# THE EFFECT OF RADISH (RAPHANUS SATIVUS) DERIVED EXOSOMES ON OSTEOARTHRITIS

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# ABSTRACT


## THE EFFECT OF RADISH (RAPHANUS SATIVUS) DERIVED EXOSOMES ON OSTEOARTHRITIS

Osteoarthritis, characterized by articular cartilage loss, is a well-established cause of impairment. Given the underlying pathology of OA, it is clear that injured cartilage has a low ability for regeneration. This means the proposed action is essential. Deterioration of articular cartilage, alterations in subchondral bone, and synovitis are hallmarks of this complex illness. Current OA treatments focus mostly on alleviating symptoms like pain and inflammation rather than stopping the disease itself from progressing. There is a significant knowledge gap when it comes to the mechanism that contribute to the OA development. Articular cartilage's extracellular matrix homeostasis is kept in check by a delicate balancing act between anabolic and catabolic pathways; abnormalities in this equilibrium are linked to OA. Chondrocytes are the special cells that make up cartilage in joints. Collagens, proteoglycans, and non-collagen proteins are all synthesized by these cells to form the extracellular matrix. Chondrocyte responses to molecular cues vary between areas and stages of illness, suggesting that mechanical stress and biochemical variables play major roles in disease development. In this regard, the utilization of plant-based products in the treatment of medical disorders is gradually becoming a preferred option due to the fewer negative effects associated with these items. In this respect, objective of this study to examine potential effects of *Raphanus sativus* derived exosomes on osteoarthritis treatment by defining proliferative, anti-inflammation potential and upregulating cartilage specific activities on protein and gene expression levels for osteoarthritis. Our results demonstrated that, *Raphanus sativus* derived exosomes has anti-inflammation effect on OA while decreasing expression of IL-1β and IL-6 levels and inducing TGF-β. Also, these exosomes has proliferative effect on OA chondryocyte cells while promoting main extracellular matrix components. Based on these findings, *Raphanus sativus* derived exosomes appear as a potential therapeutic for osteoarthritis treatment.

# ÖZET

## THE EFFECT OF RADISH (RAPHANUS SATIVUS) DERIVED EXOSOMES ON OSTEOARTHRITIS

Eklem kıkırdağı kaybı ile karakterize olan osteoartrit, iyi bilinen bir bozulma nedenidir. OA'nın altında yatan patoloji göz önüne alındığında, yaralı kıkırdağın yenilenme yeteneğinin düşük olduğu açıktır. Bu, önerilen eylemin gerekli olduğu anlamına gelir. Eklem kıkırdağının bozulması, subkondral kemikteki değişiklikler ve sinovit bu karmaşık hastalığın ayırt edici özellikleridir. Mevcut OA tedavileri, hastalığın ilerlemesini durdurmak yerine çoğunlukla ağrı ve iltihaplanma gibi semptomları hafifletmeye odaklanır. OA'nin gelişimine katkıda bulunan süreçler söz konusu olduğunda önemli bir bilgi boşluğu vardır. Eklem kıkırdağının hücre dışı matris homeostazı, anabolik ve katabolik yollar arasındaki hassas bir dengeleme eylemiyle kontrol altında tutulur; bu dengedeki anormallikler OA ile bağlantılıdır. Kondrositler, eklemlerdeki kıkırdağı oluşturan özel hücrelerdir. Kollajenler, proteoglikanlar ve kollajen olmayan proteinlerin tümü, hücre dışı matrisi oluşturmak için bu hücreler tarafından sentezlenir. Moleküler ipuçlarına kondrosit yanıtları, hastalığın alanları ve evreleri arasında farklılık gösterir; bu, mekanik stresin ve biyokimyasal değişkenlerin hastalık gelişiminde önemli roller oynadığını düşündürür. Bu bağlamda, tıbbi rahatsızlıkların tedavisinde bitki bazlı ürünlerin kullanımı, bu ürünlerle ilişkili olumsuz etkilerin daha az olması nedeniyle giderek tercih edilen bir seçenek haline gelmektedir. Bu bağlamda, bu çalışmanın amacı, Raphanus sativus türevli eksozomların osteoartrit için proliferatif, anti-inflamasyon potansiyeli ve protein ve gen ekspresyon seviyeleri üzerindeki kıkırdak spesifik aktiviteleri yukarı regüle ederek osteoartrit tedavisi üzerindeki potansiyel etkilerini incelemektir. Sonuçlarımız, Raphanus sativus kaynaklı eksozomların, IL-1β ve IL-6 ekspresyon seviyelerini düşürürken ve TGF-β'yı indüklerken, OA üzerinde anti-inflamasyon etkisine sahip olduğunu göstermiştir. Ayrıca, bu eksozomlar, ana hücre dışı matris bileşenlerini desteklerken OA kondriyosit hücreleri üzerinde proliferatif etkiye sahiptir. Bu bulgulara dayanarak, Raphanus sativus'tan türetilmiş eksozomlar, osteoartrit tedavisi için potansiyel bir terapötik olarak görünmektedir.

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| | |
|---|---|
| ACAN | Aggrecan |
| ACI | Autologous chondrocyte implantation |
| ATPS | Aqueous two-phase system |
| BMSC | Bone marrow stem cell |
| Col1a1 | Collagen type I |
| Col2a1 | Collagen type II |
| CrP | C-reactive protein |
| CSPC | Cartilage stem/progenitor cells |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| ELF3 | E74-like factor 3 |
| EV | Extracellular vesicles |
| ESCRT | Endosomal sorting complex |
| FBS | Fetal bovine serum |
| HC-OA | Human chondrocytes osteoarthritis |
| H2O2 | Hydrogen peroxide |
| IL | Interleukin |
| JNK | Jun N-terminal kinase |
| MAPK | Mitogen-activated protein kinase |
| MMP | Matrix metalloproteinase |
| mRNA | Messenger ribonucleic acid |
| MSC | Mesenchymal stem cell |
| MTS | 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4- sulfo-phenyl)-2H-tetrazolium |

| | |
|---|---|
| MVBs | Multivesicular bodies |
| NSAID | Nonsteroidal anti-inflammatory drugs |
| OA | Osteoarthritis |
| PSA | Penicillin, streptomycin, and amphotericin B |
| SMSCs | Synovial mesenchymal stem cells |
| TFF | Trefoil factor family |
| TGF-β1 | Transforming growth factor beta 1 |
| TNF-α | Tumor necrosis factor alpha |
| VEGF | Vascular endothelial growth factor |

# 1. INTRODUCTION

Osteoarthritis which is the most frequent type of arthritis is a joint disorder that affects hundreds of thousands of people and gradually worsens to the point where it severely impacts their quality of life. Subchondral bone remodeling, increasing cartilage deterioration, chronic pain, and synovitis are the hallmarks of osteoarthritis [1]. In osteoarthritis, the articular cartilage structure deteriorates. Consequently, alterations develop in the bone tissue beneath the articular cartilage. Bone growths and protrusions near the joint's edge alter the normal anatomy of the joints, causing movement restriction and pain [2].

## 1.1.OSTEOARTHRITIS (OA)

### 1.1.1. Etiology of OA

OA is frequently categorized as having primary or secondary origins. Primary OA's pathogenesis is not completely understood, but it is thought to contain a combination of systemic and local variables interacting with one another. The etiology of secondary OA has an idefined cause, including joint trauma (acute or chronic), obesity, overuse, dysfunction of muscles, rheumatoid arthritis which is inflammatory arthropathies calcium deposits inside the joints [3,4]. Growing research links OA to diabetes, syndrome of metabolism and cardiovascular disease among other chronic comorbidities. Abdominal obesity, hypertension, dyslipidemias, and insulin resistance are all linked to a higher risk of developing OA and a more rapid rate of disease progression. Furthermore, there is mounting evidence that patients with comorbidities experience clinical symptoms of OA early and more severely [5].

Age is the greatest risk factor for osteoarthritis, although many other variables have been hypothesized for its onset and development, such as abnormal mechanical loading of the joint, joint damage, and alters in genes encoding matrix molecules. Signaling molecules, including as pro-inflammatory cytokines, likely contributed to the development of OA because they stimulate matrix-degrading enzyme activities while suppressing matrix

biosynthesis. It has been theorized that chondrocyte metabolism reverts to a dedifferentiated or immature state; alternatively, chondrocytes may become hypertrophic and incur cell death. Therefore, the mechanism of cartilage deterioration has become the subject of study. Although other musculoskeletal tissues and the start of degenerative joint disease may be affected by environmental variables., it is still commonly held that wear and tear on cartilage is to blame [6].

### 1.1.2. Pathology of OA

Osteoarthritis has a very complicated and multifaceted pathology. Some of the complexities of the biological mechanisms that underlie the progression and development of illness have been elucidated thanks to recent technological developments have made it possible to identify individual genes and genetic mutations. [7]. Environmental and genetic factors both play a part in the pathogenesis of OA, making it a truly multifactorial disease. As the disease progresses, chondrocytes become damaged, are repaired, and eventually die. Lymphocytes produce inflammatory mediators such as tumor necrosis factor (TNF) and interleukin 1 in response to chondrocyte damage (IL- β). Synthesis of IL-1 by OA chondrocytes leads to upregulation of MMPs and other catabolic enzymes. And then the cartilage gets destroyed because of the proinflammatory mediators and the catabolic enzymes [8,9,10].

Most cases of OA can be attributed to natural aging and an unidentified primary cause. Young people with a prognostic indicator, including a joint injury, congenital deformity, or systemic illness, develop OA extremely rare. [11,12].

Factors in one's natural environment can include things like getting older and the biomechanical stress caused by things like body weight and joint stability. Genes and epigenetic modifications that alter gene expression are both examples of genetic factors [13]. Numerous genes and hereditary factors have been linked to OA, as shown by both family and epidemiological studies. Genes involved in the progression and development of osteoarthritis (OA) have been pinpointed by genome-wide association studies [14].

Epigenetic modifications like DNA methylation have been shown to play a significant role in the development of OA, along with alters in cytokines, growth factors, and matrix composition, according to genome-wide methylation studies. The hypomethylation in

question is related with an increase in the transcription of proteins that promote inflammation. like Trefoil Factor Family (TFF), IL-1, and IL-6. Likewise, chondrogenesis and skeletal maturation are affected by epigenetic regulation via miRNAs [15,16,17].

*1.1.2.1.Macroscopic Pathology*

Joints affected by OA typically exhibit abnormal cartilage growths known as osteophytes, as well as the loss of the normal rounded cartilage line. The articular hyaline cartilage is soft and granular in the beginning stages of OA [18]. Weight-bearing areas of the articular cartilage are lost as the disease development, and cartilage remnants can be found in the surrounding area. When two bones come into direct contact, the underlying bone will either become polished and smooth, or it will burn [19].

The bony surface may have clumps of regenerative cartilage where repair takes place. Characteristic features seen on cross-section include subchondral bone with a sclerotic appearance, subcortical cysts, and wedge-shaped necrosis. Subcortical cysts form when synovial fluid enters the subchondral bone space through small fractures in the bone surface. Multiple loose bodies are found in the joint cavity [20,21].

*1.1.2.2.Microscopic Pathology*

Whether the damage is internal or external, cartilage can be repaired or regrown. Islands of chondrocyte clones within the matrix represent intrinsic repair in the native articular hyaline cartilage [22]. Over the remaining articular hyaline cartilage and at the joint margin, an extrinsic repair manifests as a highly cellular fibrocartilage with rough and irregular collagen. After the cartilage covering a bone has been removed, the underlying bone undergoes osteoblast proliferation and new bone formation, which manifests as prominent sclerosis [23]. A subchondral cyst has been linked to the occurrence of small fractures. Hyperplastic and hypertrophic synovial extensions, chronic inflammation, and stromal fibrosis of fibroadipose tissue are all features of deteriorating synovial health [24,25].

### 1.1.3. Cells in OA Pathogenesis

*1.1.3.1.Chondrocytes*

The synovial joint is lined by cartilage, which is a kind of connective tissue and cartilage helps keep the joint lubricated and supports body weight when it's being used. Recent research has discovered that osteoarthritis (OA) cartilage contains cartilage stem/progenitor cells (CSPC), making up around 9% of total cells [26]. Previously, it was believed that articular cartilage simply contained chondrocytes. Chondrocytes are tissue-resident cells capable of producing an extracellular matrix rich in aggrecan and type II collagen. However, chondrocytes make up only 2-6% of cartilage tissue by volume [27,28].

Chondrocyte metabolic changes cause synthesis of collagen and collagen degradation, chondrosenescence, degeneration of cartilage, and an imbalance within intra-articular inflammatory environment, all of which contribute to OA [29].

Retaining chondrocytes functioning and the ECM metabolic balance in check requires a normal physiological load. Since mechanical stress is harmful to chondrocytes, it reduces their anabolic response, slows their production of extracellular matrix (ECM), and speeds up their catabolic response, all of which promote the production of matrix metalloproteinases (MMPs) [30,31]. Symptoms of the OA phenotype linked to the metabolic syndrome include elevated levels of fasting plasma glucose, triglycerides, and low-high-density lipoproteins, as well as hypertension [32]. Beneficial effects on development of OA may result from increasing synthesis of fat-derived pro-inflammatory mediators such as increased glycation end products. This latter category represents the OA phenotype that typically develops with advancing age. Activation of senescent chondrocyte catabolism and disruption of autophagy-inflammatory crosstalk are hallmarks of an inflammatory microenvironment [33,34]. In addition, decreased autophagy and increased apoptosis in chondrocytes accompany the decline in mitochondrial function of chondrocytes. Although chondrocytes' capacity to proliferate and synthesize declines with age in aging articular cartilage, their capacity to generate proinflammatory mediators and matrix metalloproteinases does not. Overall, phenotypic changes in chondrocytes have role in the progression and development of OA [35,36].

*1.1.3.2.Synovial Cells*

Growing research suggests that two-way information exchange between the various joint tissues is crucial for preserving joint homeostasis. Synovial-cartilage communication is not

only a factor that contributes to the symptoms of osteoarthritis, as well as a primary contributor in the development of the disease itself [37]. Biomarkers for OA progression frequently examine synovial morphology and cell composition. Patients with advanced OA often experience severe synovitis, even though in the of OA, synovial tissue remains unaffected. [38].

The synovium in a normal joint contains two primary types of synovial cells. Macrophage-like synovial cells (type A), that are smaller than true macrophages, primarily serve as phagocytes and produce inflammatory cytokines [39]. Nearly three quarters of the synovial cells are type B fibroblast-like cells, which are responsible for the tissue's structural integrity, nutritional support, and lubrication. Furthermore, synovial cells with properties resembling fibroblasts can move to the region of tissue remodeling. where they can interact with ECM molecules via surface receptors [40,41]. They are able to detect and react to variations in the surrounding synovial tissue's composition and structure through modulations of their interaction with ECM components and their production. Hypertrophy and infiltration of mast cells, neutrophils, fibroblasts, macrophages T and B cells, into the intimal lining layer causes a 6-12-fold amplification in density of cells. at the onset of OA [42]. Synovial fluid, an important source of these proinflammatory mediators in OA, is altered in composition as a result of the infiltrating cells' promotion of production of proinflammatory cytokines and catabolites. When compared to mast cells, macrophages are underrepresented in the synovial fluid of OA clients [43].

*1.1.3.3.Mesenchymal Stem Cells (MScs)*

MSCs, which stand for mesodermal stem cells and these become multipotent progenitor cells which come from the mesoderm. MSCs, by nature, are unable to regenerate genuine articular hyaline cartilage since this kind of cartilage is mostly fibrous and hypertrophic [44]. However, mesenchymal stem cells have been shown to support in the repair of cartilage and bone, as well as play a role in the control of the immune system and the regeneration of organs [45]. In a general sense, pluripotent stem cells, adult stem cells and embryonic stem cells are three categories of MSCs [46].

Detection of pluripotent stem cells can be observed in bone related tissues for example, synovium, adipose tissues, bone marrow, infrapatellar fat pad [47]. MSCs are produced from

bone marrow, also known as BMSCs, are the pluripotent stem cells that have been done researched the most. Stem cell treatment that is based on the in vitro growth of autologous bone marrow stromal cells (BMSCs) has been utilized to treat osteoarthritis (OA) [48]. In spite of the fact that there was no discernible disparity in the clinical results, arthroscopy and histological data revealed that the patients' symptoms had improved [49]. Synovial mesenchymal stem cells (SMSCs) have been indicated to have an exceptionally high ability for cartilage development in vitro [50]. SMSCs taken from patients with OA have been proven in research to be capable of repairing cartilage through the use of allogenic tissue created constructs in both in vitro and in vivo situations. AMSCs have the ability to stimulate cartilage repair as well as to control inflammation. AMSCs are a great source of cells for the treatment of OA because of their versatility and the ease with which they may be obtained. On the other hand, the mechanism by which AMSCs cause cartilage regeneration is still not clear. The available data suggests that AMSCs control the local milieu by secreting paracrine nutritional elements. This makes the environment more conducive to regeneration and repair, which in turn delays cartilage deterioration and improves joint function [51,52]. It is hypothesized that CSPCs are involved in cartilage regeneration because they possess characteristics that are comparable to those of BMSCs. When cartilage is damaged, CSPC migration takes place, and their powers to proliferate and regulate the immune system are increased [53].

### 1.1.4. Molecular Mechanism of OA

Articular chondrocytes, the cells responsible for making articular cartilage, keep aggrecan and collagen type II which are extracellular matrix component (ECM), the most prevalent proteoglycan, in a dynamic balance between production and breakdown under normal conditions. Nevertheless, in osteoarthritic conditions, reduction in cartilage tissue, clonal proliferation of chondrocytes in the insufficient areas, oxidative state activation in a stressed cellular environment and finally death of cells result from a disruption of matrix equilibrium. It is a general shift toward catabolism over anabolism as the disease progresses, with an increment in the destruction and ECM synthesis components inside the joint. Inadequate anabolic signaling and increased production of matrix-degrading enzymes and inflammatory cytokines by chondrocytes contribute to ECM breakdown and cartilage degeneration [54,55].

*1.1.4.1.TGF-β and OA*

Members of the transforming growth factor β (TGF-β) super family are amongst the several essential growth factors and transcription factors that control chondrocyte differentiation and maturation during endochondral ossification [56]. The TGF, which have a significant effect in the strong inhibition of articular chondrocyte hypertrophy and maturation, is a probable mechanism in the development of osteoarthritis too. The suppression signaling of TGF-β is a putative mechanism in the OA development. This is due to the fact that TGF-β prevents the hypertrophy and maturation of chondrocytes [57]. Types of TGF I and II Ser/Thr kinase which is transmembrane receptors mediate TGF initiated intracellular signaling, respectively. After TGF- β attaches to type (II) receptor, type (I) receptor is called in action. In terms of type II receptors, this one is permanently activated, and it helps to phosphorylate GS domain which is a part of type I receptor. R-Smads are phosphorylated at the C-terminal conserved SSXS motif upon activation of type I receptor [58]. Therefore, Smad2/3 that was phosphorylated separates from the receptor complex and joins forces with the ubiquitous Smad4, forming a heteromeric complex. Through nuclear accumulation and translocation, the Smad heteromeric complex combines with DNA-binding proteins to control transcription of genes [59].

TGF- β signaling has been indicated to play a critical role in OA formation by recent genetic manipulation of components of the TGF- β signaling pathway. The articular chondrocytes of transgenic mice that over express the dominant negative type II TGF- β receptor in the skeletal tissue develop hypertrophy, which is accompanied with an increase in the production of type X collagen, cartilage disorganization, and gradual deterioration [60].

However, signaling of TGF-β has some effects catabolic and protective ones can be given as examples. The pathway of TGF-β has been recognized as an important component of the signaling pathway underlying osteoarthritis. The findings demonstrate that TGF-β may have a function in the development of osteoarthritis. Given that an increase in TGF- function in the subchondral bone can be a primary factor in the development of osteoarthritis as well as a precipitating factor in pathology. therapeutic targeting may be employed to prevent and alleviate the symptoms of the condition [61].

Damage to cartilage is related with a decrease of TGF-β signaling, which suggests that the

protective function of TGF-β is lost as osteoarthritis progresses. In addition, TGF-β has a role in the early stages of the production of osteophytes [62].

*1.1.4.2.Matrix Metalloproteinases and OA*

OA causes temporary damage in the first place to the cartilage matrix, namely collagen II and aggrecan, but can cause irreparable late-stage damage [63]. Patients with OA experience excruciating pain because of the breakdown of extracellular matrix within synovial joints, especially the knee, wrists, and hips. Both joint cells and immune cells are responsible for the production of a wide variety of inflammatory mediators, some of which include tumor necrosis factor alpha (TNF-α) and interleukins (IL-1 and IL-7) [64]. These inflammatory mediators cause an increase in the matrix metalloproteinases synthesis, enzymes that may break down all ECM components. Since they constitute the one that slows down collagen production the most breakdown, the MMP-1 and MMP-13 collagenases have prominent roles in OA [65]. Synovial cells, which line joints, are responsible for the production of matrix metalloproteinase-1, whereas chondrocytes, which dwell in cartilage, create MMP-13. MMP-13 plays a double role in matrix breakdown since it both destroys collagen and the proteoglycan compound aggrecan [66]. Arthritis also causes an upregulation of the production of additional MMPs such MMP-2, MMP-3, and MMP-9, which break down the joint's non collagen matrix [67]. MMPs are a significant set of enzymes that maintain the constitution of the cell matrix. They are also regarded as the primary proteases contained in the breakdown of ECM [68]. Proteoglycans and collagens are the primary components of cartilage's extracellular matrix [69]. Examples of proteoglycans include aggrecan, which is the significant proteoglycan, and fibronectin, and bioglycan are other minor proteoglycans. Examples of collagens include fibrillar collagen type II and other minor collagens such as type IX, XI, and VI collagens [70]. MMPs can degrade all types of extracellular matrix proteins, for examples non- fibrillar and fibrillar fibronectin, laminin, collagens and basement membrane glycoproteins, and they are implicated in the cleavage of receptors on cell surfaces [71,72] MMPs also play a role in the degradation of cell surface receptors. MMPs have a significant impact on the activities of cells, including proliferation, migration, differentiation, death, and host defense [73]. MMPs take involvement in the physiological and pathological processes of tissue reconstitution, such as the healing of wounds, inflammation, and even cancer [74]. In addition, specific MMPs are responsible for

regulating inflammatory processes, cytokine and activity of chemokine can be given as examples, which can be either normal or pathological [75].

Through the breakdown of aggrecan and collagens, a group of MMPs called matrix metalloproteinases, including MMP-13, and MMP-3, play critical roles in the deterioration of cartilage that occurs in OA [76,77]. It is believed that MMP-13 which is the most significant collagenase in order to degrade collagen within cartilage as a result of that it preferentially digests collagen type II over type I and type III collagens [78]. This is one of the reasons why MMP-1 and MMP-8 are considered to be the most classic collagenases. The extracellular matrix (ECM) of cartilage is degraded by the enzyme matrix metalloproteinase-13, as well as the degenerative process that is involved in the etiology of OA [79]. Mesenchymal cells, such as fibroblasts and chondrocytes, are responsible for the majority of collagenase production. These cells synthesis and release the collagenase which are responsible for producing enzymes, a process that is influenced by the cytokines that are generated by mononuclear cells. MMP-13 has a significant role in the signal transduction and OA pathogenesis.

An abnormally high level of proteinase activity from MMP-13 can contribute to the development of OA, since it can cause AC breakdown and joint pathology similar to that of OA. Though MMP-13 expression is undetectable in healthy adult cartilage, it is highly elevated in the cartilage of those with osteoarthritis. This result is associated with MMP expression. The main collagenase in OA is MMP-13, and its activity is highest against collagen type II compared to other collagens.[80]

*1.1.4.3.Interleukins and OA*

Interleukin-β is a significant proinflammatory cytokine that induces the production of matrix metalloproteinases in chondrocyte cells. The expression of IL- 1β was indicated to be higher in OA joints, according to observations. The high levels of IL-1β cause significant reductions in the levels of SOX-9 and collagen type II expressions [81]. The anti-inflammatory cytokines OA, IL-1β and TNF are regarded as significant actors; IL-1β is believed to be a part of cartilage degradation, and TNF is thought to be implicated for feeding the inflammatory cascade [82]. These two cytokines induce the creation of several pro-inflammatory and catabolic components. Chondrocytes, osteoblasts and mononuclear and

joint tissues create them. Both IL-1β and TNF levels are raised in the synovial membrane, synovial fluid, subchondral bone, and cartilage of OA patients [83]. Both IL-1β and TNF alone or in conjunction with a number of other cytokines are capable of initiating and promoting inflammation. For examples, simultaneous injection of IL-β and TNF into the knee joints of rabbits create significantly greater cartilage degradation than administration of either cytokine separately [84]. IL-1β deletion increased the formation of OA lesions in an osteoarthritis mouse model, suggesting that this cytokine plays a complicated function in maintaining cartilage homeostasis. Regardless of the fact that IL-1β is commonly regarded as a proinflammatory cytokine. The IL-1β receptor type I is a cell-surface receptor that mediates IL-1β ‘s biological activation of cells. As compared to regular cells, human OA chondrocytes and synovial fibroblasts express this receptor at a higher level of [85,86].

High levels of triple helix type II collagen that have been crosslinked together are the primary structural component of ECM in cartilage [87]. Studies conducted in cell culture have shown that IL-1β treatment inhibits expression of collagen type II in chondrocytes derived from a variety of species. Important for cartilage's structure, aggrecan expression is reduced in IL-1β treated cartilage explants and chondrocytes [88]. As the primary enzyme in the production of glycosaminoglycans connected to core proteins of aggrecan, -1,3-glucuronyltransferase I appear to be the target of IL-1β ‘s inhibitory effect on proteoglycan synthesis in rat chondrocytes. Recent researches have demonstrated that TNF inhibits chondrocyte production of proteoglycan, link protein, and collagen type II [89]. MMPs for example; MMP-1, MMP-3, and MMP-13 are among the many proteolytic enzymes which released via chondrocytes in reply to IL-1β and TNF. Anticytokine treatment, particularly that which targets IL-1β and TNF, is an appealing technique to prevent osteoarthritis since these three proteases are critical cartilage breakdown regulators [90].

A heterodimer receptor complex consisting of a membrane-bound IL-6 receptor (IL-6r), a soluble version of IL-6 receptor (sil-6r). It is also receptor -subunit gp130 is required for IL-6 signaling. Translocation of phosphorylated Stat1 and Stat3 to the nucleus and subsequent transcription of IL-6 target genes follow ligand induced interaction of this complex [91,92]. Normally, chondrocytes secrete just a little amount of IL-6. However, certain OA specific cytokines and growth factors, including as TGF-β and IL1β, directly increase Il-6 synthesis. Furthermore, large amounts of Il-6 administered intravenously raised C-reactive protein (CrP) levels in human peripheral blood mononuclear cells in vitro and in healthy human

volunteers [93]. High levels of Il-6 and in a clinical investigation of people with osteoarthritis, higher levels of CrP at baseline were linked to a greater risk of cartilage loss. Furthermore, elevated levels of circulating IL-6 and a high body mass index (BMI) are both associated with an increased likelihood of developing radiographic knee osteoarthritis [94,95]. These associations are due to their respective impacts on catabolic and anabolic responses.

The actual function of IL-6 in osteoarthritis is debatable, since some research conducted in chondrocytes neglected to look into or employ sil-6r because it dramatically improved the affinity of IL-6 for its particular receptor. Despite this, research has implicated IL-6 in inflammatory and degenerative joint illnesses when combined with sil-6r. In both rheumatoid arthritis and osteoarthritis, inflammatory cytokine 6 and interleukin 6 receptor (sil-6r) levels are elevated in synovial fluid. With IL-1β, IL-6 enhances MMP-1 and MMP-13 expression in bovine and human cartilage explant cells [96,97]. Also, chondrocytes treated with interleukin 6 receptor and inflammatory cytokine 6 express less collagen type II, whereas mechanical damage can amplify TNF and il-6-sil-6r on proteoglycan breakdown impact [98].

Even in animal models, it is unclear what role IL-6 plays in the body. IL-6 into the joint cavity of IL-6 injection to deficient animals prevents cartilage breakdown, despite the fact that showing a lack of IL-6 in mice leads to a reduction in the number of inflammatory cells in the knee joints and a milder reaction to collagen induced arthritis [99]. In addition, IL-6-defective male mice had a higher rate of subchondral bone sclerosis and lower rates of proteoglycan production than wild-type male mice. The observed dual action of IL-6 in vivo may be due, in part, to the fact that IL-6 is considered to modulate acute phase proteins, that are either anti-inflammatory or immune suppressive as along acute phase.

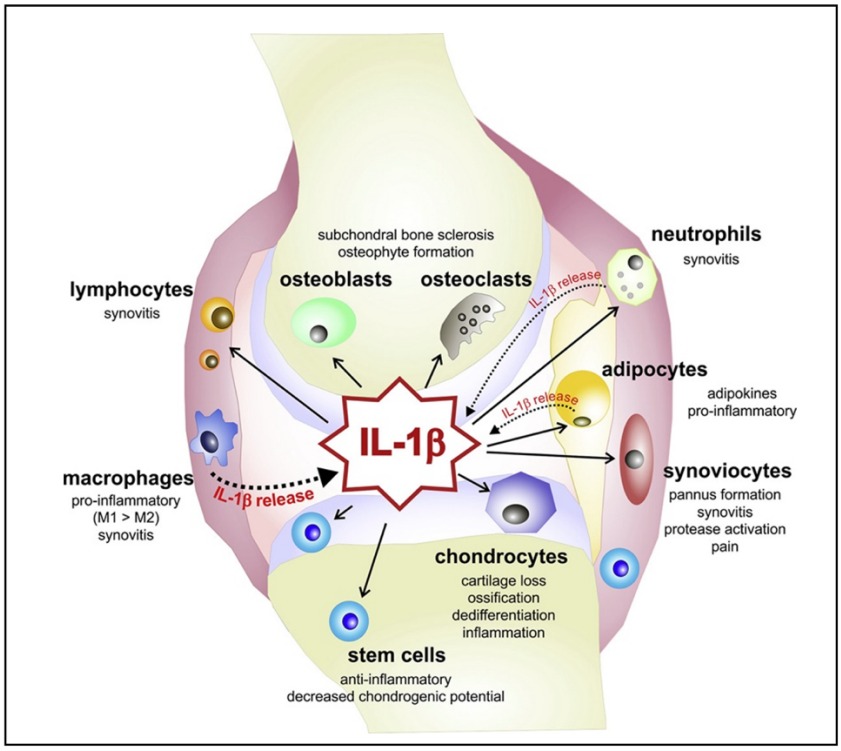


Figure 1. 1. IL-1β signaling in osteoarthritis [100].

*1.1.4.4.SOX9 and OA*

Key activators of the collagen type II gene in mice are the SOX transcription factors SOX6, SOX9, and L-SOX5, which are also necessary for the expression of the collagen type II gene in humans and mice. To create cartilage, SOX9 is typically referred to as the master chondrogenic factor. From postnatal day zero to postnatal day two, expression of the chondrogenic transcription factor SOX9 decreased [101,102]. When chondrocytes in the mouse rib cartilage dedifferentiate, mRNAs encoding the Sox9 transcription factor are repressed.

It is β -catenin, a transcription factor, that plays a central role in the Wnt signaling pathway. During chondrogenesis and dedifferentiation, its function in regulating chondrocyte phenotype was analyzed. Both supporting hypertrophic maturation of chick sternal chondrocytes via COL X expression activation and suppressing chondrogenesis of mesenchymal cells, as evidenced by an increase of SOX9 after β-catenin deletion in limb mesenchyme, are roles played by β-catenin during chondrogenesis. Furthermore, while β-

catenin inhibits SOX9 transcriptional activity, it is repressed by SOX9. SOX9 represses -catenin-mediated transcriptional activity. This indicates that Sox9 and β-catenin are both under the control of a cross control. [103,104].

Dedifferentiation of chondrocytes in the human hip caused by osteoarthritis has been linked to the buildup of β -catenin. In contrast, production of COL2 by dedifferentiated chondrocytes cultivated in alginate beads and chondrogenic development of mesenchymal cells both required low levels of β -catenin, showing that β catenin is a protein that acts as a negative regulator of differentiated chondrocytes. [106].

By definition, E74-Like Factor 3 (ELF3) is only expressed in epithelial tissues under normal circumstances. However, ELF3 levels are elevated in arthritic synovial and cartilage tissues, as well as in human chondrocytes stimulated with pro-inflammatory cytokines [108,109]. ELF3 transcription may be under epigenetic regulation in osteoarthritis illness, since in depth analysis showed a correlation between elevated ELF3 mRNA expression in OA chondrocytes and hypermethylation of the proximal promoter [110].

The transcription factor ELF3 serves a key function in the loss of phenotype in chondrocytes. Indeed, ELF3 strongly suppresses COL2A1 transcription in human chondrocytes. ELF3 not only downregulates COL2A1 expression in chondrocytes, but it also transactivates MMP-13, a collagenase that specifically cleaves type II collagen. Elevated ELF3 level inside OA cartilage coincided with higher MMP-13 protein levels and activity [106]. ELF3 is one of the key transcription factors promoting the induction of MMP-13 transcription in human chondrocytes in response to IL-1beta and TNF-α [111].

*1.1.4.5.VEGF and OA*

In addition to its angiogenic effects, VEGF controls the metabolic activity of chondrocytes. Remodeling and ossification of hypertrophic cartilage have been associated to osteoarthritis, which has been connected to the invasion of blood vessels. In osteoarthritis, VEGF is expressed by osteoblasts, hypertrophic chondrocytes, and superficial articular chondrocytes, as well as synovial fibroblasts and macrophages. Whereas, expression of VEGF is higher in osteoarthritis chondrocytes than in normal controls, this higher expression is more often found in the superficial zones of osteoarthritis articular cartilage than in areas of active osteochondral angiogenesis [112,113].

Chondrocyte phenotype in osteoarthritis is altered by inflammatory cytokines, hypoxia, and mechanical stress, all of which lead to increased VEGF expression. Jun N-terminal kinase (JNK) but not p38 mitogen-activated protein kinase (MAPK) signaling pathways were necessary for IL-1β induced VEGF synthesis. The protein known as sera amyloid, which functions as a transcription factor in response to inflammation. It is possible that VEGF expression in arthritic chondrocytes is controlled by stromal activating factor-1. Reducing endogenous SAF-1 prevented IL-1 β and TGF- β from stimulating VEGF expression in chondrocytes [115,116].

HIF-1a and VEGF have comparable localizations in osteoarthritis, and VEGF expression in chondrocytes is increased when they are exposed to hypoxia via activating HIF-1a. Chondrocytes exposed to hypoxia showed increased VEGF expression that was reliant on the p38 MAPK pathway, suggesting that inflammation and hypoxia control VEGF production in chondrocytes through distinct signaling pathways [117,118].

It is possible that HIF-1a activation is involved in the increased production of VEGF in chondrocytes that is caused by mechanical overload or stress such as compression, shear, tension, and strain. Overworked bovine cartilage disks express both VEGF and HIF-1a. Synergy between inflammatory, hypoxia, and mechanical pathways may be crucial to the tendency of diarthrodial joints to improve osteoarthritis. Inflammatory mediators may also contribute to the activation of chondrocytes by aberrant mechanical stress [119].

Under hypoxic circumstances in particular, VEGF may promote the release of MMPs while inhibiting the production of tissue inhibitor of metalloproteinases. Articular chondrocytes contribute to vascular invasion of articular cartilage and the resistivity of articular cartilage to this invasion by producing antiangiogenic substances such TIMPs. As a result, VEGF's modulation of chondrocyte activity may upregulate because of its direct angiogenic effects on the endothelial cells of blood vessels, suggesting that this is not a coincidental action of VEGF. While this may be a beneficial outcome during normal development, in the case of osteoarthritis, where the balance has already tipped toward cartilage breakdown, it may hasten the deterioration of the joint [120-121].

### 1.1.5. OA Treatment

*1.1.5.1.Surgical Methods*

This therapy to OA is typically advised at greater degrees of the illness. Although its success in avoiding numerous early and advanced stage consequences of the illness and virtually entirely removing most symptoms and indicators, this pathway should not be classed as a therapy route in the disease either. For patients with severe knee arthritis, total knee arthroplasty (TKA) is among the most effective and economical treatments. In the previous decade, annual transactions have skyrocketed, and experts predict that trend will continue [122].

Ultimate possible complications include the existence of painless, well-functioning knee arthrodesis; recent or present knee sepsis; a distant source of ongoing infection; extensor mechanism discontinuity or severe extensor dysfunction; recurvatum deformity due to muscle weakness; and the existence of a traumatic or degenerative knee injury [123] . Medical problems that reduce a patient's resistance to anesthesia, the metabolic needs of surgery and wound healing, and the extensive rehabilitation required to assure a satisfactory functional result are only a few of the relative contraindications that have been debated. Significant atherosclerotic disease of the operated leg, skin conditions like psoriasis at the operative site, morbid obesity, recurrent cellulitis, venous stasis disease, neuropathic arthropathy, and a history of osteomyelitis near the knee are also relative contraindications. The principal indication is for use in elderly people suffering from severe arthritis in the knee to alleviate knee discomfort. When less invasive treatments are not adequate to alleviate pain and enhance function to a satisfactory level, TKA may be an appropriate treatment option. Difficulty in performing activities of daily living, frequent use of analgesics, and discomfort experienced throughout the night are all common considerations. It is performed for osteoarthritis the vast majority of the time, followed by rheumatoid arthritis. However, TKA is also conducted in a variety of arthritic disorders in order to manage the pain when conventional medical treatment has been unsuccessful [125,126].

The osteotomy procedure is a method for correcting knee alignment that depends on shifting the body's weight-bearing forces away from the section of the knee that is damaged by arthrosis and onto a portion of the knee that is healthy. When compared to other treatment

techniques, the unique characteristic that distinguishes osteotomy is the force redistribution that it provides. Osteotomies are becoming less common as a result of the advent and effectiveness of knee arthroplasty in recent years, which has led to a progressive drop in the occurrence of osteotomies. There is ongoing discussion on whether or not osteotomy is truly necessary and whether or not it should be considered a preventative procedure. Osteotomy is still a beneficial treatment, but it must be performed in accordance with very specific patient criteria [127,128].

An osteotomy of the knee is a surgical technique that may be advised in cases when arthritic deterioration is evident in only a single portion of the knee joint. The surgery involves either removing or adding a fragment of bone to the proximal tibia or the lower femur in order to assist transfer your body weight away from the problematic region of your knee joint. This is done in order to alleviate pain associated with knee osteoarthritis [129].

Patients with osteoarthritis who are younger and more active may benefit from undergoing an osteotomy operation because it allows them to keep utilizing the healthy section of their knee even after their prosthetic knee has worn out. The need for a total knee replacement may be put off for as long as 10 years if this treatment is performed. Osteotomy has the potential to produce a number of difficulties; thus, it is essential to identify patients appropriately in order to reduce the risk of serious complications [130].

*1.1.5.2.Non-Surgical Methods*

Even in modern times, treatments that do not include surgery are not considered to be a definite cure for the condition. These therapies, on the other hand, can alleviate symptoms and slow down the progression of the disease, whether clinically or pathophysiologically. Examples of these approaches include rehabilitation and physiotherapy, ultrasound therapy, and different medicines such as nonsteroidal anti-inflammatory drugs, glucocorticosteroids, provided for non-therapeutic objectives merely for the treatment of symptoms and enhancement of lifestyle [131].

Physiotherapy is an essential principle that includes treatments like exercise, electrotherapy, ultrasound, and hot or cold packs. Its goal is to halt the course of the illness and reduce the patient's level of discomfort. In the application of ultrasound, both thermal and non-thermal effects must be taken into consideration. However, it is stated that although its effect is on

the joint capsule rather than the bone and cartilage tissue, it is stated that application of ultrasound can reduce pain in osteoarthritis and improve physical activity. This is despite the fact that its effect is on the joint capsule rather than bone and cartilage tissue [132].

By assisting in the prevention of obesity, joint instability, and muscular weakness, exercise can lower the risk of osteoarthritis. In addition, it may assist in a successful therapy by assisting patients in managing their weight, enhancing their muscular strength, decreasing their joint stiffness, enhancing their range of motion, increasing their functioning, and lowering the danger of falling. Patients should see a medical professional for clearance before beginning an exercise regimen and then have their fitness level thoroughly evaluated by an expert in the field of physical activity. After beginning the program with workouts of low to moderate intensity, the intensity should progressively rise until it reaches the desired level. It has been found that low-impact aerobic training and isometric or isotonic strength training are the most beneficial forms of exercise for managing the symptoms of osteoarthritis (OA). In addition, exercise programs that take place in water can make it easier for people to stick with an exercise routine and may be just as successful as exercises that take place on land related to reducing pain and improving walking and functioning [133-134].

The most prevalent symptom of osteoarthritis (OA) is pain, which may be accompanied by other symptoms such as joint stiffness, inflammatory swelling, instability, and muscle weakness. When taken together, these symptoms can lead to physical restrictions, which can then evolve into illnesses that affect older individuals' ability to live independently. Walking, housework, gardening, and climbing stairs are examples of activities of daily life that might become taxing and may require the aid of another person. Increased muscle strength, range of motion, proprioception (the sense of body stimuli and awareness of body part location, balance, and movement), and cardiovascular fitness are some of the functional improvements that may be achieved by exercise. Although physical activity might alleviate some of the discomforts associated with osteoarthritis (OA), it is powerless to change the disease's underlying structural impact [135,136].

Corticosteroids: The action of corticosteroids is to imitate that of hormones that are anti-inflammatory. Betamethasone, methylprednisolone, and triamcinolone are common medications that are injected directly into the joint area and used to treat knee osteoarthritis, specifically 11th. Pain alleviation that is only temporary, enhanced physical function, and a

reduction in joint inflammation are some of the potential advantages of receiving these injections. Corticosteroids may be useful for giving pain relief in the short term, but according to the most recent guidelines from OARSI, they are probably not an appropriate choice for managing pain in the long run [137]. Corticosteroids have been shown to be effective in alleviating pain, according to a more recent systematic review that used network meta-analysis. However, the research found that corticosteroids were not effective in improving joint function and stiffness. According to the findings of a recent comprehensive review, the clinical response to injection can be unpredictable but is still able to be anticipated based on demographic and clinical characteristics [138].

NSAIMs, also known as non-steroidal anti-inflammatory medicines, are medications that have an anti-inflammatory effect and can either be taken orally or given topically to the afflicted joint. Oral non-steroidal anti-inflammatory drugs (NSAIDs) can either be COX-2 inhibitors or non-selective choices. Despite having only mild effects on pain, there is a possibility of experiencing adverse effects with prolonged usage. It is believed that medications that target COX-2 have a lower risk of adverse effects compared to non-selective treatments. Even though it is not known how much of an effect topical NSAIDs have on the whole, it is generally accepted that they are safer and more well tolerated than oral NSAIDs. Although oral nonsteroidal anti-inflammatory drugs (NSAIDs) are typically highly helpful in managing pain, their potential for side effects, they are more likely to acceptable for occasional usage rather than frequent use [139-141].

*1.1.5.3.Regenerative Methods*

Regenerative medicine is the only potential route to a complete cure for osteoarthritis (OA), which correlates with the improvement of indications and symptoms.

Steadman was the first person to describe the process of microfracture, which is now commonly used for stimulating bone marrow. By establishing bleeding holes at the base of a cartilage defect, a clot can develop, and pluripotent cells from the bone marrow cavity can migrate to fill the deficiency with a fibrocartilage repair tissue. Cartilage defects can be repaired by creating bleeding holes at the base of the defect [142].

In this procedure, loose cartilage is removed arthroscopically using a razor and a curette in order to produce a stable edge that will hopefully restrict the ensuing clot. After that, the

layer of calcified cartilage is treated. After then, holes are drilled at spacing of 34 millimeters and to a depth of 2-4 millimeters. The presence of bone marrow fat droplets emerging from the holes is evidence that the holes are of an adequate depth [143,144]. When used to treat traumatic chondral abnormalities, microfracture has been shown in a number of studies to reliably lead to clinical improvements in the near term. However, these gains have a tendency to fade away with time. Microfracture is another technique that has been researched for potential application in early OA problems. The clinical scores of the patients in these published studies typically indicate short-term gains from their baseline values, whereas long-term ratings reveal deterioration [145]. Microbreaking is advantageous due to the fact that it is straightforward, economical, and has a track record of maintaining a high level of safety. Microfracture has a number of drawbacks, including limited durability as a result of an inability to regenerate hyaline cartilage, as well as unsatisfactory results when applied to major lesions [146].

Autologous chondrocyte implantation, more commonly known as ACI, was initially performed in Sweden. It was intended to cause a defect in the articular cartilage that was present in the patient. In order to carry out the procedure, chondrocytes will first need to be harvested from a functional part of the knee by means of arthroscopy [147]. Before being made ready for re-implantation, these cells are first separated and then cultivated for anywhere between 15 and 22 days. During the second operation, the defect is prepared by removing all of the injured cartilage and carving a vertical edge from the good cartilage using a scalpel. This is done so that the defect may be repaired. After that, a ring curette is utilized to remove debris from the calcified cartilage layer. After the flaw has been produced, a template will be utilized to determine its exact dimensions [148]. After that, a periosteal flap is removed from the proximal medial tibia, and it is subsequently sutured over the defect in an ACI of the first generation. After that, autologous chondrocytes are injected under the flap, which has to have a watertight cover placed on it for protection. Collagen I is utilized in the second generation of ACI. One of the many benefits of ACI is that it may create permanent cartilage similar to hyaline even in big (more than 2 $cm^2$) abnormalities. Concerns about the possibility of chondrocytes differentiating into cartilage while the joint is being enlarged, as well as the possibility of graft rejection, are among the drawbacks of this surgery. hypertrophy (highest with initial generation procedures) (highest with first generation techniques). In addition, both first- and second-generation procedures need an

open arthrotomy; however, matrix-supported autologous chondrocyte implantation does not have this limitation (MSCI) [149].

Some of the mechanisms involved in tissue healing are regulated by platelet-derived growth factors. At the moment, these components are extracted from a sample of the patient's own blood and then injected directly into the joint cavity once the necessary preparation has been carried out. There is currently a lack of consensus in the scientific literature on the degree to which these therapies are successful in clinical settings. A meta-analysis of eight publications with data ranging from poor to intermediate quality came to the conclusion that the influence of platelet-rich plasma (PRP) is still unknown. Conversely, a comprehensive study and meta-analysis of data with a reasonable level of quality demonstrated that platelet-rich plasma (PRP) provided effective pain relief for at least six months following injection [150]. Furthermore, PRP injections were shown to improve pain and function in a study of three meta-analyses of varying quality two months after treatment, with symptomatic relief lasting for up to 12 months and the greatest improvement occurring at six months. According to the findings of several studies, those who only have mild to moderate osteoarthritic alterations might think about using this as a potential therapeutic option [151].

In light of the fact that ACI is a two-stage process and that cultivated chondrocytes undergo differentiation, the researchers investigated the possibility of employing mesenchymal stem cells (MSC) to regenerate cartilage. 1998 marked the beginning of the use of mesenchymal stromal cells (MSC) in the treatment of cartilage defects [152]. The use of MSC as part of a tissue engineering strategy for the treatment of traumatic cartilage defects was shown to have promising results in a recent study; however, further research is required before procedures can be created. Investigations are still being conducted into the mysteries surrounding the creation of MSCs as well as the optimal ways to cultivate and eventually implant an MSC-seeded scaffold. In a similar vein, research on stem cell therapy as a treatment for osteoarthritis of the knee is still in its early stages. In the same vein as traumatic flaws, osteoarthritic defects (OA defects) could be treatable using an MSC seed scaffold, however this is not yet known. Direct injection of MSC into the joint itself is an additional alternate treatment method. Early findings from the application of this method, which have been reported by a number of writers, are positive; nevertheless, long-term controlled research is required before a judgment can be formed regarding the effectiveness of this method. Recent findings from a randomized controlled experiment explored the use of MSC in conjunction

with microfractures [153]. It was discovered that the group that received an injection of MSC three weeks after surgery had considerably superior short-term functional scores and MRI results than the group that received an injection of hyaluronic acid three weeks after surgery. At the moment, it is impossible to determine if injections on their own are capable of restoring cartilage integrity for localized abnormalities [154].

When utilized as part of a tissue engineering plan, MSC therapy eliminates the requirement for chondrocyte extraction, and when performed as an intra-articular injection, it is substantially less invasive than other treatment options. These are two of the potential benefits of the treatment. The fact that MSC treatment is still mostly in the experimental stage is now the most significant drawback associated with it. In order for MSC therapy to be recognized as a valid treatment option for the management of knee osteoarthritis (OA), further research is required [155].

## 1.2.EXOSOMES

Most extracellular vesicles are (EVs) fall into one of three categories: exosomes, microvesicles, or apoptotic bodies. Extracellular vesicles (EVs) are nano-sized communication mediators that are released by cells. These messengers transfer biological signals from one cell to another. In the realm of regenerative medicine, researchers have been focusing more and more of their attention on EVs. Numerous studies have demonstrated that there may be potential utility in reducing inflammation and enhancing regeneration and repair of tissue [156].

Nanosized vesicles having a bilayer lipid membrane with a size range of 30 to 2000 nm are known as extracellular vesicles. These vesicles are capable of transporting a wide variety of biologically active molecules, including RNA subtypes such as mRNA, microRNA, and lncRNA, DNA fragments, lipids, proteins, and enzymes. The vast majority of cells, including those found in connective tissues and MSCs, are able to create EVs and release them into a variety of biological fluids, including as blood and synovial fluid. The effects of EVs might begin as soon as they are discharged or they can be moved to a different location. EVs are able to communicate recipient cells by causing the identical effect to be produced as their source cells [157,158].

### 1.2.1. Exosome Biogenesis

Rab GTPases, annexin, integrin, and fibronectin are known as membrane fusion proteins and they also indicate membrane transport protein feature. In addition, tetraspanins such as CD9, CD63, CD81, and CD82, heat shock proteins (Hsp27 and Hsp20), and lipid-related proteins are typical components of exosomes [159]. Double lesion of the plasma membrane triggers the synthesis of exosomes, which thereafter develop into intracellular multivesicular bodies (MVBs), intermediates in the endosomal system. Exosomes, or ILVs for short, are generated during this stage of MVB development within the luminal space. Cargoes are sequestered on MVB limiting membrane microdomains via a continuum of processes and specific sorting machinery [160]. This is then followed by budding of the membrane inward and the subsequent fission of very small vesicles containing the separated cytoplasm. The endosomal sorting complex needed for transport (ESCRT) lies at the heart of these machines and is responsible for structuring and severing ILV membranes. ESCRT-0, -I, -II, and -III are the four primary subcomplexes that make up the ESCRT machine. The MVB membrane's microdomains are where cargo aggregation occurs, and ESCRT-0 and ESCRT-I play a role there. In order for ILVs to be budded and shattered into the MVB lumen, ESCRT-III must be assembled and recruited, both of which need the presence of ESCRT-II. Because of ALIX's capacity to connect the ESCRT-III subunit and cargo at the neck of ILVs, the classical ESCRT route can crosstalk with the syntein/ALIX axis to control cargo clustering and membrane budding. Exosome formation persists even after depletion of the ESCRT complex, indicating the existence of a mechanism for exosome synthesis that does not rely on ESCRT. Some researchers showed that the EGFR phosphorylates Rab GTPase 31 (RAB31) in response to epidermal growth factor (EGF) activation, and that Rab31 then communicate flotillin proteins in lipid raft microdomains to induce EGFR entrance into ILVs in an ESCRT-independent fashion. Thus, several machines can work on the endosomal system to target and encapsulate secreted cargoes, and the proportions of these machines acting on any one endosome will vary depending on the kind of cell and the signals or stimuli it is exposed to [161-164].

It is unclear what mechanism regulates the relative rates of MVB secretion and degradation during maturation. MVBs are either carried via microtubules to the plasma membrane for secretion, or they are directed to lysosomes for breakdown. Exosomes, which are mostly

dependent on the common secretory machinery and are able to be encouraged by numerous Rab family members including Rab27a and Rab27b, are released from the plasma membrane when the cumulative nondegradable MVBs dock there and fuse with the membrane [165]. In addition to preventing their contents from degrading too quickly on route to their destination cells, exosomes' bilayer phospholipid membrane also facilitates site-specific attachment toward the recipient cells after they have been secreted into the extracellular space. Therefore, the ability of exosomes to communicate with their target cells in a wide range of ways, which are in turn dependent on the types of cells involved and the exosomes' origins. They can be secreted into the body or kept on the surface of recipient cells, where they make activation of surface receptors to provide a functional response. They can also be taken up by the receiving cells by endocytic routes such micropinocytosis, clathrin mediated endocytosis, phagocytosis, and or lipid raft-mediated uptake, or through direct fusion with the cell plasma membrane. Following the aforementioned routes of internalization, exosomes empty entering the cytoplasm of receptor cells and are subsequently destroyed and recycled by lysosomes. [166-169]

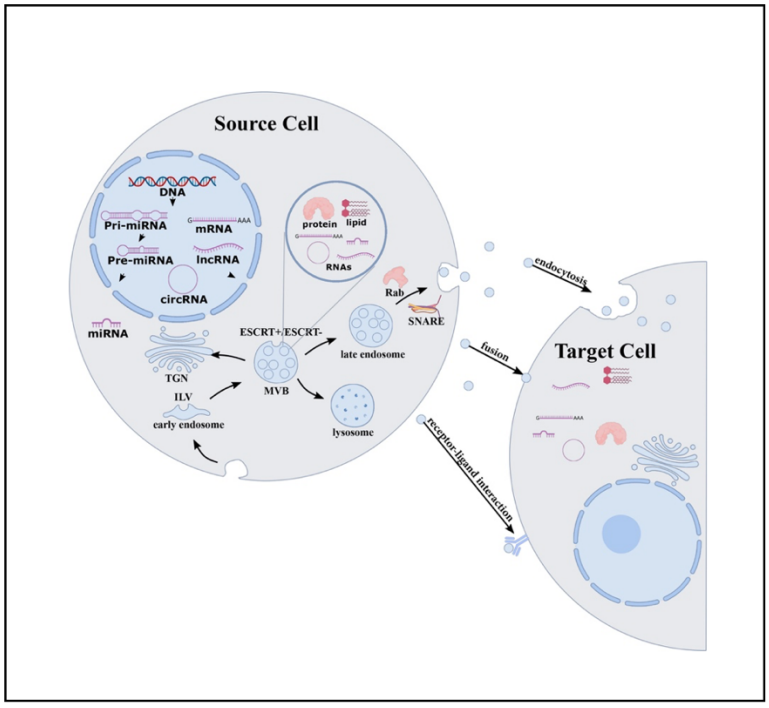


Figure 1. 2. Biogenesis of Exosomes [170].

### 1.2.2. Exosome Uptake Mechanism

It is currently unknown whether non-immune and immune cells must internalize exosomes in order for them to induce biological responses. For instance, RNA-induced cellular responses depend on internalization. Comparatively, cellular responses caused by membrane-bound or soluble FasL and TRAIL from exosomes do not need internalization, but are based on location and transient adherence for paracrine or soluble signaling. Exosome internalization may occur by fusion, receptor-mediated endocytosis, macropinocytosis, or phagocytosis, according to varying perspectives in the scientific literature. Nevertheless, the latter two methods might be mechanisms for the clearing. of exosomes as opposed to inducing a physiological response [171,172].

*1.2.2.1.Fusion*

Fusion of a vesicle with a cell membrane is known as vesicle-cell fusion. Microvesicles produced from monocytes were shown to fuse and bind with the activated platelets plasma membrane, transferring proteins including tissue factor and P-selectin glycoprotein ligand-1 (PSGL-1). This was confirmed using a fluorescent lipid-mixing test and a membrane fusion experiment. An analogous investigation showed that filipin might have blocked the fusion of cells with metastatic melanoma release exosomes that fuse with the plasma membrane. It was shown that proteins have a modest, possibly structural function, in the fusion process. The interaction of exosomes with cytoplasmic vesicles was indicated by their co-localization with Rab53 or Lamp-1[173, 174].

The process of cell-cell fusion is not completely clear, in contrast to endocytosis, for which numerous molecular pathways have been defined. Tetraspanin complexes on target cells may control the occurrence. There is evidence that tetra- spanins have a role in activation of T cell as well as membrane fusion in sperm-oocyte, myoblast, mononuclear phagocyte, and mammalian viral-cell fusion [175].

*1.2.2.2.Phagocytosis*

Scientists discovered that the actin cytoskeleton, phosphatidylinositol 3-kinase (PI3K), and dynamin2 were all necessary for the phagocytosis of exosomes. Clathrin-mediated

endocytosis and phagocytosis have been linked to the protein's actin, PI3K, and dynamin2. Lamp-1, lysobisphosphatidic acid, and Rab7 were found co-localized with internalized exosomes in late autophagosomes, endosomal, and lysosomal vesicles. Additional study is needed to determine if phagocytosis is a real process of exosome internalization for the goal of intercellular communication or simply a way of removal [176].

*1.2.2.3.Macropinocytosis*

In the process of macropinocytosis, protrusions of the plasma membrane are forced by actin filaments to produce an invagination, that then endocytoses extracellular fluid and tiny particles in a non-specific manner. Phosphatidylserine found on the surface of oligodendrocyte-derived exosomes was shown to trigger macropinocytosis in a subpopulation of microglia and macrophages that lacked the ability to deliver antigens. Macropinocytosis of exosomes is reliant on and PI3K and Na+, and the pharmacological inhibitors EIPA and LY294002, respectively, reduce uptake of exosomes by inhibiting the Na+-H+ ion exchange and activity PI3K, respectively [177].

### 1.2.3. Plant-Derived Exosomes

Since exosomes were found for the first time in mammalian cells, the field of exosomal study has blossomed. Regrettably, exosome research on plant areas has been neglected for a number of years due to a lack of interest. Researchers began working on unraveling the processes that control the death of plant cells. This led to the beginning of the active study on plant exosomes. After doing research on Arabidopsis thaliana, the researchers found that there was a consistent presence of spherical structures between the plasma membrane and the plant's cell wall. Unbelievably, investigations that date back to the 1970s have also revealed up the existence of similar structures inside of plants [178,179]. Perhaps even more astounding than the existence of these vesicles that are attached to membranes is that it was indicated that fusion occurs between plasma membrane and membrane bound vesicles and releasing their contents in the space between the cell wall and its membrane. The resemblance between mammalian exosomes and these membrane-bound plant vesicles stimulated curiosity in the existence of plant exosomes. This is because mammalian exosomes are released when MVBs fuse with the plasma membrane. Mammalian exosomes

have been investigated in considerably greater depth compared to plant exosomes; as a result, there are many reports for their separation and use. In spite of the widespread recognition of the role of exosomes in the process of cellular communication in mammalian organisms, very little was known about the importance of plant extracellular vesicles (EVs), other than the fact that ancient microscopic photographs revealed their presence. However, it is now recognized that plant exosomes have a role in the immune response, which finally led to the research of several strategies for isolating EV from plant cells [180-184].

It has also been observed that exosomes and nanoparticles that behave similarly to exosomes and are created from plants exhibit anti-cancer action. Raimondo et al. explored the use of nanovesicles generated from citrus limon (lemon) juice as a potential therapy for chronic myeloid leukemia (CML) [185]. The nanovesicles derived from lemons significantly inhibited the growth of tumors in a CML model, which was the desired effect, and they inhibited the secretion of multiple cytokines that are connected with angiogenesis through TNF-alpha-related-apoptosis-inducing-ligand (TRAIL)-mediated cell death. Both of these effects were caused by the elimination of cancer cells. Based on the findings of in vivo biodistribution experiments, the nanovesicles were able to target the cancerous tissue within fifteen minutes, and their effects persisted for more than twenty-four hours [186]. The fact that the nanovesicles were successful in mediating communication across different kingdoms and in eradicating cancer cells via TRAIL signaling lends credence to the possibility that they may be used as chemotherapeutic agents. Transmission electron microscopy is the primary method of investigation utilized in research concerning plant exosomes. According to the findings of these studies, EVs in plant tissue begin to proliferate and congregate near the site of assault when they are subjected to fungal or pathogen attack. Because EVs are so prevalent during infection, the potential roles that plant EVs may play are particularly fascinating [187].

Because plant derived exosomes, in their capacity as nanovesicles, are able to transport drugs in a risk-free manner and maintain their ability to circulate in the blood for a considerable amount of time following systemic administration, they are regarded as potentially useful targeted delivery vectors for tumorigenic disorders and chronic illnesses. Zhang et al. employed ginger derived exosomes that were capable of targeting tumors in order to analyze the toxicity of plant derived exosomes [188]. Specifically, they looked at whether or not there was apparent tissue damage in organs. The ability of ginger derived exosomes to target

tumors resulted in a reduction in the accumulation of ginger derived exosomes in the spleen and liver. This reduction in accumulation had the potential to elicit a lowered the overall systemic toxicity of medications to healthy tissues while simultaneously extending the time that pharmaceuticals were circulated in the blood. Histological examination of the heart, liver, spleen, lung, or kidney revealed no significant damaging effect when compared with the control group. This suggests that plant derived exosomes have the potential to be utilized as a drug delivery nano-platform, which would result in an increase in drug efficacy while simultaneously lowering the risk of drug toxicity [189,191].

In the most recent few years, there has been an ever-increasing need for the development of environmentally friendly, biocompatible, and sustainable products. Extracellular vesicles, which may originate from either plant or mammalian cells, have received a lot of interest as of late. Considering all of these factors, medicinal plants assume a particularly prominent position due to the healing effects they possess [192].

Radish is a common ingredient in home remedies for treating a wide variety of illnesses, including jaundice, gallstones, liver problems, rectal prolapse, and various types of stomach pain. This practice originates from folk medicine. In addition, it was discovered that radish contains its own distinct bioactive chemicals, which have just very recently been identified as possibly having positive effects on human health [193]. In recent years, a number of scientific investigations have uncovered the numerous beneficial impacts that radish has on human health, and some of these studies have even been able to define or propose, albeit in a limited capacity, the molecular underpinnings of these effects [195]. In addition, Suh et al. (2006) conducted an in vitro investigation in which they revealed that the extract from the white radish taproot was able to suppress the aberrant growth of vascular smooth muscle cells [196]. According to research carried out by Kim et al. (2014), the Asian white radish, which is a kind of edible solid taproot, possesses both anti-inflammatory and anti-cancerous properties. Sulforaphane, an isothiocyanate that may be found in radish, was shown in research that was published relatively recently to suppress the multiplication of breast cancer cells [197].

The significance of exosomes in medicinal plants is currently being examined with a much larger interest for their potential involvement in complementary medicine as a result of the ever-increasing interest in medicinal plants as well as the existence of plant exosomes.

## 1.3.THE AIM OF THE STUDY

The main purpose of this study to examine whether *Raphanus sativus* derived exosomes has significant effect on in vitro model of human chondrocyte osteoarthritis cells. Although the utilization of plants for medical purposes is not a recent idea, the study of plant exosomes is a recently found field that requires further analysis and research. A level of interest in the utilization of medicinal plants that has never been seen before is currently present.

In order to accomplish this goal several studies were performed such as isolation and characterization of *Raphanus sativus,* anti-inflammation effect of *Raphanus sativus* based on gene and protein levels which are responsible for cartilage regeneration.

# 2. MATERIALS

## 2.1.Chemicals

Dextran (9004-54-0, Sigma-Aldrich, USA), Polyethlyene glycol (81310, Sigma-Aldrich, USA), Dimethyl Sulfoxide (Santa Cruz Biotechnology sc-202581, USA), Trypsin-EDTA (Gibco 25200056, Thermo Fisher Scientific, USA), Phosphate Buffered Saline (Lonza BE17-517Q, Switzerland), cDNA synthesis kit iScript™ (#1708890, BioRad, USA), Chondrocyte Growth Medium (Sigma Aldrich # 411-500), TRIzolTM Reagent, (15596-018, ThermoFisher Scientific, USA), Human VEGF ELISA Kit (KHG0111, Invitrogen, USA), QuantiTect SYBR Green PCR Ki (204145, Qiagen, USA), Human MMP-13 ELISA Kit (# EHMMP13, Invitrogen, USA), RayBio. Human TGF- β ELISA Kit (#ELHTGFb1, RayBiotech, USA), TRIzol™ Reagent (15596-018, ThermoFisher Scientific, USA), RIPA Lysis and Extraction Buffer (#89900, ThermoFisher Scientific, USA), Anti-Collagen II antibody (#ab34712, Abcam, USA), Anti-Aggrecan Antibody (#AB1031, Sigma-Aldrich, USA), Goat anti-Rabbit IgG (H+L) Secondary antibody FITC (# 65-6111, Thermo Fisher, USA), MycoZap™ Plus-PR (#VZA-2021, Lonza, Germany)

## 2.2.Equipments

T-25, T-75, T-150 cell culture flasks (#90025, #90075, #90150, TPP, Switzerland), Countess Cell Counting Chamber Slides (#C102288, Thermo Fisher, USA), 2.5,10,20,200,1000 µL of micropipettes (2231300002, Eppendorf, Germany), 5,10,25,50 mL of serological pipettes (S, CAPP, Germany), 100, and 1000 mL of graduated cylinder (Isolab, Germany), 2 mL cryotube (091.11.102, Isolab, Germany), Falcon tubes 15,50 mL (LB.IS.078.02.003, Isolab, Germany),

## 2.3.Instruments

Confocal Laser Scanning Microscope (Zeiss LSM 700, Germany), Weighing Machine (Shimadzu, AUW220D, Japan), Fluorescence Microscope Axio Vert. A1(Zeiss, Germany), Laminar Flow Cabin (Heal Force Class II Biosafety Cabinet A2, China) Inverted Microscope

(Nikon Eclipse, TS100, Netherlands), $CO_2$ incubator (In-Vitro Cell ES NU-5800, NuAire, USA), Low-Speed Centrifuge (Gyrozen 416, Korea), -80°C freezer (New Brunswick,U410, Germany), High-Speed Centrifuge (Sigma Aldrich 3-18KS, Germany), Nano-Drop 2000 (Thermo Fisher Scientific, USA), CFX96 Real-Time PCR (Bio- Rad, C1000 Touch, USA), Thermal Cycler (Biorad Mycycler 1709703, USA), Microplate reader (Bio Tek ELx800, USA), ELISA plate reader (Bio Tek, USA),

**2.4.Cell Lines**

Human Chondrocytes Osteoarthritis (HC-OA) (402OA-05) (Cell Applications. INC. San Diego)

# 3. METHODS

## 3.1.Exosome Isolation

Exosomes were isolated from radish juice via aqueous two-phase system (ATPS). Radish juice was obtained by using blender and centrifuged at 10000 g for 1 hour in order to get rid of pulp. After centrifuge supernatant were collected and filtered with 0.45 μm filter. Then it mixed with DEX-PEG solution with 1:1 ratio. This mixture was centrifugated at 1000 g for 10 minutes. Then 80% of total volume was discarded and %20 of volume was filled with 80% of wash solution (DEX-PEG 50% (v/v), $dH_2O$ 50% (v/v)). Centrifugation step was repeated with same conditions. In the final part, upper phase was discarded and bottom phase were collected. Collected bottom phase was filtered with 0.45 μm and 0.22 μm respectively in hood.

## 3.2.Exosome Characterization and Quantification with Nanoparticle Tracking Assay (NTA) and Zeta Sizer

In order to make quantification and identify the size of the isolated molecule, NTA method was applied. According to manufacturer's protocol, isolated samples were diluted $dH_2O$ with 1:40 ratio. Result was determined with 488 nm laser. For at least five takes, nanoparticles were recorded to the video at 60-second intervals, and NTA software version 3.3.301 was used for the analysis.

## 3.3.Exosome Characterization by using Scanning Electron Microscope (SEM)

Scanning electron microscopy was used to examine isolated exosomes (SEM). Samples were switched around on a glass microscope slide and dried at -20 °C for the entire night. The slide was then covered with a coverslip and analyzed using a SEM after that.

### 3.4.Cell Culture and Cell Passaging

Human Chondrocytes Osteoarthritis (HC-OA) (402OA-05) (Cell Applicaions. INC. San Diego) were culture in Chondrocyte Growth Medium (1 ml MycoZap$^{TM}$ Plus-PR,).Cells were seeded on T25, T75, T150 flasks and they were incubated in Nuaire NU5510/E/G incubator with specific conditions (at 37$^{o}$C, 5%$CO_2$, humidified 80% RH and 95% $O_2$). Medium was changed with fresh medium every other day. When confluency reaches between 70-80% cells were passaged. First of all, trypsin (with 0.05 percent (w/v)) was applied to cells in order to detach them and incubate them at 37$^{o}$C for 5 minutes. Then fresh medium was added to flask with the same amount of trypsin and centrifugated at 300g for 5 minutes. After centrifuge supernatant was discarded and pellet is resuspended with fresh medium. In order to count cell, hemocytometer was used. 10 µl of resuspended cells was put on hemocytometer and counting was done via inverted light microscope.

### 3.5.Cellular Uptake

First of all, HC-OA cells were seeded on 96 well plate with 5x10$^{3}$cells/well concentration. Diluent C and isolated exosome were mixed with 1:1 ratio. Then 4 µl of PKH26 dye were added for each 1 ml of mixture. This mixture was put on the tube which has special column (10 kDa) and centrifuged for 2 minutes at 750 rcf. Dyed exosome was passed through the column and collected. In order to dye cell membrane, Cell Tracker Green (CMFDA) dye was used with 1:1000 ratio. Hoechst dye which is for nucleus was also used with 1:1000 ratio. After 24h from cell seeding process, medium was discarded and replaced with fresh medium which has CMFDA and Hoechst dye with 1:1000 ratio. Cells were incubated for 20 minutes at dark condition. After incubation, prepared medium was discarded and replaced with fresh medium. Then exosomes which were dyed previously, were given to cells. Images were taken each 1$^{st}$, 6$^{th}$, and 12$^{th}$ hours under fluorescence microscope.

### 3.6.Cell Toxicity and Viability

The MTS assay was utilized to analyze Human Chondrocytes Osteoarthritis (HC-OA) viability after exosome treatment. Cells were seeded 5x10$^{3}$ cells/well into 96-well plates and

incubated them for 24 hours at the temperatures and humidity levels described in section 3.4. After 24 hours, DMEM medium was discarded fresh medium which was prepared with different concentration from 25 µg/ml to 200 µg/ml of radish exosome were added to attached cells. Cell viability after exosome treatment was measured using MTS test on days in a row 24, 48, and 72. The MTS solution was added to the PBS-glucose solution to reach the desired final concentration of 10%. The cells were fed a PBS-glucose solution that had been prepared with MTS. The cells were cultured in a PBS-glucose solution for an hour at 37C$^{o}$ in a humidified environment (RH 80%), containing 5% $CO_2$ and 95% (v/v) air. In an ELISA plate reader (Biotek, USA), the absorbance values were measured at 490 nm.

### 3.7. Scratch Assay

Using a scratch assay, it was examined for evidence that radish exosomes influence cell migration and proliferation. First of all, $3x10^5$ cells were seeded on 6-well plates and incubated at 37$^{o}$C for 24 h. After a day, medium was replaced with sterile PBS in order to discard cells after scratch was applied. Then cells were scraped with a sterile 1000 micropipette tip and examined under a light microscope. PBS was discarded and wells were rinsed. Fresh medium which was prepared with optimum dose of radish exosome were given to cells. Control group was given only medium without any exosome. The cells were subsequently cultured under the conditions described in 5.4, which included incubation at 37 °C. Images were taken after 24h and 48 with light microscope (Carl Zeiss Microscopy, LLS, Thornwood, NY, USA). Image analysis software (ImageJ, NIH, Bethesda, MD) was used to compute the closure rate by calculating the ratio of the final distance (Af) between the wound edges to the beginning distance (Ai), then multiplying the result by 100 to get the percentage by which the wound had healed.

Percentage of closure = [(Ai-Af)/Ai] x 100 is the overall formula employed.

### 3.8.Anti-Inflammation Assay

The effect of hydrogen peroxide on radish exosomes under inflammatory conditions is being researched. This was accomplished by seeding $5x10^3$ HC-OA cells per well in 96-well plates and then treating the cells with 1.0 mM of $H_2O_2$, 100 µg/ml Radish exosome, or both $H_2O_2$

and radish exosome for 24 hours at 37°C. At the end of 24 hours, the cell viability assay which is MTS was performed as described in Section 3.6.

### 3.9.RNA Isolation

HC-OA cells were seeded on 6 well plate with $2x10^5$ cells per well. After 24h, cells were treated with 100 µg/ml Radish exosome and control group was left to grow with only fresh medium. After 48h of treatment cells were collected and centrifugated at 300g for 5 minutes. Supernatant was discarded and pellets were dissolved with 400 µL of Trizol and incubated for 5 minutes. After incubation, 100 µL of chloroform was added and incubated 2 minutes. Then, samples were centrifugated at 12,000g for 15 minutes at 4 ºC. After centrifugation, there were three phases. The colorless phase which is on the top was replaced to new tube. Then isopropanol was added to the tube with same amount of upper phase and after 10 minutes of incubation samples were centrifugated 12,000g for 10 minutes at 4 ºC. Supernatant were discarded and pellet was dissolved with %75 of ethanol with twice amount of isopropanol were added previously. After a quick vortex, samples were centrifugates 7,500g for 5 minutes at 4 ºC. Supernatant were discarded and samples were incubated for air dry for 5-10 minutes. Finally, samples were dissolved with 20 µL of RNase free water and concentration of isolated RNA were measured with nanodrop.

### 3.10. Reverse Transcriptase Polymerase Chain Reaction

BioRad iScript™ cDNA Synthesis kit (#1708891, BioRad, USA) was used on order to obtain complementary DNA (cDNA). The master mixture was put together in accordance with the following table. At 37 °C, the PCR reaction ran for 60 minutes. The concentration of cDNA samples was determined using a Nano Drop once the reaction was complete.

Table 3. 1. BioRd iScript cDNA Synthesis kit

| **Component of Kit** | **Volume for each reaction (µL)** |
|---|---|
| iScript Reaction Mix (5x) | 4 |
| iScript Reverse Transcriptase | 1 |
| Nuclease-free water | Variable |
| Template of RNA | Variable |
| Total Volume | 20 |

Table 3. 2. Protocol of Reaction

| | |
|---|---|
| **Priming** | 5 min at 25°C |
| **Reverse Transcription** | 20 min at 46°C |
| **RT inactivation** | 1 min at 95°C |
| **Optional Step** | Hold at 4°C |

### 3.11. Real-Time PCR (qPCR)

In order to examine OA pathogenesis related mRNA expression levels qPCR were applied. After isolation of cDNA, 1µl (for one well) of cDNA, 5 µl (for one well) of QuantiTect SYBR Green PCR Kit (#204145), Rnase free water and 10 mM of each primer (Table 3.4) were mixed for preparing master mix. Final volume was 10 µl for each well. GAPDH was used as a housekeeping gene. The gene expression data was adjusted against their corresponding GAPDH values and shown as fold changes. Protocol setting of qPCR and primer sequences were given below (Table 3.3)

Table 3. 3. Protocol setting of qPCR

| Cycle | Repeat | Step | Duration | Temperature |
|---|---|---|---|---|
| Initial Denaturation | 1 | 1 | 5 mins | 95 °C |
| Denaturation | 42 | 1 | 30 sec | 95 °C |
| Annealing | | 2 | 1 min | 58 °C |
| Extension | | 3 | 30 sec | 72 °C |
| Final Extension | 1 | 1 | 1 min | 95 °C |
| Melte Curve | 110 | 1 | 10 sec | -0.5 °C/cycle |
| Hold | 1 | 1 | Infinite | 4 °C |

Table 3. 4. Sequences of Primers

| Gene | Species | Orientation | Sequence |
|---|---|---|---|
| ACAN | Human | Forward | 5’-TGAGTCCTCAAGCCTCCTGT -3’ |
| | | Reverse | 5’-CCTCTGTCTCCTTGCAGGTC -3’ |
| MMP-13 | Human | Forward | 5’-GAAAAAGTCGCCACGTAAGC-3’ |
| | | Reverse | 5’-CGACAATGAGTCCAGCTCAA-3’ |
| COL2A1 | Human | Forward | 5’-ATTTCAAGGCAATCCTGGTG-3’ |
| | | Reverse | 5’-GGCCTGGATAACCTCTGTGA-3’ |
| MMP-9 | Human | Forward | 5’-GCCACTACTGTGCCTTTGAGTC-3’ |
| | | Reverse | 5’-CCCTCAGAGAATCGCCAGTACT-3’ |
| SOX-9 | Human | Forward | 5'-TTCATGAAATGACCGACGA-3' |
| | | Reverse | 5'-CGCTCTCCTTCTTCAGATCG-3' |
| IL-1β | Human | Forward | 5’-ATGACTTACTGCAGCGGCAAT-3’ |
| | | Reverse | 5’-GTCTTGGAAGCTGCCCTTCA-3’ |
| IL-6 | Human | Forward | 5’-TGCTGGCTAAGCTGCATTCA-3’ |
| | | Reverse | 5’-GGAAATCCTCAAGGCTTCGAA-3’ |
| Col1A1 | Human | Forward | 5’-CCACGCATGAGCGGACCCTAA-3’ |
| | | Reverse | 5’-ATTGGTGGGATGTCTTCGTCTTGG-3’ |

| | | | |
|---|---|---|---|
| GAPDH | Human | Forward | 5'-TTCGACAGTCAGCCGCATCTTCTT-3' |
| | | Reverse | 5'-ACCAAATCCGTTGACTCCGACCTT-3' |

## 3.12. Enzyme-Linked Immunosorbent Assay

### 3.12.1. Human MMP-13

According to manufacturer's protocol (Human MMP-13 ELISA Kit, # EHMMP13, Invitrogen, USA). 100µl from each samples including standards which were prepared previously were added to ELISA with three replicate and incubated for 3 hours at room temperature. After incubation, samples were discarded and washed with 1X wash buffer for 3 times. Then 100 µl of biotin conjugate was added to wells and left for incubation for 1 hour at RT. Solutions were discarded and 100 µl of streptavidin-HRP solutions were added to each well and incubated for 50 minutes. After incubation solution were replaced with 100 µl TMB substrate and incubated for 30 minutes at dark environment just before adding 50 µl of stop solution. Plate was read at 450 nm after 30 minutes of incubation.

### 3.12.2. Human Collagen Type II

According to manufacturer's protocol (Human Collagen Type II ELISA Kit, # MBS263555, Mybiosource, USA), prepared standards and samples were added to ELISA plate with 100 µl of volume. Then incubated at 37°C for 90 minutes. After incubation, solutions were replaced with wash buffer 4 times. 100 µl of Biointylated antibody solution was added to wells and incubated for 37 °C for 60 minutes. Wells were washed 3 times and enzyme working solution was added and incubated at 37°C for 30 minutes. Washing step was repeated for 5 times before adding color reagent and incubated at 37°C up to 30 minutes. Finally, plate was read at 450 nm.

### 3.12.3. Human VEGF

According to manufacturer's protocol (Human VEGF ELISA Kit #KHG0111, Invitrogen, USA), to initiate the experiment, 50 µl of the Incubation Buffer was dispensed into the wells, followed by 100 µl of the standard sample in each well. Fifty microliters of sample and the same volume of Standard diluent buffer were poured into each sample well. For 3 hours, samples were kept at room temperature. The wells were then washed four times with 1X wash buffer to get rid of any remaining protein. The wells were then incubated at RT on a moderate shaker for 1 hour after 100 µl VEGF Biotin Conjugate solution was added. 100 µl Streptavidin-HRP solution was added after the wells were washed four times with 1X wash buffer. After being incubated at RT for 30 minutes. Once more, 1X wash buffer was used to wash the wells four times. After 30 minutes of incubation at room temperature in the dark, wells were treated with 100 µl of Stabilized Chromogen. The substrate reaction was terminated by adding 100 l of stop solution to each well. The absorbance values of the wells were measured with an ELISA plate reader set at 450 nm. Standard values were used to produce a standard curve, which was then used to calculate the wells' individual findings.

### 3.12.4. Human TGF- β

According to manufacturer's protocol (Human TGF-β ELISA Kit #ELHTGFb1, RayBiotech, USA) the components of the human TGF- β1 ELISA kit include a microplate coated with TGF- β1, an HRP-streptavidin concentrate, a standard protein, a 20X wash buffer concentration, a stop solution, a TGF- β detection antibody, a TMB one-step substrate reagent, a diluent, and a plate cover. Samples and standards were each put to their appropriate wells. After 3 hours of incubation at RT on a gentle shaker, the samples were considered ready to be analyzed. Four times, 300 µl of 1X wash buffer were used to thoroughly clean the wells. The 1X biotinylated antibody was added to each well for 1 hour at RT. Following that, 1X wash solution was used to wash the wells four times. After that, 100 µl of Streptavidin was put into each well, and they were left there for 45 minutes. After that, 1X wash buffer was used to clean the wells four times. The wells were filled with 100 µl of a TMB one-step substrate. This substrate was incubated in the wells at RT for 30 minutes with moderate shaking while the room was kept dark. Fifty microliters of stop solution were

added to the wells to terminate the substrate reaction. The absorbance values of the wells were measured with an ELISA plate reader set at 450 nm. Standard values were used to produce a standard curve, which was then used to calculate the wells' individual findings.

### 3.13. Immunocytochemistry Staining (ICC)

HC-OA cells were seeded on six well plate with $2x10^5$ cell/well concentration. Then 100 µg/ml of radish exosome were given to cells control group were changed with only fresh medium. After 2 days medium was discarded and all wells were washed with PBS-T (PBS+0.1 %Tween 20). After washing step, 4% of paraformaldehyde were added and incubated 30 minutes at 4°C. After incubation, all wells were washed with PBS-T 3 times for 5 minutes. Then, %0.1 of Triton-X was prepared and added to wells and incubated at room temperature for 10 minutes. Washing step was repeated with PBS-T and blocking step was done with % 2 of goat serum (incubated at 4°C for 20 minutes). After blocking step, wells were washed with PBS and Collagen type II and Aggrecan antibodies were prepared with 1:200 dilution and given to wells for overnight at 4°C. After 24 hours, washing step was repeated and secondary antibodies were diluted with PBS 1:200 ratio and added to wells and incubated at 4°C in the dark for 2 hours. After 2 hours, washing step was repeated and DAPI (1:1000) were added to wells in order to dye nucleus for 15 minutes at 4C. Final step, by using mounting media slides were closed and images were taken with Confocal Laser Scanning Microscope (Zeiss LSM 700, Germany).

# 4. RESULTS

## 4.1. Exosome Characterization and Quantification with Nanoparticle Tracking Assay (NTA), Zeta Sizer and Scanning Electron Microscope (SEM)

Quantifying exosomes often involves using nanoparticle tracking analysis. Based on the study of Brownian motion, NTA is a nanoparticle characterization approach that allows for the visualization and measurement of suspended particles with sizes ranging from 10 to 1000 nm. Particle size was determined by measuring Brownian motion using Zetasizer. Diffused light was studied by lighting the particles with a laser and then measuring how their intensity changed over time. Furthermore, Scanning Electron Microscope were used to identify size of isolated exosomes. As a result of series of characterization experiments Figure 4.1 indicated that isolated exosome as an average with a mode at 112.8+/-7.2 nm and mean of 110.2+/-0.9 nm. In addition, concentration of isolated exosomes was calculated as 1.80e+09 +/- 7.52e+07 particles/ml.

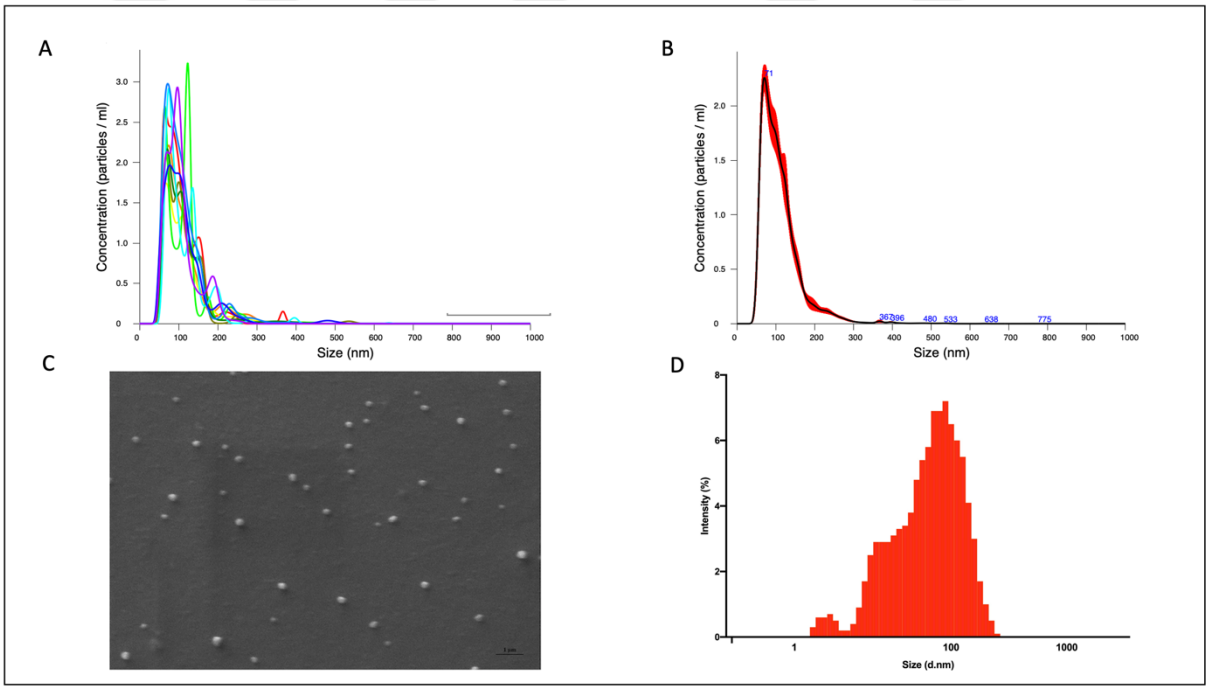


Figure 4. 1. Exosome characterizations based on size and shape. (a) Nanoparticle Tracking Assay (NTA) FTLA concentration/Size graph. (b) Averaged FTLA Concentration/Size graph. (c) Scanning Electron Microscope (SEM) image. (d) Size distribution of *R. sativus* exosome with zeta sizer.

### 4.2.Cell Viability

In order to detect toxicity of radish exosomes and detect optimum dose for HC-OA cells, MTS assay was performed. Different concentrations between 25-200 µg/mL were applied to HC-OA cells and absorbance values were determined for 3 days. As a result of Figure 4.2, 100 µg/mL of radish exosome increase proliferation after 48 h. Low dosages of exosomes up to 75 µg /ml did not induce a significant increase in cell number within 24 hours. However, 100 µg /ml exosome administration caused a considerable rise in cell number during the first day. Exosome concentrations between 25 and 100 µg /ml result in a substantial increase in cell number over a 48-hour period compared to their growth medium-treated samples.

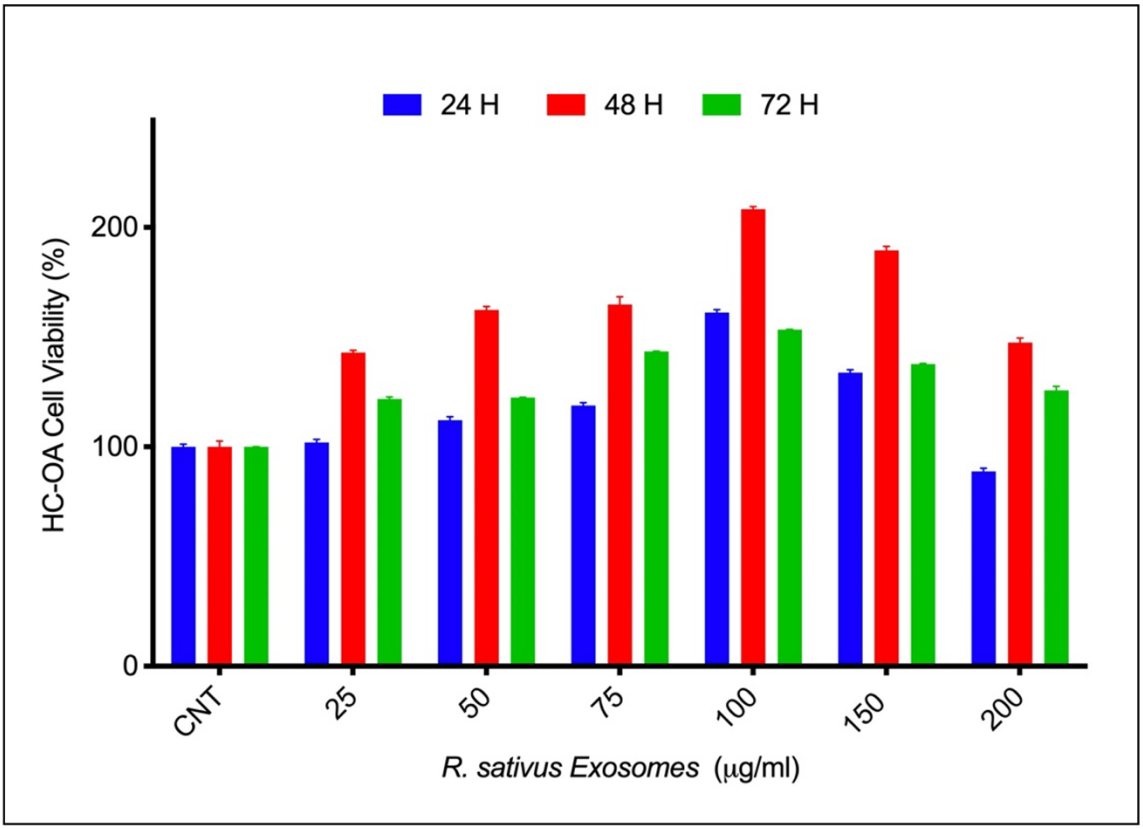


Figure 4. 2. Different doses of *R.sativus* exosomes between 25-200 µg/mL effects on HC-OA cells for 3 days and proliferation percentages compared to control group. The results are the mean standard deviation of three separate experiments.

### 4.3.Cellular Uptake of Exosomes

To find out whether HC-OA cells actually take up exosomes produced by *R. sativus* exosomes and if this is an indication of inter-kingdom communication, researchers used time-dependent cellular uptake to test the activity of exosomes. Exosomes were isolated and then administered to HC-OA cells while stained with the PKH26 dye. Using fluorescent microscopy, it was seen that exosomes being taken up by cells at different times. Based on the findings, HC-OA cells begin internalizing exosomes after 6 hours of treatment, and the concentration of exosomes steadily rises over the next 12 hours (Figure 4.3)

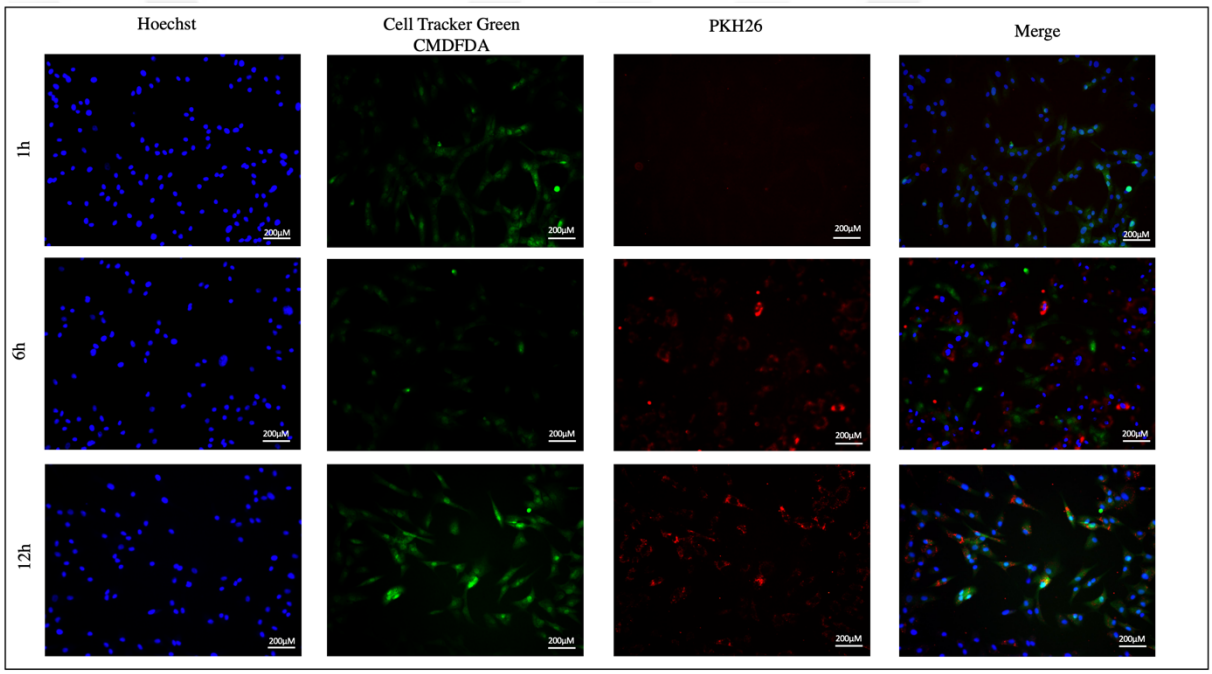


Figure 4. 3. Uptake images HC-OA cells with 100 μg/mL of *R. sativus* exosomes for 1st, 6th and 12th hour (20X magnification)

### 4.4.Anti-Inflammation Assay

Using $H_2O_2$, the anti-inflammatory impact of radish exosomes is assessed. Radish exosomes at a concentration of 100 μg/mL, hydrogen peroxide at a concentration of 1 mM, or a combination of the two are used to treat cells. The MTS test is performed 24 hours following application. Using 100 μg/mL radish exosome treated data as a positive control, a graph of

the percentage of cell viability is generated. Results indicated that 1 mM $H_2O_2$ has an inflammatory impact on HC-OA cells, decreasing their viability by almost thrice and inducing cytotoxicity. In contrast, cells treated with radish exosome and hydrogen peroxide exhibit the same results as the positive control, bringing the cell viability rate back to the control level, indicating that radish exosome might inhibit the inflammatory impact of $H_2O_2$. Radish exosomes have demonstrated anti-inflammatory properties on HC-OA cells.

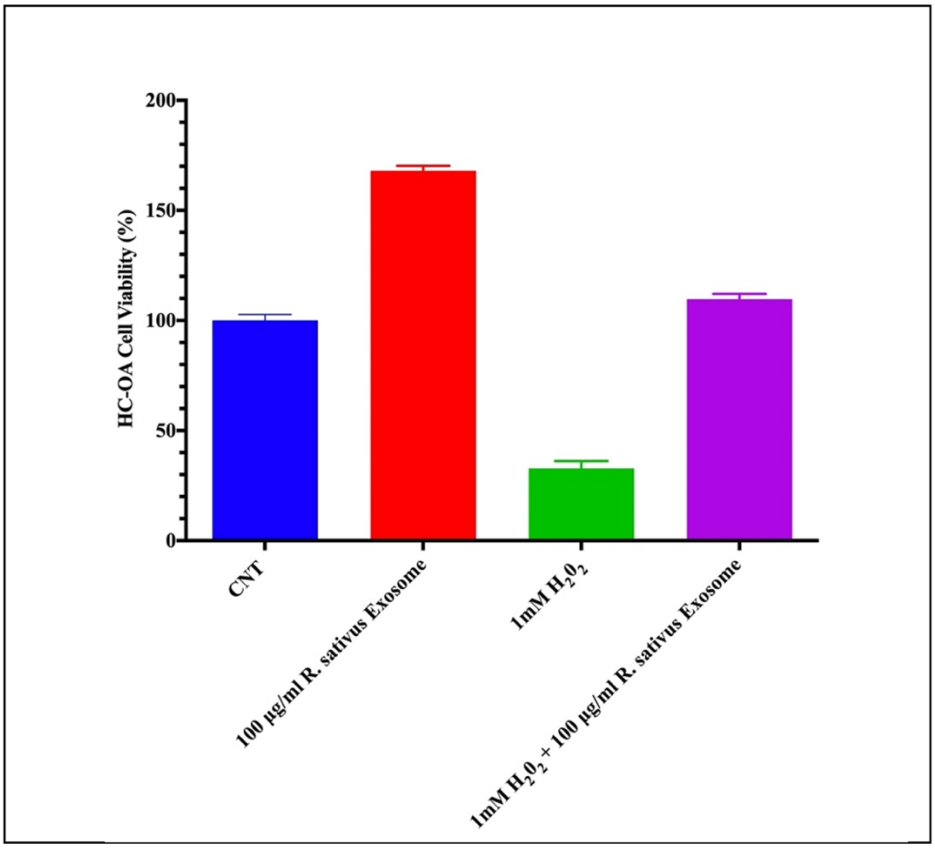


Figure 4. 4. Result of anti-inflammation effect of *R. sativus* exosomes (100 µg/mL) on HC-OA cell line by using 1mM of $H_2O_2$. The data shown is the average standard deviation from three separate studies.

### 4.5. Scratch Assay

Using a scratch experiment, it was examined for evidence that radish exosomes influence cell proliferation. Within 16 hours, the closure rate of HC-OA cells treated with 100 µg/mL of exosomes were 98.712.924 percent, whereas those of control cells treated with growth media were only 52.732.816 percent. There was a statistically significant difference between the control group and radish exosome treatment groups, with the 100 µg/mL treatment.

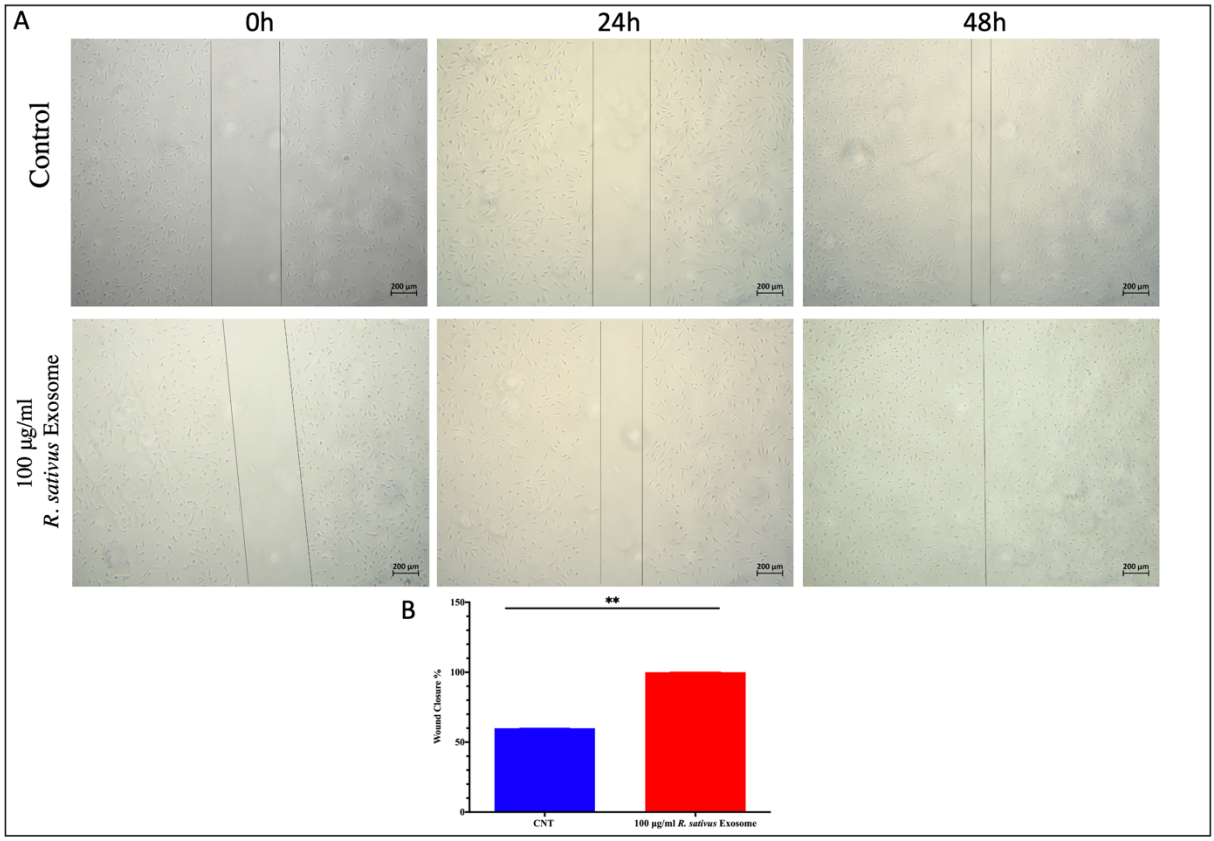


Figure 4. 5. (a) Images of scratch assay first 2 days under light microscope with 200 µm scale bar for control and 100 µg/ml radish exosome treated HC-OA cells. (b) Rate of closure HC-OA cells with radish exosome treated and untreated. Data are indicated with ± SD, *P < 0.05.

Scratch experiment results showed that whereas cells in the control group somewhat healed the scratched area after 48 hours, HC-OA cells treated with radish exosomes were observed to migrate quicker, resulting in a wound closure rate that was about 60% higher.

**4.6.RT-PCR**

To investigate whether radish exosomes have a significant effect on the mechanism underlying their proliferative activity, HC-OA cells were treated with 100 μg/ml radish exosome for 48 hours before RT-PCR analysis to determine the levels of anti-inflammatory action, stimulatory activity of cell proliferation and matrix proteins homeostasis related genes expression. The results of this analysis are presented in the table below. After being treated with radish exosomes at a concentration of 100 μg/ml, HC-OA cells exhibited approximately three folds rise in Col II mRNA expression levels, while ACAN mRNA expression levels decreased almost four-fold. These findings were based on research findings. On the other hand, the levels of gene expression for Col I, IL1β and IL-6 were significantly reduced to half for all of them compared to control group. In addition, matrix metalloproteinases MMP 13 and MMP 9 gene expressions were shown to have decreased by 1.8 and 1.9 respectively following treatment of HC-OA cells with radish exosomes. In conclusion, it was discovered that the amount of mRNA for SOX 9 was notably upregulated by 3.4 times after radish exosome treatment.

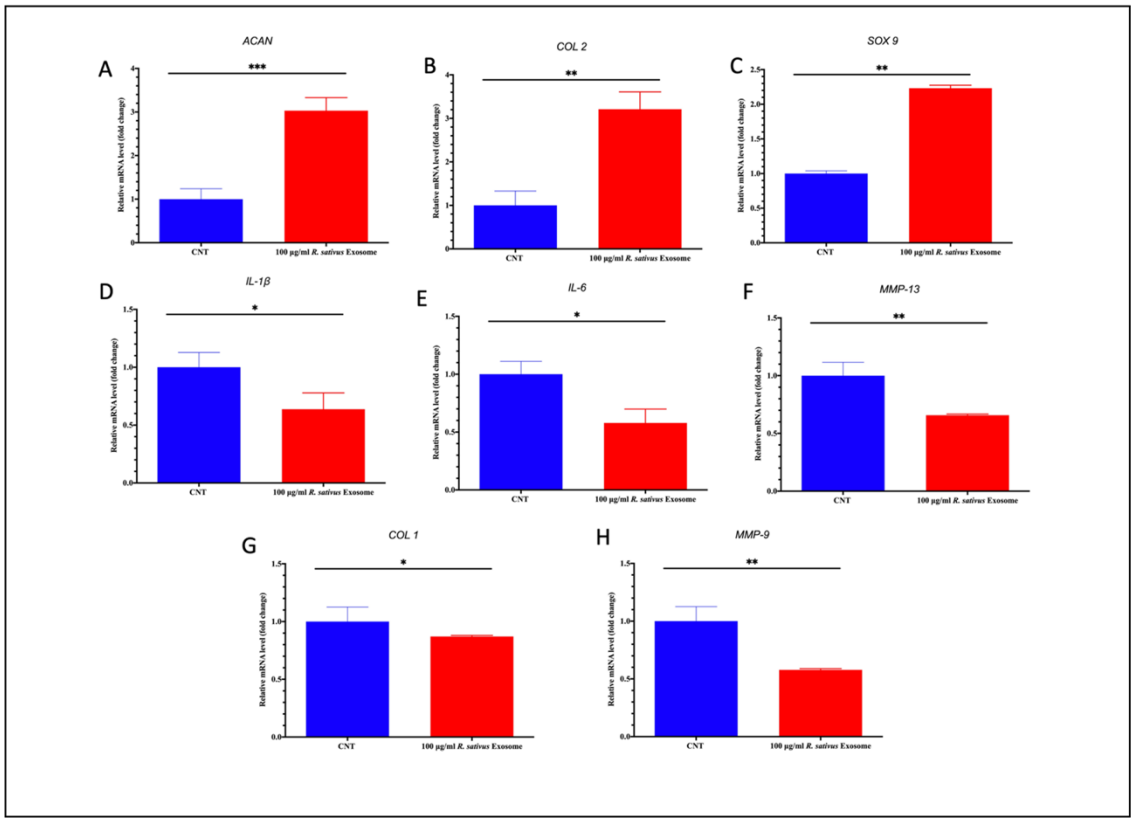


Figure 4. 6. Relative mRNA expression levels by fold change for HC-OA cells after 100 μg/ml of radish exosome treatment. (a) ACAN: Aggrecan, (b) Col 2: Collagen type II, (c) SOX-9: Transcription factor SOX-9. (d) IL1β: Interleukin 1-beta, e IL-6: Interleukin, (f) MMP13: Matrix metalloproteinase-13, (g) Col 1: Collagen type I (h) MMP9: Matrixmetalloproteinase-9, Multiple t tests with two-tails were used to analyze the results. Data are indicated with SEM, n=6, and *P0.05

## 4.7.Enzyme-Linked Immunosorbent Assay

### 4.7.1. Human Collagen type II

Human Col II ELISA was used to assess Col II levels on HC-OA cells following exosome treatment. When treated with 100 µg/ml of radish exosomes, the Col II expression level increased almost 100 percent, as shown by the test findings.

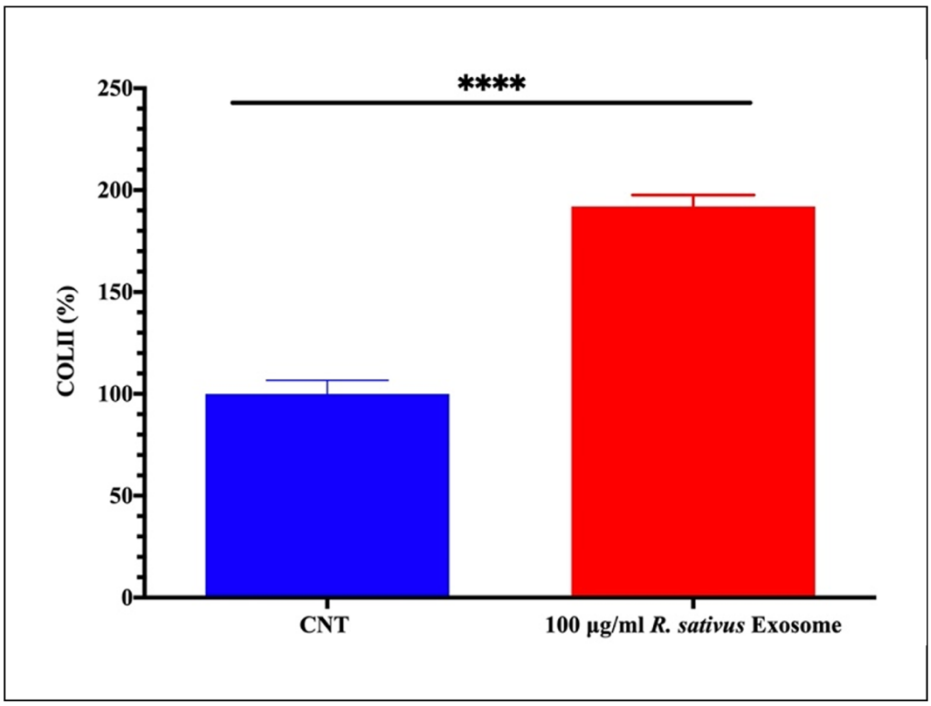


Figure 4. 7. Col II expression levels of HC-OA cells. Multiple t tests with two-tails were used to analyze the results. The data are shown with SEM, n=3, *P0.05 and **P0.01

### 4.7.2. Human MMP-13

The effects of radish exosomes for MMP-13 levels on HC-OA cells were measured using an enzyme-linked immunosorbent assay (ELISA) in humans. Assay findings showed that treatment with radish exosome resulted in a substantial decrease in MMP-13 expression levels.

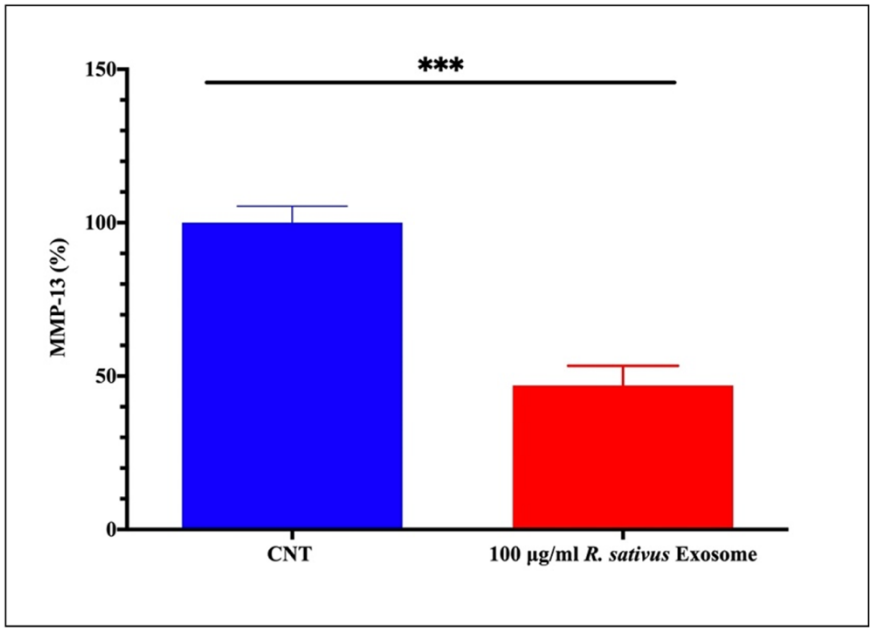


Figure 4. 8. MMP-13 expression levels of HC-OA cells. Multiple t tests with two-tails were used to analyze the results. The data are shown with SEM, n=3, *P0.05 and **P0.01

### 4.7.3. Human TGF- β1

Human transforming growth factor beta 1 enzyme-linked immunosorbent assays were used to measure TGF-β1 in HC-OA cells treated with radish exosomes. The findings of the experiment showed that TGF-β1 expression was dramatically increased in HC-OA cells. TGF-β1 expression was increased more than 100 percent.

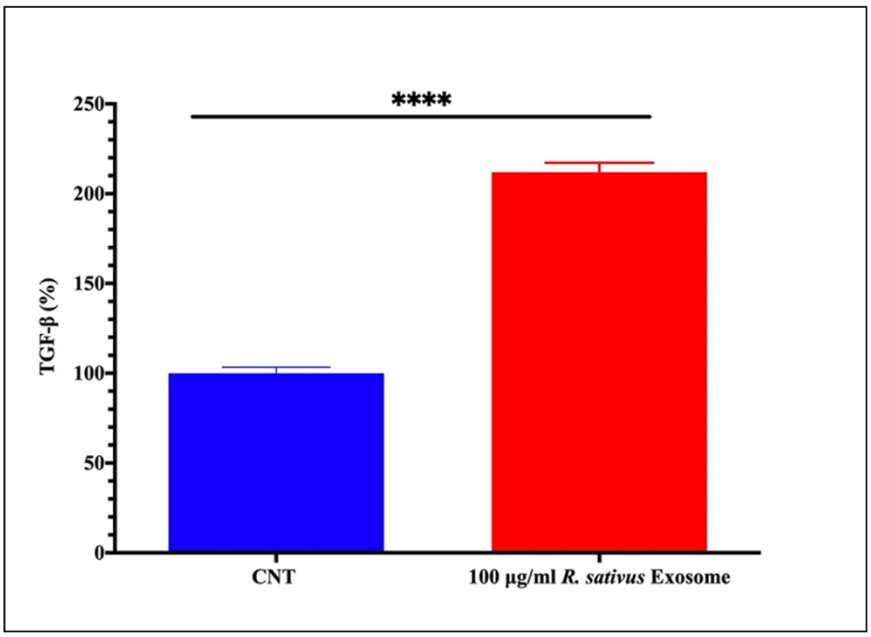


Figure 4. 9. TGF-β1 expression levels of HC-OA cells. Multiple t tests with two-tails were used to analyze the results. The data are shown with SEM, n=3, *P0.05 and **P0.01

### 4.7.4. Human VEGF

Human VEGF ELISA was done to determine VEGF levels on HC-OA cells after application of radish exosomes. After application of 100 µg/ml of radish exosomes VEGF levels on HC-OA cells considerably increased. VEGF gene expression level were increased almost two-fold compared to control group.

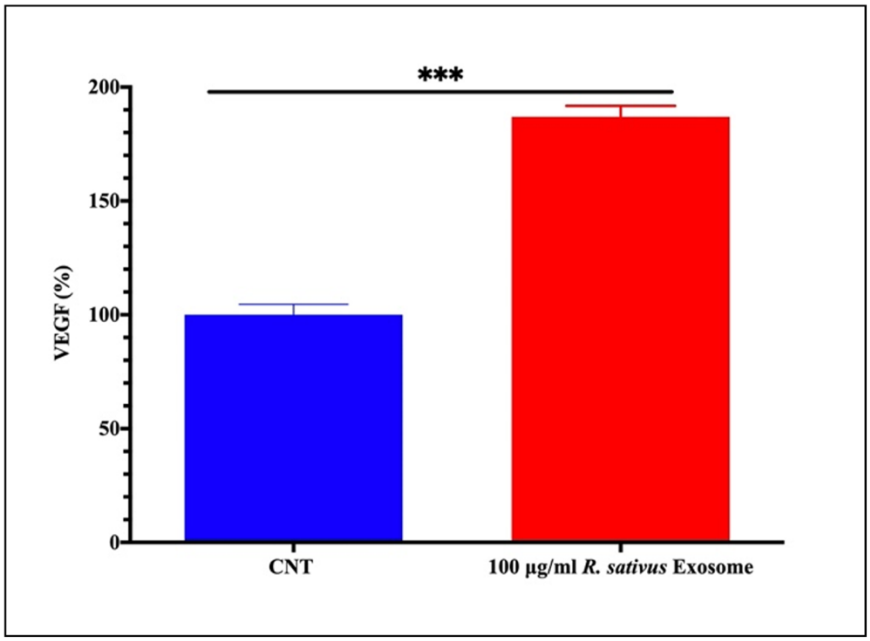


Figure 4. 10. VEGF expression levels of HC-OA cells. Multiple t tests with two-tails were used to analyze the results. The data are shown with SEM, n=3, *P0.05 and **P0.01

## 4.8.Immunocytochemistry

In order to identify and quantify protein expression levels on cells, immunocytochemistry analysis was utilized. Aggrecan (ACAN) and Col II which have great importance for cartilage formation and strength therefore these proteins were examined by using ICC. According to confocal images of ICC, following treatment Col II and ACAN protein expression levels in HC-OA cells increased.

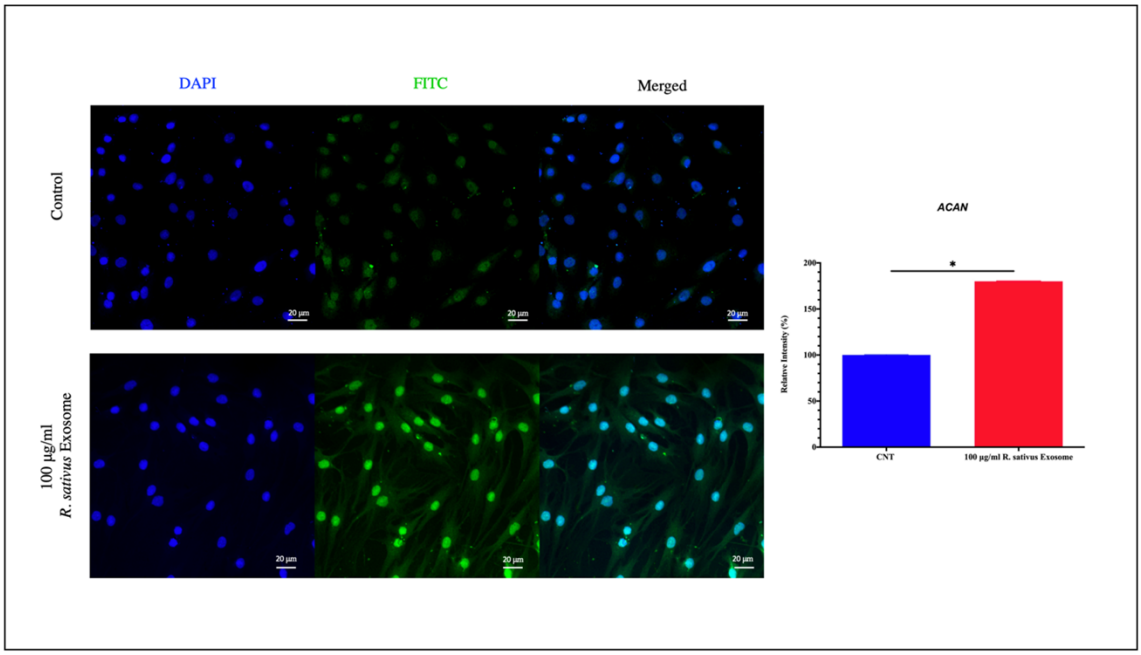


Figure 4. 11. ICC quantification and location of ACAN protein expression of HC-OA cells by confocal microscopy. All results were analyzed by two tailed multiple t tests. Data are shown with ± SD, n=3.

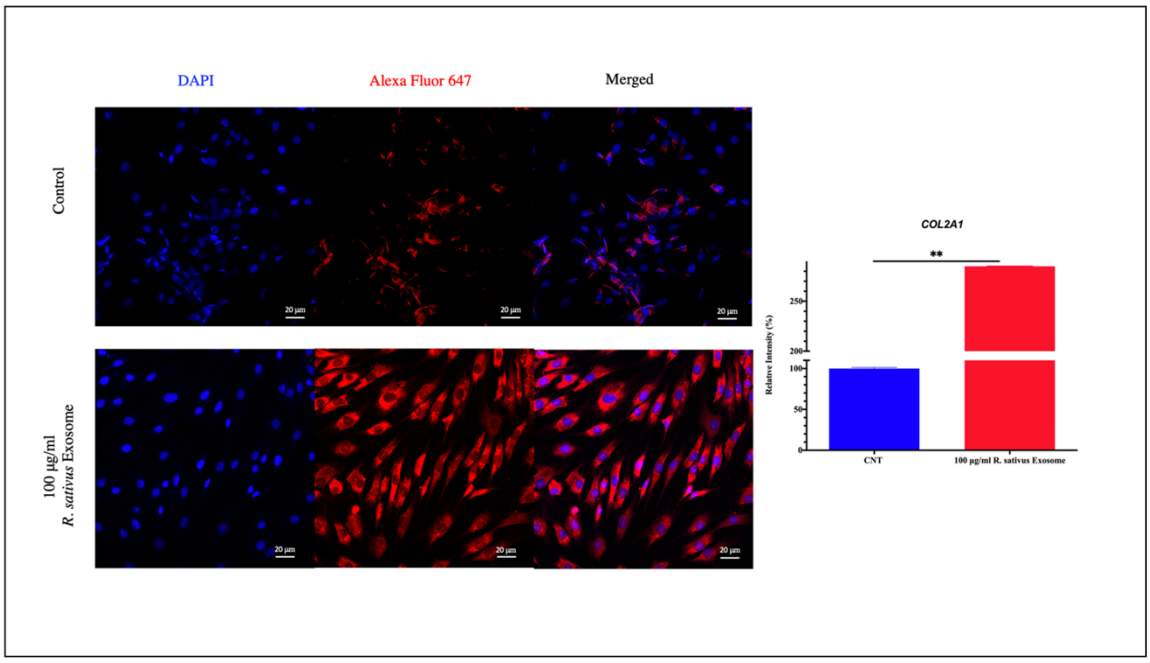


Figure 4. 12. ICC quantification and location of COL2A1 protein expression of HC-OA cells by confocal microscopy. All results were analyzed by two tailed multiple t tests. Data are shown with ± SD, n=3

# 5. DISCUSSION

Osteoarthritis, often known as OA, is a degenerative joint disease that does not cause inflammation and primarily attacks the cartilage that is found within all components of the joint. Both clinical and radiographic data are required to make a definitive diagnosis of osteoarthritis. The risk of developing this condition rises with age, and the peak incidence occurs in obese adults around the age of 40. When compared to adults over the age of 65, individuals under the age of 30 have an overall incidence rate of OA that is around 1%, while those over the age of 65 have an incidence rate that is approximately 70-80%. According to statistics provided by the World Health Organization, around 25 percent of persons over the age of 65 suffer with discomfort and a loss of function related with this statistic [198].

Non-pharmacological therapies, pharmacological treatments, intra-articular treatments, and surgical treatments were the therapy modalities that were categorized and grouped together. With the advancements in medicine that have been made in recent years, it is now feasible to lessen symptoms such as pain and impairment, as well as to improve functioning and enhance the range of motion.

Treatment for osteoarthritis should now be oriented at minimizing intra-articular stress in a way that protects all joint tissues. In the past, the preservation of articular cartilage was the primary focus of treatment for OA.

Exosomes are nano-sized extracellular vesicles that are produced by cells. They include nucleic acids, proteins, lipids, and other bioactive compounds, and they are thought to have a part in both physiological and pathological processes that occur within the body. Exosomes' endogeneity and variability set them apart from synthetic carriers like liposomes and nanoparticles. confer substantial and distinct benefits in the detection and treatment of illness. According to their size and formation mechanisms, these extracellular vesicles differ [199].

Nanoparticle Tracking Assay (NTA), Scanning electron microscope (SEM) are two techniques that are needed to be performed in order to characterize isolated nanovesicles and called them exosomes based on MISEV [200]. In this study *R. sativus* exosomes were isolated and characterization methods were applied based on its size and shape. In order to quantify isolated exosomes NTA were utilized and total concentration of exosomes were

calculated. According to literature exosome size should be between 50-200 nm. As it can be seen in Figure 4.1, the average exosome size as measured by NTA remained within the exosome size limitations. Furthermore, isolated exosomes were examined under SEM and its intended size were indicated (Figure 4.1.f). Consistent result for size of isolated exosomes were observed via zetasizer. Exosome uptake were also demonstrated in order to provide knowledge communication (cross kingdom) between exosomes and HC-OA cells. Exosomes were visible after 6h and they were increasing visible within cells after 12 hours.

It is already common knowledge that inflammation may be seen in OA joints many years before substantial radiographic change appears. The use of sensitive imaging techniques in conjunction with direct arthroscopic visualization has led researchers to the conclusion that osteoarthritis (OA) is already an inflammatory disease even in the early stages of the condition, before any apparent cartilage deterioration has taken place. Inflammation is a main risk factor for advancement of cartilage loss and signs and symptoms of illness, such as joint pain, swelling, and stiffness, which are hallmarks of synovitis [201]. The reason that OA is inflammatory disease, anti-inflammation effect of isolated exosomes was examined. As it can be seen from Figure, 4.3 *R. sativus* exosomes has anti- inflammation effect on HC-OA cells.

In order to observe optimum dose for cell viability and measure toxicity MTS assay were applied and 100 µl with 24h of treatment was chosen for further experiments. The scratch wound experiment was utilized to determine the effect *R. sativus* exosomes on chondrocyte migration which is one of the most important features for OA treatment. Figure 4.5 indicated that after 24 hours of *R. sativus* exosomes application, wound was almost completely closed (90%) compared to control group which has no treatment. Scratch wound experiments revealed that *R. sativus* exosomes substantially increased the motility of chondrocytes.

Matrix metalloproteinases (MMPs) are well recognized as the primary proteases responsible for ECM breakdown. The extracellular matrix (ECM) of cartilage is mostly made up of proteoglycans like aggrecan and smaller proteoglycans like decorin, fibronectin, etc., as well as collagens such fibrillar collagen type II and smaller collagens. Since they represent the rate-limiting step in collagen breakdown, the collagenases MMP-1 and MMP-13 play disproportionately large roles in OA. Synovial cells lining joints are principally responsible for MMP-1 production, while chondrocytes in cartilage secrete MMP-13. The matrix degradation role of MMP-13 is dual, since it destroys both collagen and the proteoglycan

molecule aggrecan. Arthritis also causes an upregulation of the production of additional MMP such MMP-9, which break down the joint's non-collagen matrix [202]. When it comes to triggering OA, MMP-13 is a major player. Furthermore, MMP-13 plays a crucial role in OA development and the inflammatory process by activating transcription factor 3 (TCF3), which indirectly impacts MMP13 production by degrading the collagen matrix of cartilage ECM [203]. According to ELISA and qPCR results of this study, MMP-13 was downregulated by study *R. sativus* exosomes both protein and mRNA level expression.

In addition, cartilage specific genes such as collagen types II and aggrecan, were also examined via ELISA and qPCR. The three basic components of articular cartilage's graded tissue structure are chondrocytes, a collagen type II network, and proteoglycan. During the pathological development of osteoarthritis, aggrecan is very important in the tissues' capacity to endure compressive stresses (OA). Exposure of the collagen matrix, which can be broken down by metalloproteases, can occur if aggrecan gradually disappears from cartilage. The decreased synthesis of aggrecan and collagen type II and the increased production of matrix-degrading matrix metalloprotease-13 are both hallmarks of OA [203]. Furthermore, these two main cartilage related genes (collagen type II and aggrecan) expression were identified with immunocytochemistry and both intensities and images indicated that when HC-OA cells treated with *R. sativus* exosomes these levels were increased.

As mentioned before, differentiation of chondrocytes and maturation are regulated by a number of important growth factors and transcription factors, including members of the transforming growth factor β super family. Because of their potential to stimulate matrix production, growth factors have been the subject of substantial research for both the pathophysiology of osteoarthritis and cartilage healing. It has been claimed that pharmacological stimulation of the TGF-β pathway can protect articular cartilage integrity in patients with osteoarthritis. This is because overgrowth of chondrocytes occurs when TGF- signaling is disrupted in cartilage, which in turn cause cartilage degeneration. The result of this study also shows that TGF-β expression levels are increased with *R. sativus* exosomes. It is well agreed upon that the overproduction of inflammatory cytokines plays essential roles in the progression of osteoarthritis. The pro-inflammatory cytokine interleukin-1 beta (IL-1 β) has been found to have a crucial role in osteoarthritis (OA). Matrix metalloproteinases (MMPs) play a critical role in the irreversible degradation of cartilage matrix by digestion of type II collagen, and IL-1 β-induced overexpression of

MMPs, in particular collagenase (MMP-3 and -13), plays a major role in this process. This matrix proteoglycan release from cartilage has damaging effects. In the etiology of cartilage injury and degradation that occurs in arthritis, interleukin-1 beta, often known as IL-1 β, plays a major role. The synovial-derived IL-1β has been linked to the progression of the illness in noninflammatory arthropathies such osteoarthritis (OA). It has been demonstrated that blocking interleukin 6 (IL-6) is useful in treating a variety of musculoskeletal illnesses. The impact of systemic IL-6 suppression on cartilage degradation, a characteristic feature of osteoarthritis (OA), has not yet been explored in OA animal models or in people, despite in vitro and animal studies suggesting that IL-6 is a significant cytokine in the OA pathogenesis. This is despite the fact that in vitro and animal studies have suggested that IL-6 is a major cytokine in the pathogenesis of OA. In the early stages of the illness, large amounts of IL-6 may be observed in the synovial fluid and surrounding tissues of patients with knee osteoarthritis; these levels are also related with the development of knee osteoarthritis following meniscectomy. IL-6 may be produced by a variety of joint tissues, including fibroblast-like synoviocytes, chondrocytes, and subchondral bone osteoblasts. These cells and osteoblasts release large quantities of this cytokine in response to various stimulations, such as mechanical loading. Patients who suffer from knee osteoarthritis often have an infrapatellar fat pad that can increase the release of IL-6 by synoviocytes. [205,206]. As it can be seen from results of anti-inflammation and qPCR, constituents of the radish exosomes have shown an in vitro inhibitory activity against inflammatory molecules.

Moreover, the relevance of the transcription factor Sox9 for the differentiation of chondrocytes, the transcriptional upregulation of Col2a1, and the preservation of the chondrocyte phenotype has been proven by a series of independent lines of evidence. Sox9 is a member of a large family of regulatory molecules that are related to the sex determination factor SRY. These molecules are distinguished by the presence of a 79-amino acid motif known as an SRY-like high mobility group box that is able to bind sequence-specifically to increasement elements in the Col2a1 gene. It has been shown the fact that Sox9 is essential for chondrogenesis to be induced in the adult organism by the fact that there is a remarkable increase in the expression of the Sox9 gene during the early stages of fracture healing in mouse models [207]. PCR studies revealed that exosome application significantly increases Sox9 mRNA expression in HC-OA cells which correlates with increased levels of Col2a1 expression.

Exosomes are popular area for many diseases, plant derived exosomes which are also the subject of rapidly increasing studies in this context, are new area for researches. The majority of the earlier studies in the field of osteoarthritis had initially concentrated on inflammation mechanism and cartilage development signaling.

Over the past decade, researchers have investigated mesenchymal stem cells (MSCs) as a potential novel therapy for osteoarthritis (OA). In regenerative medicine, exosomes generated from mesenchymal stem cells have received a lot of attention because of their potential to heal damaged tissues. Direct cell-cell contact and the secretion of soluble molecules both contribute to MSCs' ability to restore tissue homeostasis. Downregulating inflammatory signals in osteoarthritic cartilage was observed by the research that made with exosomes of MSC. However, there are some limitations such as, despite the extensive research demonstrating the efficacy of MSCs in repairing damaged joints and treating OA, direct cell transplantation has a number of drawbacks. These include poor cell survival following injection, an inability to predict long-term improvements in cell behavior and cell-cell interactions, and a lack of a sufficient cell storage bank to allow for on-demand treatment [208]. According to the findings of our research, the anti-inflammatory impact of exosomes obtained from plants is comparable to that of exosomes derived from mammals.

Despite this, current research into the molecular mechanisms of osteoarthritis is continuing to grow beyond the scope of these studies. The findings of this study have shed light on the fact that exosomes generated from *R. sativus* enhance ECM synthesis, and have anti-inflammation qualities.

# 6. CONCLUSION

In conclusion, exosomes generated from *R. sativus* accelerate the proliferation of cells which are degraded from osteoarthritis. In particular, these exosomes stimulate the pro-inflammatory and pro-degradative context capabilities of HC-OA cells through the induced through IL-1β stimulation. Additionally, they increase the stimulation of the TGF-β pathway can protect articular cartilage integrity. When treated with exosomes, HC-OA cells exhibit greater levels of proliferative qualities, which can also be ascribed to increased amounts of extracellular matrix (ECM) proteins. Exosomes generated from *R. sativus* have been shown to exhibit anti-inflammation characteristics, in addition to increasing cartilage regeneration, enhancing ECM proteins. These findings suggest that the exosomes produced by *R. sativus* might be useful in the treatment of osteoarthritis. However, further research is required in order to determine the impact that *R. sativus* exosomes have on osteoarthritis in both in vitro and in vivo settings.

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