lower OARSI score and much lower cartilage degradation when compared with placebo.

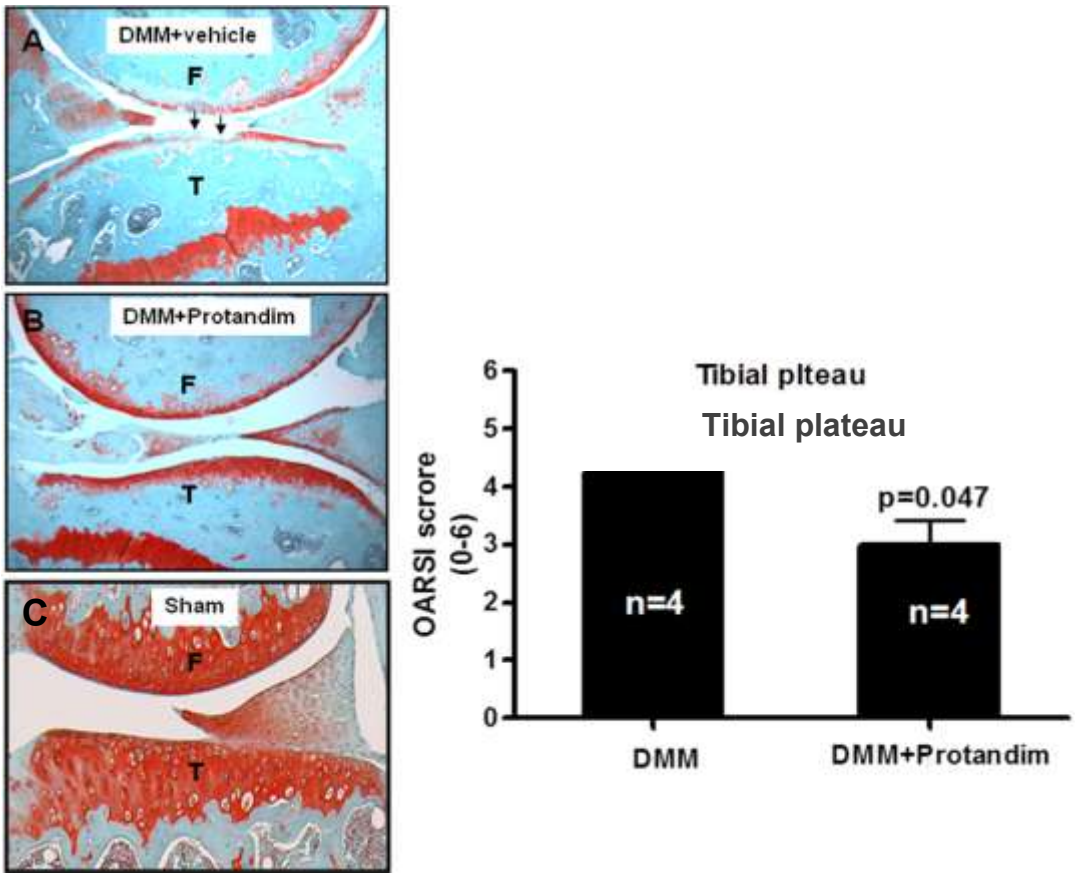


Figure 11: Protandim®-activating Nrf2 reduces cartilage degradation in DMM mouse model of OA.

Histological section of the medial tibial plateaus and femoral condyles performed in DMM wild-type (WT) mice treated with (A) vehicle, (B) 10 mg/kg/day Protandim® for 8-weeks post surgery. (C) Sham mice 8-weeks post-surgery. Arrows indicate areas of fibrillation with loss of chondrocytes and Safranin-*O* staining (loss of aggrecan). F indicates femoral condyle and T tibial plateau (original magnification X40). Statistics: P values were assessed by Mann-Whitney U test comparing DMM mice + drug to the DMM mice + vehicle. (n=4).

5- Cartilage from human OA patients and mouse model of OA show profound decline in the expression of Nrf2 and GSTA4-4

The transcription factor Nrf2 is an important modulator of transcription for cellular antioxidant enzymes. The expression of the important HNE-detoxifying enzyme GSTA4-4 is also modulated by Nrf2 transactivation. Loss of the protective role of GSTA4-4 and its modulator Nrf2 can leave the cell susceptible to the deleterious effects of HNE and oxidative stress [166]. In OA, we postulate that the decrease of Nrf2 and GSAT4-4 may contribute to HNE accumulation and consequently to cartilage damage. The objective was to evaluate the level of Nrf2 and GSTA4-4 expression in OA cartilage. Figure 12A unveils significant decrease in (A) Nrf2 and GSTA4-4 protein level in human OA cartilage samples as compared to normal and (B) Nrf2 and GSTA4-4 mRNA levels in cartilage from DMM mouse model of OA as compared to sham. Collectively, obtained results reveal an important decline in both Nrf2 and GSTA4-4 at both mRNA and protein levels in OA cartilage.

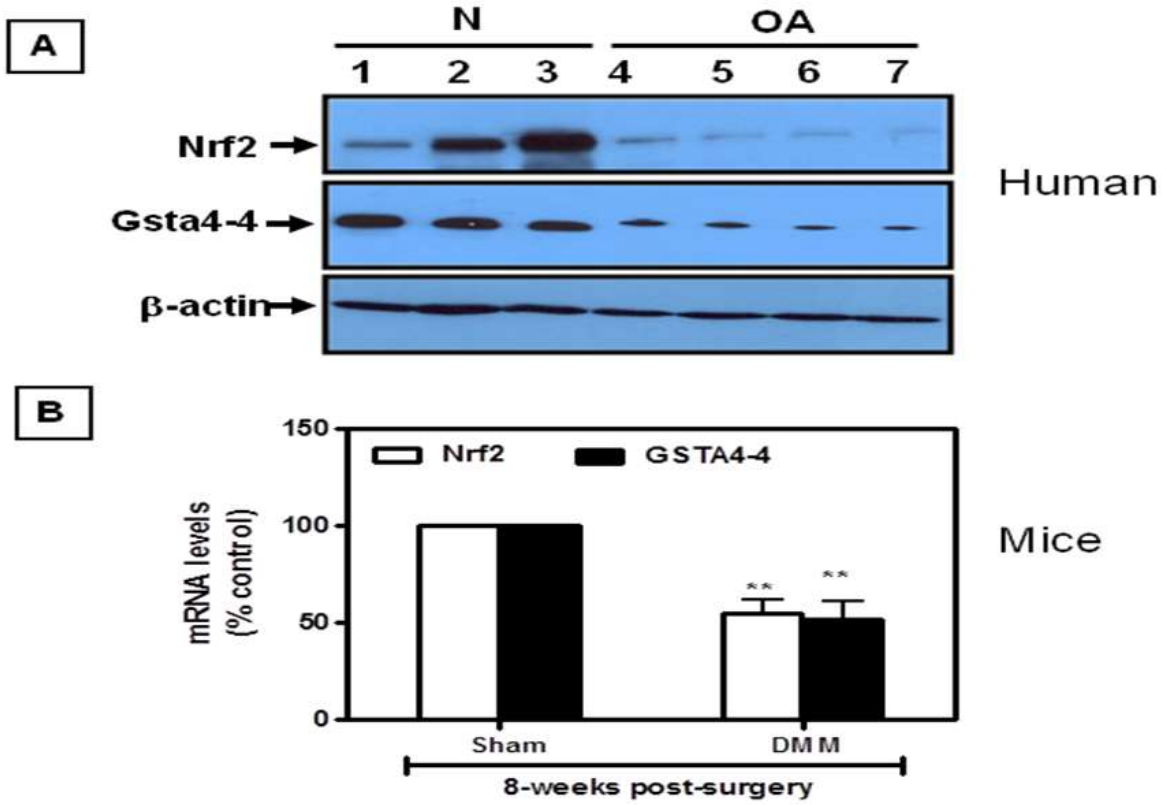
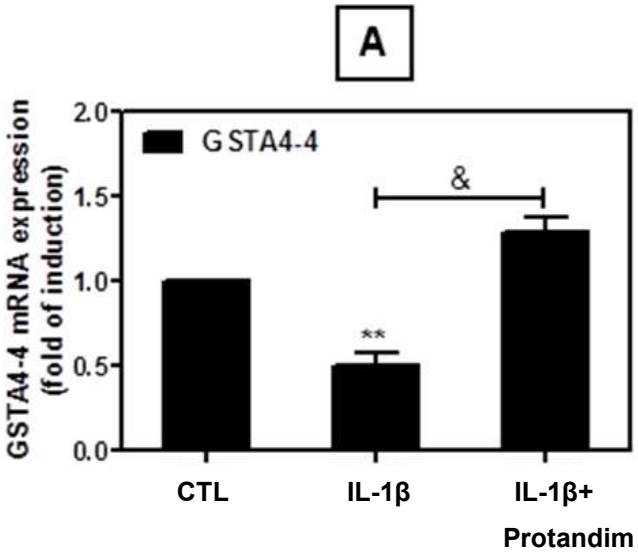


Figure 12: **(A)** Expression of Nrf2, GSTA4-4 was decreased in human OA cartilage (n = 3-4). Cartilage extracts from healthy subject and OA patients are subjected to Western blot using monoclonal anti-human Nrf2 and GSTA4-4. **(B).** Nrf2 and GSTA4-4 mRNA levels were determined by real-time PCR in cartilage from sham and DMM WT mice 8-weeks post-surgery. Statistics: Student's unpaired *t*-test (n=3-4): $^{**}P < 0.01$ (vs. sham).

6- Increased Nrf2 activity reverses the effect of IL-1β on the expression of GSTA4-4, MMP-13 and on the production of HNE

IL-1β plays an important role in OA by triggering the release of catabolic and inflammatory mediators as well as oxidative stress markers [30]. In the next series of experiments, we investigated whether Nrf2 activation abolishes IL-1β induced MMP-13 and HNE production and GSTA4-4 inactivation in human OA chondrocytes. To do so, cells were pre-treated with 10 mg/ml Protandim® for 1 hour followed by incubation with 1 ng/ml IL-1β for 24 hours. As illustrated in Figure 13, Protandim® restores GSTA4-4 activity (A) and blocks IL-1β -induced MMP-13 and HNE production (B, C).



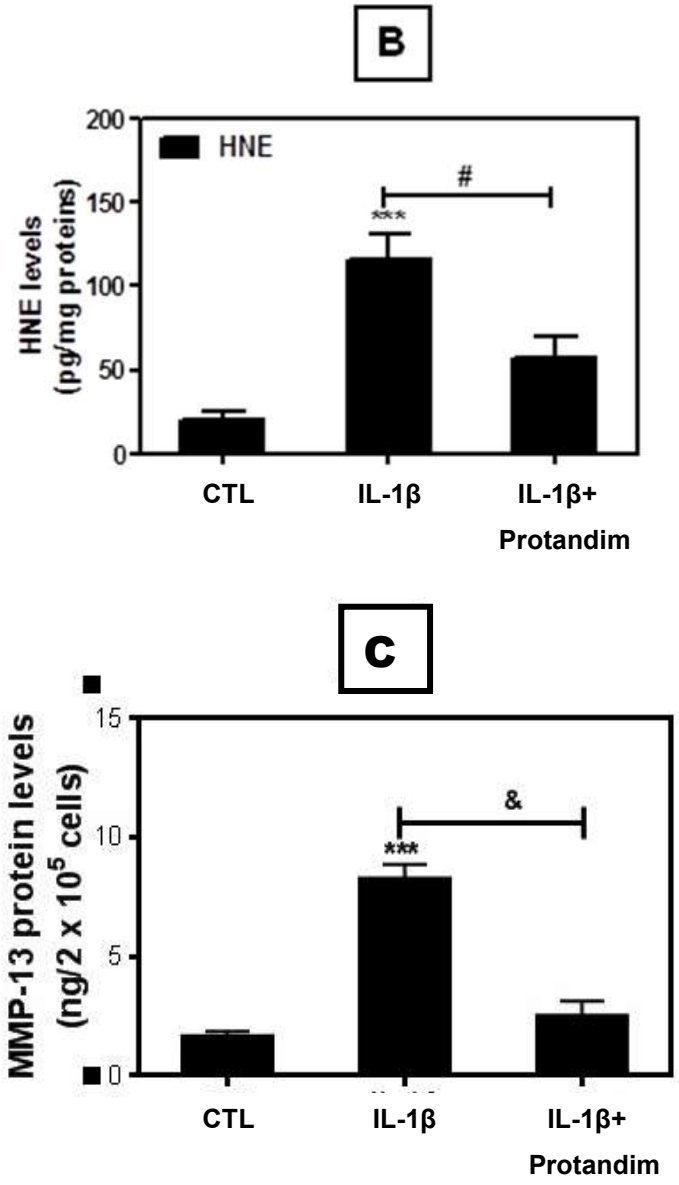


Figure 13: The activation of Nrf2 by Protandim® abolished IL-1β -induced GSTA4-4 down-regulation (A), HNE production (B), and MMP-13 release (C).

Isolated chondrocytes were pre-incubated for 1 h with 10 μg of Protandim® followed by incubation for 24 hours in the presence or absence of 1 ng/mL IL-1β. GSTA4-4 mRNA expression was determined by real-time PCR (A). HNE (B) and MMP-13 (C) levels were

measured by ELISA in the cellular extracts and culture media, respectively. Statistics: Student's unpaired *t*-test (n=3): $^{**}P < 0.01$, $^{***}P < 0.01$ (vs. CTL); #$P<0.05$, &$P<0.01$ (vs. IL-1β).

7- The effect of IL-1β on the levels of Nrf2, GSTA4-4, MMP-13 and HNE can be overcome by Nrf2 overexpression

This experiment is designed to investigate whether Nrf2 overexpression is essential to block IL-1β induced GSTA-4-4 down-regulation as well as MMP-13 and HNE production. Briefly, human OA chondrocytes were transiently transfected with Nrf2 expression vector or empty vectors and then treated or not with 1 ng/ml IL-1β for 24 hours. As shown in figure 14A (upper panel), IL-1β reduced Nrf2 expression in a dose-dependent manner. Furthermore, the overexpression of Nrf2 (Fig. 14A lower panel) significantly abolished IL-1β-induced GSTA4-4 down regulation (Fig. 14B), HNE generation (Fig. 14C), and MMP-13 release (Fig. 14D). Altogether, our data confirm that Nrf2 is essential to regulate redox status and cartilage-degrading enzymes.

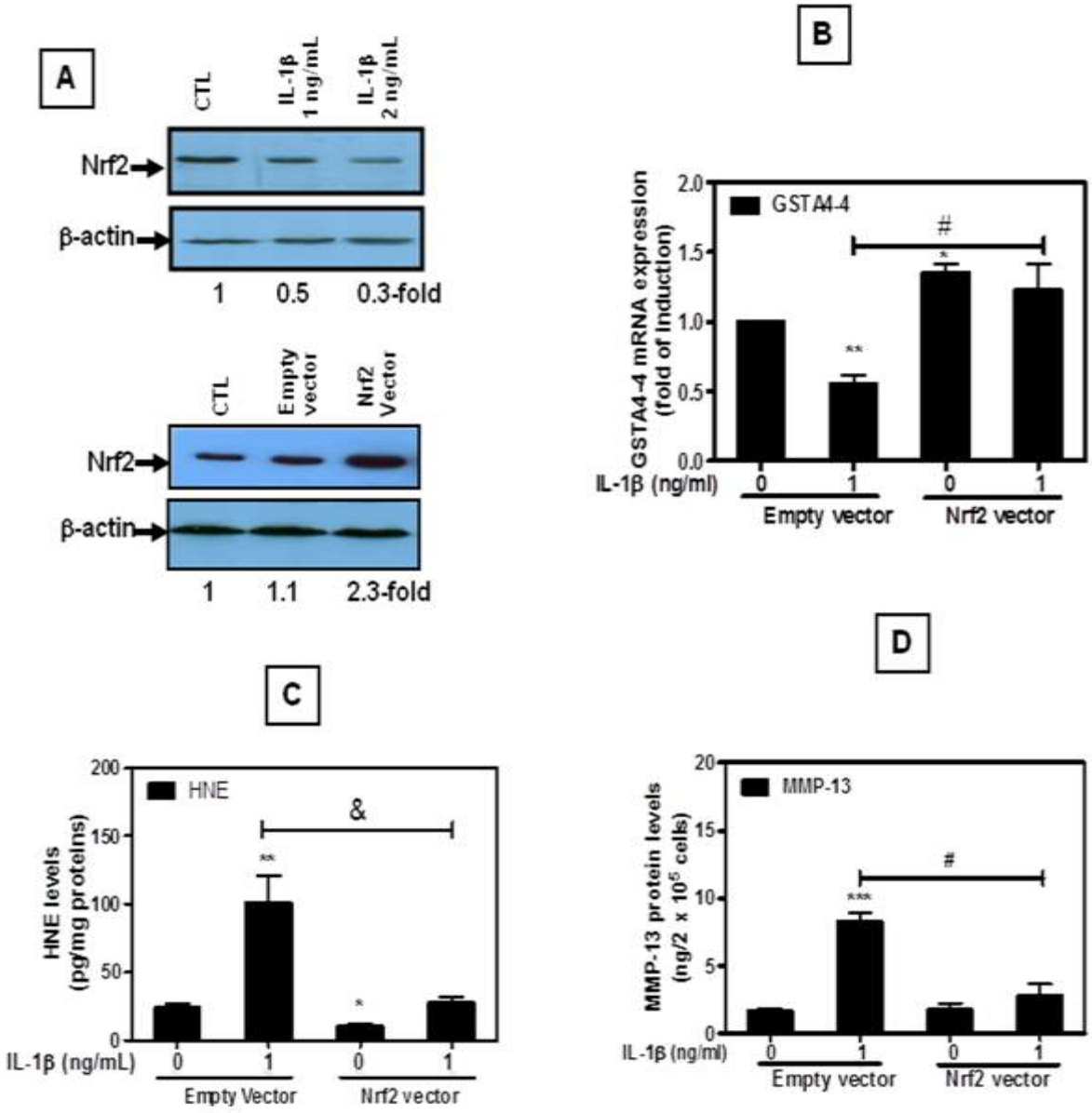


Figure 14: (A) IL-1β inhibits Nrf2 expression in human OA chondrocytes. Cells were treated with 1 and 2 ng/mL IL-1β for 24 hours. (B-D) Overexpression of Nrf2 (A, lower panel) prevented the IL-1β-induced GSTA4-4 down-regulation (B), IL-1β-induced HNE generation (C) and MMP-13 (D) release. Isolated chondrocytes were transiently transfected with empty or Nrf2 expression vector (1 ng/10^6 cells) and treated than after for 24 hours with or without 1 ng/mL IL-1β. Cellular extracts were subjected to the determination of Nrf2 by Western blot (A

lower panel), GSTA4-4 mRNA expression by real-time PCR (B), and HNE level by ELISA (C). MMP-13 level was measured in the culture media by ELISA (D). Statistics: Student's unpaired t-test (n=3): *P < 0.05, **P < 0.01, ***P < 0.001 (vs. empty vector; #P<0.05, &P<0.01 (vs. empty vector + IL-1β).

CHAPTER IV: DISCUSSION

OA is the most common joint disorder that significantly limits physical activity and productivity. Its prevalence is expected to increase significantly in the upcoming decades, as a result of increased life expectancy and population aging. Consequently, it is of greatest social importance that we facilitate advances in the treatment of this musculoskeletal disorder through research, the goal of which should be to improve disease prevention and treatment. There is currently no effective treatment to prevent or stop cartilage destruction during OA and the primary goal is to alleviate pain and other associated symptoms. Therefore, identification of agents that down-regulate the expression of cartilage degrading enzymes may prove useful for the prevention or treatment of OA.

Over the past decades, ROS have been at the center of the pathophysiological scene of OA disease. Since its inception, this project has evolved along with the research fields of ROS and OA. What was striking in the last decades was the discordance between the enormous volume of data supporting the involvement of ROS in OA development and the inconclusive clinical results of exogenous antioxidants therapy. These results highlight the existence of other reactive species that are not taken into account. In this light, our research is focused on clarifying the role of a secondary, potentially toxic product, namely, HNE, which is produced from ROS-induced LPO. HNE is a signaling mediator with wide-ranging biological effects. Initial emphasis was on the development of immunological methods using ELISA in order to detect this aldehyde in biological samples. Using this method, we were able to detect and quantify protein-bound HNE in synovial fluids and articular tissues from OA patients [67]. These measurements represented a major milestone toward the assessment of the functional significance of these HNE-protein adducts and provide a reliable marker of ROS-induced LPO in tissue. Moreover, our research group has made major advances in understanding how it

participates in signal transduction and post-translational modification (PTM) of proteins in OA [45, 52, 67, 97, 125]. Recently, we reported that biochemical changes similar to those seen in OA were induced with an intra-articular injection of HNE into dog joints, suggesting the direct involvement of HNE in OA. In this study, we also showed that HNE injected into dog joints resulted in a significant decrease in proteoglycan content and an increase in MMP-13 and aggrecanase-2 levels [167].

HNE in the pathology of OA

Although oxidative stress is thought to be involved in the pathogenesis of OA, there is no conclusive experimental evidence supporting the idea that defective antioxidant responses in joints lead to increased cartilage destruction. Oxidative stress has an important role in the pathology of several diseases such as OA. In the presence of ROS and pro-inflammatory mediators such as H_2O_2 and IL-1β, respectively, there is an important increase in the production of LPO-end products, in particular, the toxic aldehyde HNE [67]. The structure and properties of HNE make it a target for close investigation in several labs including ours to better understand its unique role as pathophysiological modulator in OA.

Production of HNE in OA articular tissues

The accumulation of HNE in OA articular tissues were indicated by various reports. Recently, we have documented a significant increase in the level of HNE in synovial fluid samples from OA patients as compared with those from controls [67]. These data are consistent with the results reported by Grigolo et al [168] and Shah et al. [169] who found

higher levels of both MDA and HNE in human OA articular tissues as compared with control. However, factors promoting increased accumulation of HNE-protein adducts in OA remain to be clarified. These factors include the inactivation of antioxidant enzymes, the up-regulation of NADPH oxidase, and the regeneration of GSH [103]. In addition, another factor to be considered is the intrinsic capacity of cells to metabolize HNE, whether free or bound, to nontoxic products. This can occur through conjugation with GSH by the GSTA4-4 followed by export [170]. In another study, we demonstrated that GSTA4-4 overexpression in chondrocytes offered significant protection against HNE-induced cell cytotoxicity [97]. These data support the critical role of this enzyme in OA through its ability to regulate HNE level in cartilage.

HNE induces catabolic and inflammatory responses in OA.

In OA chondrocytes, we reported that HNE inhibits type II collagen (Col II) expression [67]. In OA cartilage explants, HNE increases the degradation of Col II, as determined by the measurement of Col II fragments and hydroxyproline release. These data could be attributed to the spontaneous Col II degradation or to MMPs up-regulation. Indeed, it clearly showed that HNE increases the MMP-13 activity and expression, via p38 MAPK activation. On the other hand, HNE increased PGE_2 release and COX-2 expression in OA chondrocytes [52]. The HNE-induced COX-2 production required the activation of p38 MAPK signaling pathway and ATF-2 and CREB-1 transcription factors transactivation. These results were supported by others studies indicating that HNE is a potent inducer of COX-2 and PGE_2 release in various cell lines [171, 172]. The upregulation of COX-2 by HNE contrasted with that of inducible

nitric oxide synthase (iNOS) expression [52]. HNE reduced dramatically IL-1β-induced NO and iNOS production through inactivation of the NF-κB pathway. In a recent study, we have also demonstrated that HNE depletion in chondrocyte is responsible for the switch from COX-2 to 5-lipoxygenase pathway [45].

HNE-induced protein modification in OA

One major effect of HNE in OA is its ability to form adducts with specific proteins. We reported that MMP-13 activity was regulated by HNE binding [67]. The activation of MMP-13 by HNE could occur by conformational change without proteolytic cleavage of its pro-domain, probably via the cysteine switch mechanism [173]. In addition, we showed an increase in HNE-Col II adducts within OA cartilage. The modification of Col II by HNE binding increased the vulnerability of HNE-modified Col II degradation by MMP-13. Col II has been identified also as a potential target for MDA binding in chondrocytes [169]. These results are consistent with the findings of other studies indicating that incubation of chondrocytes with calcium ionophore results in the accumulation of oxidized proteins and, in turn, in matrix degradation [174]. Squier reported that oxidatively modified proteins are highly sensitive to proteolytic breakdown [175]. The fact that HNE increased the proteolytic susceptibility of Col II suggests the presence of changes in the structure of the protein. The significance of increased HNE-Col II adducts in OA cartilage was recently clarified. We showed that interactions between chondrocytes and HNE-Col II adducts, via adhesion molecules, strongly evoking a panoply of signaling pathways that trigger cell adhesion and viability as well as MMP-13 and COX-2 expression [125], as illustrated in Figure 15.

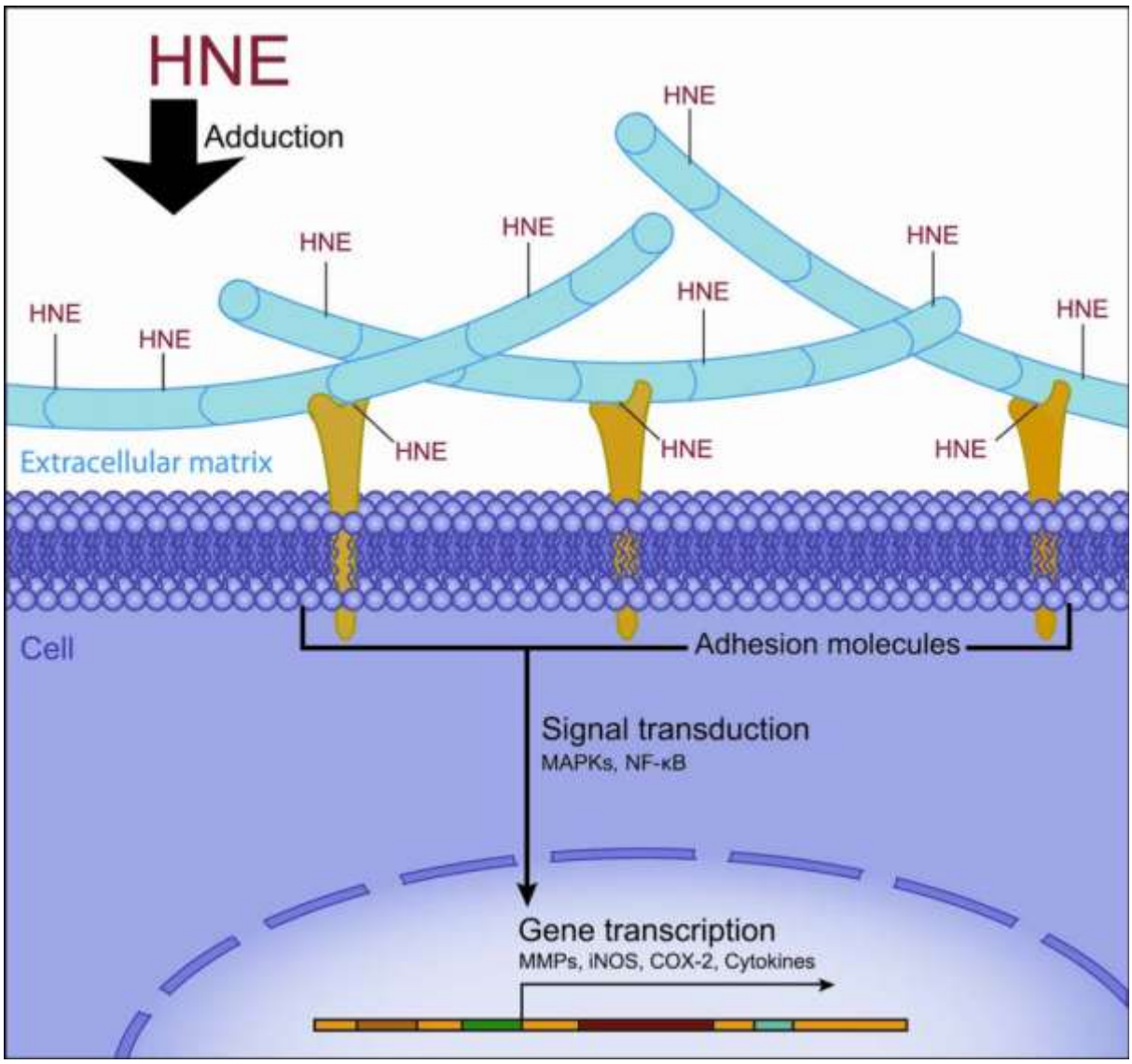


Figure 15: Effect of HNE-modified ECM components on chondrocytes.

Scheme represents signaling pathway affected by cell interaction with HNE-modified Col II in ECM [125] .

Nrf2 and GSTA4-4 in OA

To manage the level of HNE and other LPO-end products the cell has an antioxidant system to keep HNE below pathological concentrations. Mechanisms promoting increased

HNE accumulation in OA remain to be clarified. The GSTs are major determinants of intracellular HNE concentration and can influence susceptibility to toxic effects, particularly when HNE and GST levels are altered in disease states [127]. GSTs comprise a family of versatile enzymes that catalyze the nucleophilic conjugation of GSH with HNE [176]. The GSTA4-4 isoform is selective for GSH conjugation of LPO products and is well-recognized for its catalytic efficiency with HNE [177, 178]. Disruption of this gene enhances HNE tissue levels, as verified experimentally in 129/sv GSTA4-4-/- mice [179]. GSTA4-4 null mice have a short lifespan, supporting the possible relationship between HNE and aging.

By forming adducts with the aldehyde, GSTA4-4 helps detoxify free HNE molecules and prevent its accumulation and interaction with cellular components. In fact, in a previous study we have reported that the overexpression of GSTA4-4 protects chondrocytes from the HNE-induced apoptosis [97]. However, when the production of HNE extends the capacity of GSTA4-4 for detoxification, the cell becomes susceptible for the toxicity of HNE. Transcription factor Nrf2 act as a master redox switch that can trigger the expression of several antioxidant agents within the cell in the presence of redox-agents. Therefore, the activation of Nrf2 is important for protecting the cell against the accumulation of oxidative agents by triggering the expression of antioxidant defense mechanisms [92, 147].

Nrf2 is a redox-sensitive transcription factor which binds to ARE located in the promoter region of genes encoding detoxifying enzymes such as GSTA4-4 and related stress-responsive proteins [180]. Induction of these enzymes via Nrf2–ARE signaling hence provides an effective means for achieving cellular protection against a variety of electrophilic molecules [142]. Under the basal resting condition, Nrf2 is sequestered in the cytoplasm by the cytoskeleton-associated protein, Keap1[149]. Keap1 functions as a negative regulator of

Nrf2 by promoting ubiquitination and proteasomal degradation of Nrf2. When liberated from its repressor Keap1, Nrf2 translocates into the nucleus and forms a heterodimer with a small Maf (sMaf) protein to binds to ARE localized in the promoter regions.

In addition to protection against oxidative and electrophilic stresses, recent studies have demonstrated that Nrf2 responds to pro-inflammatory stimuli and rescues cells/tissues from inflammatory injuries [181]. Other studies showed that Nrf2 also inhibits catabolic responses through MMP-9 down-regulation [182]. Nrf2-/- mice develop complex pathogenic manifestations, including lupus-like autoimmune syndrome, pulmonary fibrosis [183, 184]. In a recent study, the involvement of this nuclear factor in cartilage degradation was reported in Nrf2-/- mice [185]. A high level of cytokines, chemokines, adhesion molecules and COX-2 was observed in these mice than that of wild-type animals [186-188]. In contrast, all these inflammatory mediators are suppressed by Nrf2 activation and/or overexpression, suggesting an anti-inflammatory role of Nrf2. From these data, we believe that changes of Nrf2 expression could be significantly relevant in OA pathogenesis, contributing to the regulation of catabolic and inflammatory mediators.

In addition, Nrf2-/- mice showed greater activation of NF-κB than in their wild-type counterparts [187]. Moreover, the activation of Nrf2 has been shown to directly modify the DNA binding domain of NF-kB and AP-1, thereby inactivating these transcription factors [189-191]. On the other hand, major systems that orchestrate Nrf2 inactivation involve different signaling pathways, including p38 MAPK, ERK, NF-kB and Src kinases [192-195].

Interestingly, data from our current study indicates a profound reduction in both Nrf2 and GSTA4-4 at protein and mRNA level in human OA and animal model of OA samples

when compared with control. These findings led us to hypothesize that loss of Nrf2 in articular cartilage would induce a reduction in GSTA4-4 expression and activity and consequently contribute to induce an oxidative-stress phenotype in chondrocytes. Until now, the role of Nrf2 and GSTA4-4 in OA was not yet reported. Moreover, the role of Nrf2 in RA is still controversial. On one hand, several studies recorded a protective effect against joint inflammation associated with the administration of Nrf2 activators such as Rebamipide and the activation of Nrf2 antioxidant pathway [196]. This observation indicates a loss or deficiency in Nrf2 activity .On the other hand, Wruck et al. reported an increase in Nrf2, which occurs in response to oxidative stress within RA joints. However, they indicate that this increase is insufficient to fully counteract the progression of the disease [185]. They hypothesize, however, that a deficiency in the Nrf2-mediated antioxidant defenses plays a central role in the pathogenesis of RA. Nrf2 deficiency significantly enhanced the severity of arthritis in their RA model. The difference between Nrf2-knockout mice and Nrf2-wild-type mice with regard to the level of joint destruction was clear on histological sections stained with H&E and safranin-O. The evaluation of hyperplasia, pannus formation, erosion of cartilage and infiltration in the joint showed a significantly higher rate of destruction in all four categories in the Nrf2-knockout mice compared with wild-type mice. We hypothesize that an increased oxidative burden resulting from defective oxidative defense may lead to the increase in cartilage destruction in arthritic Nrf2-knockout mice. In another study with regard to RA, Maicas et al [197] showed that Nrf2 deficiency accelerated the incidence of arthritis, and animals showed a widespread disease affecting both front and hind paws. Therefore, the inflammatory response was enhanced, with increased migration of leukocytes and joint destruction in front paws. They observed an increased production of TNF-α, IL-6, and

chemokine (C-X-C motif) ligand 1 (CXCL-1) in the joint, with small changes in eicosanoid levels. Serum levels of CXCL-1 and receptor activator for nuclear factor κB ligand were enhanced and osteocalcin decreased in arthritic Nrf2-/- mice. The expression of COX-2, inducible nitric oxide synthase, and peroxynitrite in the joints was higher in Nrf2 deficiency, whereas heme oxygenase-1 was downregulated. Where OA is a degenerative disease, RA on the other hand is a systemic one. Though both diseases have different pathologies, they share the presence of high level of inflammation. Overall, published studies indicate an important role of Nrf2 antioxidant activity in inflammation process and inflammatory diseases.

Nrf2 activators

Since the identification of its role in the activation of antioxidant gene transcription, researches have been looking at transcription factor Nrf2 as an important redox switch in the biological system. Moreover, changes in the level of Nrf2 and/or its activity is under investigation for a potential role in the pathology of several diseases especially of inflammatory nature [149]. In an attempt to control and modulate the activity of Nrf2, which can be of great benefit in protecting cells against oxidative stress, several natural and synthetic compounds are being studied and developed. In our study we are testing two natural and one synthetic activators of Nrf2; Protandim®, 6-Gingerol and AI-1, respectively. Our results indicate that pre-treating cells with Nrf2 activator was associated with an interesting increase in total Nrf2 protein level. Moreover, the use of Nrf2 activator not only enhanced both mRNA and protein levels of GSTA4-4, but it also prevented the inhibitory effect of IL-1β on the expression of GSTA4-4. In addition, pre-treatment with Protandim® significantly reduced IL-

1β-induced production of HNE within the cell. Interestingly, the increase in Nrf2 and GSTA4-4 within the cell was accompanied with marked reduction in the levels of inflammatory and catabolic mediators; PGE_2, MMP-13 and NO in the presence of pro-inflammatory cytokine IL-1β. This decrease is most likely due to the lower HNE production. Another test performed on histological section of the medial tibial plateaus and femoral condyles performed in DMM mouse model of OA provided histological evidence that the administration of 10 mg/kg/day of Protandim® for 8 weeks post-surgery significantly protected the cartilage from degradation. This finding indicates an important possibility to benefit from early treatment with Nrf2 activators as a protective approach in people with high risk of developing OA after knee surgery.

In a recent study, we tested our hypothesis that carnosine, an HNE trapping compound, prevents cartilage damage [167]. Carnosine has recently attracted much attention as a naturally occurring antioxidant agent. We demonstrate that carnosine, given as prophylactic or in pre-existing OA, is able to reduce cartilage damage most likely through its distinctive combination of antioxidant properties. These findings may suggest the involvement of HNE in OA process. In keeping with the findings of our study, several recent studies have indicated that carnosine abolishes the generation of HNE and other oxidative stress-related products [198, 199]. HNE is of particular interest because of its high chemical reactivity, evidenced by its production of such varied biological effects [200]. The chemical characteristics of this aldehyde revealed that it can also react with reduced GSH at much higher rates that are further accelerated by GSTA4-4 [127, 201]. Indeed, its reaction with carnosine may be a second line of defense under conditions of GSH depletion. The fact that the administration of exogenous carnosine prevents HNE production as well as inflammatory and catabolic factors suggests that despite

its slow reactivity, carnosine offers significant protection against HNE toxicity and its biological effects, even in GSH-depleted cells and tissues. Furthermore, the oral intake of carnosine reduces MMP-13, ADAMTS-5, and COX-2 expression in cartilage and synovium explants from the anterior cruciate ligament transection (ACLT) dog model of OA. Our observations revealed that carnosine can reduce the initiation and progression of OA structural changes by removing HNE from the knee joint and attenuating the expression of factors known to be involved in cartilage degradation. These findings imply that HNE is a significant contributor to OA cartilage degradation

Regulation of Nrf2 and GSTA4-4 function by acetylation

At posttranslational level, it has been reported that Nrf2 transactivation and GSTA4-4 activity are regulated by a process namely acetylation. This process is an important posttranslational regulatory mechanism involved in regulating enzyme activity and controlling different cellular functions such as protein stability, protein localization, cell viability and metabolism [202, 203]. In our unpublished data, we identified two distinct mechanisms involved in GSTA4-4 regulation at transcriptional and PTM levels in isolated human OA chondrocytes: One involving the suppression of Nrf2 transactivation by acetylation, and one involving inhibition of GSTA4-4 activity by acetylation, most likely via down-regulation of Sirt2-mediated deacetylation, a member of a deacetylase family of enzymes that has seven mammalian homologues. In particular, Sirt1 and Sirt2 hold special importance due to their presence in the nucleus and the cytoplasm where they can affect coregulators, enzymes and transcription factors involved in gene expression. The activity of Sirt is thought to be involved directly and indirectly with certain pathological conditions such as cancer, atherosclerosis and

cardiovascular diseases [204, 205]. In OA, recent studies revealed that disease development is associated with a decrease in Sirt1 expression which may cause chondrocytes death and the loss of ECM [206]. However, the role of acetylation in the regulation of Nrf2 stability and transcriptional activity is still controversial. A study by Kawai et al. [207] proposed an important role for acetylation in the regulation of location and transcriptional activity of Nrf2. The study proposed that Nrf2 undergoes acetylation in the nucleus which enhances its binding to ARE and subsequently gene transcription and that the deacetylation of Nrf2 by Sirt1 will trigger its release from ARE and relocalization to the cytoplasm, thereby terminating Nrf2-dependant gene transcription [207]. On the other hand, another study by Mercado et al. [208] proposed that increase in Nrf2 acetylation causes reduced Nrf2 stability ultimately impairing the transcription of antioxidant genes by Nrf2 and cellular antioxidant defense mechanism in the presence of oxidative stress [208].

Due to the importance of the reported disturbance in the level of Nrf2 and GSTA4-4, better understanding of the possible mechanism causing this reduction and the factors involved can provide important insights on OA. Moreover, factors that can modulate the activity of Nrf2 and GSTA4-4 may also indirectly regulate the level of HNE within articular tissue and therefore serve as a potentially important target in OA.

CONCLUSION

Our observations revealed promising Nrf2 activators outcomes in the prevention of OA cartilage lesions induced by joint instability. Nrf2 activators treatment seems to impact oxidative stress and the catabolic and inflammatory pathways of OA by activating GSTA4-4 and inhibiting HNE production. We strongly believed that we are on the brink of uncovering a key aspect underlying the role of HNE in OA onset and the fundamental aspects involved in its pathogenesis. In any case, this research project will more directly assess the role of HNE and its detoxifying enzyme, GSTA4-4, in OA etiopathogenesis. As well, this study results in an increased understanding of the biochemical and molecular mechanisms associated with decline of GSTA4-4 as well as Nrf2, an important transcription factor involved in GSTA4-4 regulation and cell function. Therefore, it is most likely that the future studies will lead to the development of the first molecular diagnostic tools for identifying individuals at risk of developing of OA. Furthermore, another approach includes developing innovative therapeutic approaches to maintain or reactive Nrf2 expression and consequently GSTA4-4 in articular cartilage by pharmacological interventions.

In our study we were able to measure the level of Nrf2 in human OA sample. However, we do propose running a large cohort study on patients with OA. Such study can provide solid proof for diminished Nrf2 level and/or activity in OA. For future work, we suggest the use of DMM mouse model of OA using Nrf2 knockout mice. This can help prove the involvement of Nrf2 in OA and the outcomes associated with the loss of its activity on disease development. Furthermore, Nrf2 knockout mice can help demonstrate the influence of reduced or

diminished Nrf2 level on the concentrations of HNE and GSTA4-4 in articular tissue. We also propose the use of canine model of OA; different OA models in dogs are comparable to human OA. Moreover, the use of such model permits performing macroscopic examination of lesions in OA cartilage, which is not possible with DMM mouse model.

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