

AMERICAN UNIVERSITY OF BEIRUT

MAINTAINING THE CARTILAGE PHENOTYPE OF LATE
PASSAGE CHONDROCYTES USING SALIDROSIDE, TGF- β
AND SULFATED ALGINATE FOR CARTILAGE TISSUE
ENGINEERING APPLICATIONS

by
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ABSTRACT

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Title: Maintaining the Cartilage Phenotype of Late Passage Chondrocytes Using Salidroside, TGF- β and Sulfated Alginate for Cartilage Tissue Engineering Applications

Cartilage self-repair following injury or osteoarthritis is limited due to its low regenerative capacity, avascular and aneural nature. Therefore, a promising treatment of cartilage injury and disease may involve engineering cartilage tissue in vitro by combining cells (e.g. chondrocytes), scaffolds (e.g. alginate sulfate (AlgSulf)), and signals (e.g. Salidroside and TGF- β). Autologous chondrocyte transplantation (ACT) is one of the most commonly used techniques for the treatment of cartilage defects. In ACT, chondrocytes are expanded in vitro till passage 3 or 4 and are then injected in the defect site. However, late passage chondrocytes undergo a process called dedifferentiation whereby cells lose their phenotype assuming a fibroblastic phenotype, thereby secreting type I collagen (Col1) instead of type II collagen (Col2) among other key changes. We have previously shown that chondrocytes encapsulated in three-dimensional (3D) biomimetic sulfated polysaccharides such as AlgSulf exhibit increased proliferation while maintaining their cartilage phenotype. The effects of alginate sulfate on cell growth and matrix deposition may be linked to enhanced binding of growth factors such as TGF- β . Moreover, Salidroside was found to upregulate the expression of cartilage specific genes such as Col2, aggrecan (Acan) and Sox9, while down-regulating the expression of the fibroblastic gene Col1 by acting on the TGF- β /Smad3 signalling pathway. This thesis aims to study the effect of treatment of P2 and P4 chondrocytes seeded on AlgSulf 2D films with Salidroside, TGF- β and their combination. Therefore, P2 and P4 chondrocytes were seeded on 2D films of AlgSulf of three different degrees of sulfation (DS=1, 1.5 and 2), in the presence or absence of Salidroside, TGF- β and their combination, and then, cell viability, proliferation, phenotype, and collagen secretion were assessed. Trypan Blue was used to study cell proliferation, while a live/dead assay was performed to assess cell viability. Quantitative real time PCR and Western Blot were also performed to evaluate the expression and synthesis of chondrogenic markers. The proliferation assay showed that a combination of Salidroside and TGF- β on AlgSulf films of DS=2 (AlgSulf2.0) resulted in the highest chondrocyte proliferation compared to all other treatments ($p < 0.0001$). Under this same treatment, chondrocytes exhibited a round morphology when observed using Ethidium homodimer and Calcein AM, and displayed the highest levels of Col2A1, Acan and Sox9, while down-regulating the expression of Col1 and MMP13 ($p < 0.0001$). The Western Blot also showed a prominent expression of Col2 and downregulation of Col1 when tested on P2 chondrocytes subjected to Salidroside on AlgSulf2.0. Therefore, this work shows that the treatment of chondrocytes seeded on AlgSulf2.0 films with a combination of Salidroside and TGF- β significantly promoted

the proliferation of late passage chondrocytes while preserving their phenotype. The results of the current study may therefore be used in cartilage engineering applications.

Keywords: osteoarthritis - cartilage tissue engineering - Salidroside - sulfated glycosaminoglycans - TGF- β - collagen type II - alginate sulfate

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CHAPTER 1

INTRODUCTION

1.1 Definition of Osteoarthritis

Osteoarthritis (OA) is one of the most widespread diseases globally affecting around 600 million people.¹ OA affects various joints such as the hip, knee, hand, and ankle. The frequency of OA has surged, partially due to longer lifespans and higher overall body weights. A recent study in the United States has shown that the prevalence of OA in knee joints has more than doubled since the 1950s. It is also estimated that by 2032, the prevalence of OA will increase from 26.6% to 29.5%.^{2, 4}

1.1.1 Risk Factors

Some of the most encountered risk factors of OA include age, obesity, muscle weakness, joint instability, female gender, and knee injuries.² In fact, aging is widely known as one of the primary risk factors for osteoarthritis (OA). Besides the increasing exposure to various risk factors with time, age-related molecular and biological variations could significantly affect joint structure disorganization. Recently, there has been multiple impending pathological processes that highlight the effect of aging on the start and progress of OA.⁴ The cellular senescence-associated secretory phenotype was found in the degenerative articular cartilage and synovial joint tissue in OA progression.^{5,6} Besides, the dysfunction of the mitochondria due to aging-related factors, which leads to oxidative stress following the excessive accumulation of reactive oxygen species and imbalanced energy metabolism in chondrocytes, could trigger the apoptosis of chondrocytes and the destruction of cartilage.⁷ In addition, inflammation in the synovial

joint damages the extracellular matrix (ECM) of the articular cartilage, hence inducing OA.

Besides, obesity expressively increases the risk of developing knee and hip osteoarthritis (OA). It is a primary risk factor for knee OA because of the excessive weight applying high mechanical stress on knee joints, thus weakening the articular cartilage and ligaments, ultimately leading to OA. Obesity also increases the risk of developing OA in non-weight-bearing joints such as the hands.⁸ In fact, adipocytes release cytokines which are known as adipokines (such as leptin, omentin, resistin, visfatin, adiponectin, and retinol-binding protein), and have been found to be related to the initiation and development of OA.^{9,10} Moreover, cytokines like TNF- α , IL-6, IL-1, and IL-8 cause joint inflammation, leading to the destruction of the ECM and the degeneration of the cartilage.¹¹

Osteoarthritis (OA) affects over 600 million people globally, with a higher occurrence among women compared to men.¹² Women were found to be more prone to the onset and progression of OA due to the decrease in sex hormone levels during menopause.¹³ Furthermore, in contrast to male OA patients, female patients are more disposed to suffer from joint inflammation, thinner articular cartilage, greater clinical pain, and more severe joint movement problems.¹⁴

Additionally, knee injuries are an important risk factor for developing knee osteoarthritis (OA). Post-traumatic OA can happen in joints formerly injured. Research proves that injured joints are five times more prone to develop OA than unharmed joints.¹⁵ Clinical statistics in the U.S show that post-traumatic OA plays a role in approximately 12-42% of OA cases, depending on age, with the actual percentage possibly being

higher.¹⁶ Joint trauma has been shown to provoke significant changes in gene expression on different levels of the knee joint. Besides, excessive joint loading because of sports can higher the risk of OA. Professional athletes have a higher incidence rate of early OA in their knee joints, in contrast to non-professional athletes and the non-athletic people.¹⁷

1.1.2 Clinical Symptoms of OA

OA involves several pathophysiological processes that result from a dysregulation in the role of cytokines and growth factors, neuropeptides, prostaglandins, reactive oxygen intermediates, cartilage matrix fragments, protease inhibitors, and proteolytic enzymes.³ This dysregulation consequently leads to the degeneration of ligaments, bones, cartilage and synovium that results in an inflammatory response and a central and peripheral nervous system sensitization.³ The main symptoms involve joint pain and stiffness, due to the loss of articular cartilage, attrition of subarticular bone, ligamentous laxity, weakening of periarticular muscles, and synovial distension and inflammation.⁴ OA patients often suffer from enlargement of the bones, as well as inflamed and swollen joints.¹⁸ Some other OA-related changes, comprise marginal osteophytes, physical modifications in the osteochondral tissue, and joint space narrowing.¹⁸ Pain is the most protruding symptom and the main reason patients seek medical care. Pain usually designates inflammation, tissue damage, or nervous system disorders.¹⁹ Since blood vessels and nerves are absent in the articular cartilage, OA pain can happen both before and after the damage of the cartilage becomes detectable on imaging, proposing that cartilage destruction is not the sole cause of OA pain.²⁰ However, OA pain is believed to be related to synovitis, bone modifications in subchondral bone, marrow wounds, osteophyte development, and ligament lesions all containing multiple sensory nerves.²¹

1.1.3 Pathogenesis of OA

The pathogenesis of OA has been the center of numerous studies.²² Even though its risk factors were identified, and the physiological changes of the synovial joint caused by OA are well known, the pathological mechanisms of the start and progression of OA are still very complex.

Multiple studies showed that subchondral bone sclerosis is one of the primary causes behind aging-related OA and that the remodeling of the abnormal bone, linked to dysregulation of osteoblasts and osteoclasts is primarily responsible for OA onset and its progression.^{23,24} Higher subchondral bone remodeling and porosity, lower bone density, and bone mineralization along with unbalanced matrix organization, were recognized in the early stage of OA.^{25,26} These modifications at the level of the subchondral bone were found to be occurring simultaneously with the early breakdown of articular cartilage.^{27,28,29} Besides, the late stage of OA revealed architectural modifications of the subchondral bone categorized by a decrease of bone remodeling and improved subchondral bone densification resulting in sclerosis.^{30,31}

In addition to the dysregulation of the subchondral bone, the synovium tissue also showed important modifications at the early stage of OA, even before the cartilage breakdown takes place.³² At the beginning of OA, histological modifications of the synovium consist of the hypertrophy of the synovial lining and hyperplasia, amplified angiogenesis, a reduced level of synovial inflammation and synovial fibrosis.^{33,34} Synovitis accompanied by a high level of macrophages was detected at the last phase of OA.³⁵ Low-grade synovial inflammation was spotted in more than half of OA patients at the early and late phases of the disease.³⁶ Hence, it is considered that the pro-

inflammatory factors that the synovial tissue releases, induce the breakdown of the articular cartilage's ECM.³⁷

Obesity plays a key role in the onset and progression of OA but that is not solely due to the excessive body weight loading onto knee joints.³⁸ For instance, the pathogenesis comprises a complex network of interactions between tissues and cells. Adipokines that are released by adipose tissue are found to be critical in OA pathogenesis.^{39,40} An experiment was done on a completely fat-free transgenic lipodystrophy (LD) mouse model to prove the contribution of adipose tissues to OA induction and progression. LD mice with a total lack of adipose tissue were found to be resistant to DMM-induced or spontaneous OA. The vulnerability to posttraumatic OA was detected following the implantation of mature fat depots in the LD mice. Hence, it was concluded that the adipose tissue and factors it releases, are able to induce OA lesions.⁴¹

1.2 Articular Cartilage Biology

Articular cartilage covers the surfaces of joints, ensuring almost frictionless movement and absorbing mechanical shock caused by daily activities. It is made up of an ECM and a sole type of specialized cells, the chondrocyte. The articular cartilage tissue lacks blood vessels, lymphatic vessels, and nerves.⁴² Chondrocytes differ in shape and metabolic activity based on their location within the cartilage tissue. The ECM is structured into four distinct zones going from the surface and reaching the subchondral bone: the superficial zone, transitional zone, deep zone, and calcified cartilage zone.⁴³ The superficial zone includes a thin, acellular layer of collagen fibrils. Beneath this layer, ellipsoid-shaped chondrocytes are arranged parallel to the tissue surface. This zone is

characterized by a higher tensile strength than the deeper layers, which enables it to resist high shear forces. The dense collagen layer also plays the role of a barrier, avoiding the outflow of cartilage molecules and the access to antibodies and cytokines. Disorder of this collagen layer is one of the key factors provoking induced cartilage breakdown.⁴⁴ Chondrocytes in the superficial zone synthesize various types and quantities of matrix proteins in contrast to deeper layers, including superficial zone protein (SZP) which plays a major role in cartilage lubrication.⁴⁵

The transitional zone, which is much thicker than the superficial zone, has a matrix structure and cell shape that is located between the superficial and deep zones. Chondrocytes in this layer have a spheroidal shape and synthesize thicker collagen fibrils and a larger quantity of proteoglycans than those in the superficial zone. As for the deep layer, it encompasses the highest amount of proteoglycans and the thickest collagen fibrils. Chondrocytes in this zone are known to be spheroidal and are often arranged in columns perpendicular to the cartilage surface. The calcified cartilage zone, which is present between the deep zone and the subchondral bone, plays the role of a physical barrier that separates the cartilage from the bone. The cellular microenvironment considerably influences the functions of cells. In chondrocytes, many factors have an impact over migration, proliferation, and the production and degradation of the ECM. Each zone in the cartilage tissue consists of a specific microenvironment for chondrocytes, as illustrated in Figure 1.1. Chondrocytes are implanted in an ECM majorly composed of type II collagen and proteoglycans.⁴² These cells possess membrane receptors called integrins that enable interactions with matrix proteins. In fact, integrin $\alpha 10\beta 1$ binds to GFOGER sequences in collagen⁴³, while the CD44 receptor interacts with chondroitin sulfate and hyaluronic acid. Oxygen levels within the cartilage are different;

they range from 7 to 10% at the superficial zone to 0.1% next to the subchondral bone⁴⁴. Mechanical stimulation also varies in type and strength through the cartilage layers.⁴⁵ In stem cells, the microenvironment is subject to important modifications during chondrogenesis. Originally, mesenchymal stem cells (MSCs) synthesize fibronectin, accompanied by an amplified expression of type I collagen, which improves cell motility and results in condensation, which forms an primordial step in MSC chondrogenesis. As differentiation continues, cells lower fibronectin expression and substitute type I collagen with type II collagen and aggrecan.⁴⁶

Cellular modifications in multiple cell types in the synovial joint tissue affected by osteoarthritis (OA) have been the center of attention of multiple studies.⁴⁷ Even though interactions between different tissues are important for OA's development; the breakdown of articular cartilage is a primary characteristic in most cases of OA, especially in the knee. Chondrocytes, the only cell type present in articular cartilage, suffer from an amplified cell death in degenerative cartilage in contrast to normal cartilage.⁴³ For instance, apoptosis is significantly higher in OA-affected chondrocytes during both early and late phases.⁴⁸ Studies have identified various triggers for chondrocyte apoptosis such as death receptors (e.g., TNFR, FAS), cytokines (e.g., IL-1 β , TNF- α), exposure to ultraviolet radiation, and abnormal mechanical stress.⁴⁹ There are three particular pathways promoting chondrocyte apoptosis in OA: the death receptor pathway, the mitochondrial-mediated caspase-dependent pathway, and the ER stress-induced unfolded protein response mechanism.

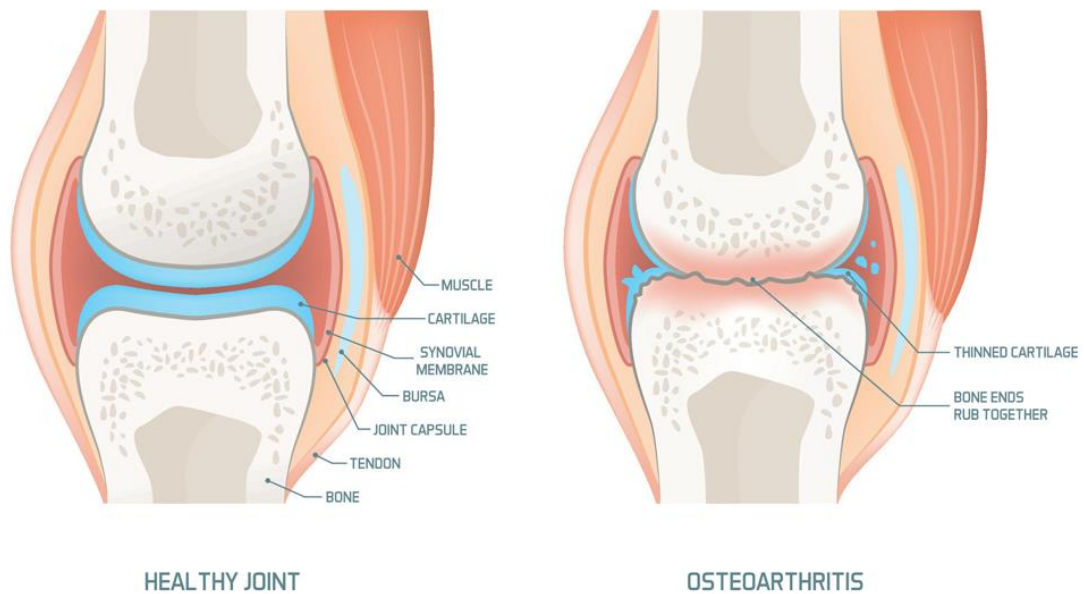


Figure 1: Comparison between the normal structure of articular cartilage with its structure in OA affected patients⁸⁷ (reproduced from Healthdirect, 2024).

The superficial layer of articular cartilage consists of 10 to 20% of its total thickness, with flat, densely packed chondrocytes, while the middle and deep layers are made up of rounder, sparser, and larger chondrocytes that are derived from the chondrocytes present in the outermost layer.⁵⁰ OA is comprised of an amplified production of enzymes that break down the ECM and the destruction of articular cartilage. This abnormal hypertrophic differentiation of chondrocytes plays a key role in the progression of OA.⁵¹ Multiple systemic and local factors, including growth hormone, parathyroid hormone, Indian hedgehog, insulin-like growth factor, fibroblast growth factor, transforming growth factor families, RUNX2, Wnt signaling pathways, and MEF2 transcription factors, were found to regulate chondrocyte hypertrophy and function.^{52,53}

Recent studies have highlighted the critical role of metabolism in preserving energy balance in articular chondrocytes, with metabolic abnormalities leading to the occurrence of OA.⁵⁴ Accelerated catabolism in OA cartilage causes ECM degradation to surpass

synthesis. Inflammation in the synovial membrane and abnormal blood vessel invasion result in a hypoxic stress, autophagy, and oxidative stress among chondrocytes of the synovial membrane further inducing OA pathogenesis.⁵⁴

1.3 OA Treatments

1.3.2 Non-surgical treatments

Currently, no medications can change the progression of osteoarthritis (OA) to prevent long-term disability.⁵⁵ International guidelines advise the use of medications that mainly relieve symptoms and could slow or stop the biological processes that are leading to tissue damage.⁵⁵ Non-pharmacological OA treatments include weight loss since excessive body weight highly promotes the development of osteoarthritis (OA), by adding extra load during daily activities and leading to the increase of the synthesis of enzymes that destroy joints or provoke inflammation.⁵⁶ Moreover, The International Association for the Study of Osteoarthritis (OARSI) states that exercise is a fundamental treatment for OA, suitable for all patients.⁵⁷ Studies have shown that exercise alleviates painful movements and enhances physical function.⁵⁸ Physical therapy also provides OA patients with important benefits, including treatments like therapeutic ultrasound, electrical stimulation, magneto therapy, cryotherapy, phototherapy, hydrotherapy, and thermotherapy.^{59,60,61,62,63} These therapies can considerably reduce OA symptoms such as pain, swelling, and joint mobility issues.⁶²

Currently available pharmacological OA treatments include acetaminophen, cyclooxygenase 2 (COX2) inhibitors, non-steroidal anti-inflammatories, and intra-articular hyaluronic acid or steroid injections. Paracetamol is the first-line analgesic for alleviating OA pain but it is linked to higher risks of gastrointestinal, cardiovascular, and

renal illnesses.⁶⁴ NSAIDs are frequently used for their anti-inflammatory and analgesic properties, along with medications such as ketorolac, and COX-2 inhibitors like ibuprofen, diclofenac, and rofecoxib.⁶⁵ Acetaminophen has also been efficient in treating OA especially when NSAIDs are contraindicated.⁶⁶ Glucocorticoids are another important drug class in treating OA, with their intra-articular injections offering rapid relief from pain and inflammation, such as methylprednisolone acetate, betamethasone sodium phosphate, hexamethonium trenbolone, and dexamethasone.^{67,68,69} They function by exerting anti-inflammatory and immunosuppressive effects, halting the production of pro-inflammatory cytokine, and preventing leukocyte recruitment and activation.^{70,71} As for chondroprotective agents, such as hyaluronic acid, montelukast, glucosamine, and chondroitin sulfate, they aim to inhibit the progression of osteoarthritis.⁷² These drugs function by slowing down inflammation and receptor blockade to effectively modifying the viscoelastic properties of cartilage.⁷³ Moreover, anti-cytokine drugs work on the pro-inflammatory and matrix-breakdown effects of cytokines.⁷⁴ Other approaches consist of anti-TNF- α antibodies and anti-inflammatory enzymes such as IL-4, IL-10, IL-13, and TNF- β .⁷⁵ Nevertheless, these drugs have only shown moderate efficacy, often resulting in substantial pain burden and gastrointestinal side effects for the patients, after chronic administration.²⁵

1.3.3 Surgical treatments

As OA progresses and available treatments and medications are not enough to provide efficient relief, surgical intervention becomes crucial. The main goals of surgery for OA patients are to inhibit the pain, reduce disability, and improve the quality of life. For articular cartilage, surgical procedures consist of scraping such as chondroplasty and

debris removal, repair such as perforation and microfracture, or restoration like autologous chondrocyte graft, allogeneic osteochondral graft, and autologous osteochondral graft.⁷⁶ Joint arthroplasty is used to replace arthritic bone with artificial joints represented by metal implants. It significantly reduces pain, corrects joint distortions, and improves knee function.⁷⁷ However, clinical studies have revealed that some patients do not reach full recovery after surgery and may suffer from difficulty to fully straighten their knee.⁷⁸ Cases such as injuries in the cartilage and ligament, deep vein thrombosis in the lower limbs and infections post-surgery, can affect the success of the operation.⁷⁸ However, this procedure was limited by the infections that occurred following the surgery, and the common need of re-operations due to implants wearing out over time.²⁶ Those implants are also unable to fully achieve the native anatomy or mechanics of the replaced bone.²⁶ Cartilage repair or restoration leads to short-term success with osteochondral autograft. Since the lifespan of prosthetic devices is limited, new treatment modalities have risen to repair the damage OA has caused to the patients. Microfracture of subchondral bone is a minimally invasive arthroscopic surgical procedure that stimulates fibrocartilage growth through the breach of the subchondral bone, releasing osteoprogenitor cells into the defect.²⁷ It has shown a slight improvement in pain and defect filling, and could be used to treat lesions smaller than 2.5 cm², however, the tissue starts degenerating after a short time into fibrous tissue or could result in the replacement by intralesional osteophytes in the treated lesion, by the formation of endochondral and recurrence of disease symptoms and signs.²⁸ Therefore, microfracture remains prescribed only to young and active patients.²⁸ Besides, autologous chondrocyte transplantation (ACT) was another promising option to alleviate the consequences of OA. ACT uses autologous cells for the repair of cartilage defects, and was associated with

more durable clinical results than microfracture.²⁹ In addition, large lesions (greater than 4.5 cm²) and old patients show a higher risk of failure and reoperation after ACT²⁹, with large and multifocal lesions portending degenerative changes on the long term.³⁰ As for osteochondral autograft transfer, it could provide a faster improvement of the clinical results than that obtained with ACT, and could be used to treat lesions from 1-4 cm², but it is significantly restricted by donor-site morbidity, and graft-site mismatch which may not recreate the adequate joint mechanics and it is also often accompanied by a lack of integration with surrounding tissues.²⁷

1.4 Autologous Chondrocyte Transplantation

ACT is the first technique that follows the principle of tissue engineering for the treatment of cartilage illnesses.

1.4.1 Development of ACT

A tissue-engineered cartilage needs to survive the harsh conditions it might get subjected to and is meant to contain the same structurally similar materials of an articular cartilage to withstand normal joint loading. ACT requires the culture of healthy articular cartilage chondrocytes and implanting them through surgery.³² ACT was first established in the late 1980s by Peterson and co-workers and was developed in humans by Brittberg et al. in 1994^{79,80,81}. In this operation, a biopsy of healthy cartilage is extracted from a low-weight-bearing location of the knee. The tissue extracted is enzymatically broken down in the laboratory to separate the cells which are then isolated in a convenient environment to grow efficiently *in vitro* to get a sufficient amount of cells. A periosteal flap of a size that is convenient to cover the defect is collected from the proximal medial tibia and is then stitched to the cartilage's surface with a small gap left for cells injection.

Fibrin glue is then injected in the spaces existing between the stitches, in order to create a water-tight seal that avoids cell leakage. Next, cells are injected to seal the defect site (Figure 2). Although ACT constituted an important improvement in cartilage therapy, it still has lots of limitations.

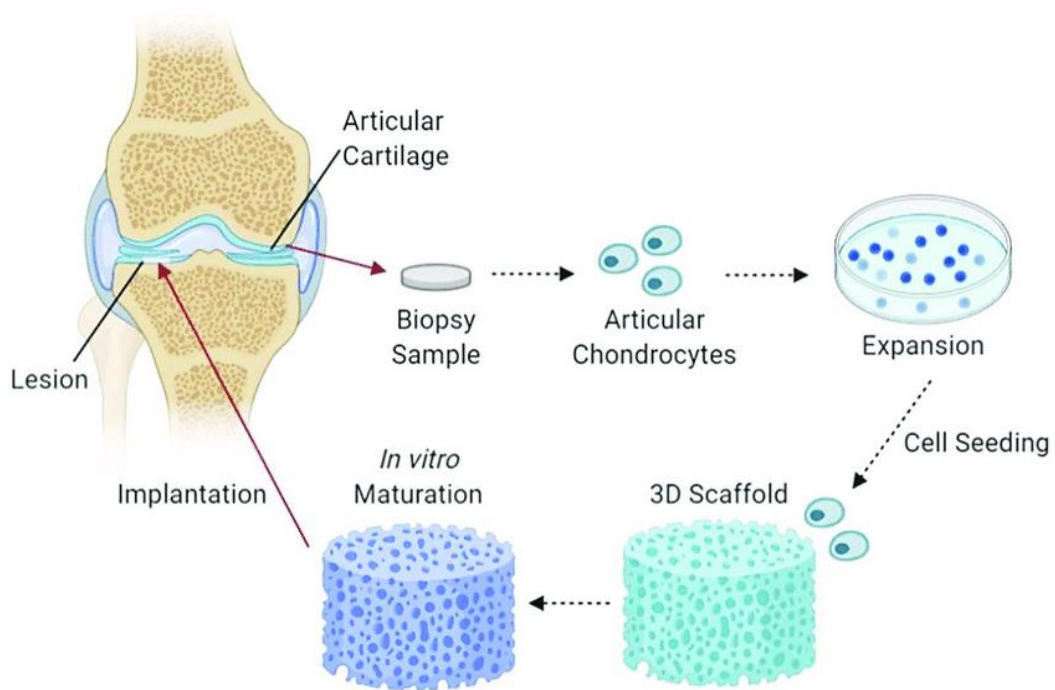


Figure 2: ACT procedure⁸⁸ (reproduced from Pharmaceutics, 2021)

1.4.2 Limitations of ACT

ACT requires the culture of cells in the laboratory as monolayers, which would lead consequently to a loss of the cartilage phenotype, referred to as dedifferentiation. Dedifferentiated chondrocytes show modifications in shape and gene expression, resembling fibroblasts.^{82,83} These cells synthesize type I collagen rather than type II collagen, which leads to the formation of fibrous tissue instead of hyaline cartilage when implanted during ACT operation.⁸⁴ Another important drawback of ACT is its reliance

on articular chondrocytes, which are limited in quantity. Studies are currently focusing on alternative cell sources that can offer the same characteristics and functions as chondrocytes.^{85,86} Stem cells are being recognized as a promising alternative, with multiple research findings showcasing the promotion of chondrogenesis using TGF β .⁸⁷ Materials that can induce cell proliferation while maintaining the original phenotype of chondrocytes would hence be highly important for the ACT application. Therefore, extensive studies are currently undertaken to develop adequate procedures that would prevent the dedifferentiation of chondrocytes upon passaging, through the combination of chondrocytes along with scaffolds and signalling molecules. Scaffolds could be categorized into protein-based polymers (e.g. fibrin, silk...) or carbohydrate based scaffolds (e.g. alginates, agarose...). These hydrogels are important as they induce the growth of chondrocytes, and promote the natural remodelling of ECM, and prevent chondrocytes' dedifferentiation.^{35,36}

1.5 Biomimetic Scaffolds

Sulfated glycosaminoglycans (GAGs) are crucial elements making up the structure of ECM since they largely contribute to the physiological functions and stimulate signalling pathways by interacting with multiple growth factors. For instance, proteoglycans constitute 10 to 20% of the cartilage's ECM, and they get altered to a significant extent by GAGs like chondroitin sulfate (CS), heparin sulfate (HS), and kearatan sulfate (KS).³⁷ Due to limitations related to the high expenses and purification of sulfated GAGs, recently, the synthesis of biomimetic sulfated polysaccharides has been utilized as a reliable model to the engineering of cartilage. Therefore, alginates were considered as biocompatible polysaccharides that could be sulfated and then applied in therapeutic

procedures. The majority of heparin sodium salt (HN) binding growth factors have shown a high affinity to alginate sulfate.³⁸ Since alginate sulfates have demonstrated properties that are similar to those of cartilage ECM's sulfated GAGs, they can be employed to resolve problems involving chondrocyte dedifferentiation, as well as the development, resistance and formation of cartilage tissue after OA onset. Besides, the use of alginate sulfates hydrogel was proved to significantly help the proliferation of chondrocytes.¹⁸

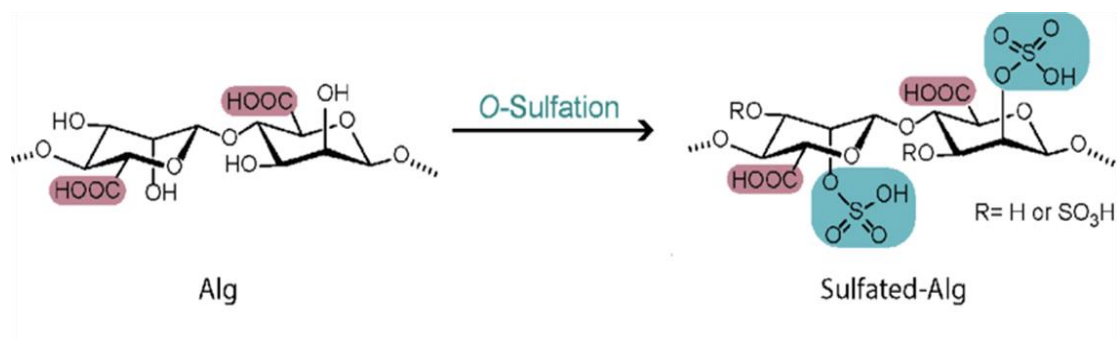


Figure 3: Alginates Sulfation Process⁸⁹ (reproduced from ResearchGate, 2020)

1.6 Salidroside

Salidroside [2-(4-hydroxyphenyl) ethyl beta-Dglucopyranoside], is a phenylalanine glycoside extracted from a plant known as *Rhodiola rosea*. It was found that Salidroside has pharmacological effects related to boosting the immunity, being hepatoprotective, having anticancerous and anti-oxidative properties, as well as eliminating melancholy.³⁹ Besides, it could inhibit the apoptosis of immune cells located in the thymus and spleen, and could halt pro-inflammatory mechanisms.⁴⁰ Salidroside has also been able to block the interleukin-1B (IL-1B) induced inflammatory reaction in chondrocytes affected by OA, through the inhibition of NF-kB.⁴¹ In fact, IL-1B, which is a pro-inflammatory cytokine released by chondrocytes, is detected in high amount in the cartilage tissue of patients affected by OA, as it promotes the breakdown of ECM, and leads to the apoptosis

of chondrocytes.⁴⁷ The PI3K/AKT pathway is activated during the inflammatory response, and gets triggered in the conditions of OA.⁴⁸ Research has proved that Salidroside could play an important anti-inflammatory role, which would promote the delay of OA development by inhibiting the PI3K/AKT pathway thus protecting chondrocytes.⁴⁹

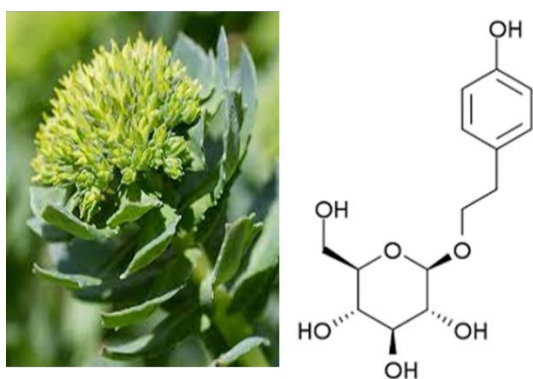


Figure 4: Rhodiola Rosea vs. Salidroside Molecule⁹⁰ (reproduced from iStock.com / ALLEKO)

Moreover, Salidroside was found to increase the expression of cartilage specific genes such as Col2A1, Acan and Sox9, while decreasing the expression of Col1A1, which is the dedifferentiation gene.⁵⁰ Col2A1's expression was found to be highly regulated by Sox9 protein during the differentiation of chondrocytes.⁵⁰ Salidroside was also able to significantly promote the secretion of GAGs in chondrocytes, to strengthen the ECM network, hence contributing to the protection of chondrocytes' phenotype and enhancing their proliferation.¹² While the TGF- β /Smad3 signal has an essential function in the ECM deposition, proliferation and phenotype maintenance of chondrocytes, TGF- β inhibits the rate of maturation of chondrocytes, and Smad2 and Smad3 get moved to the nucleus because of the TGF- β signal.⁵¹ Salidroside was found to promote the surge of the levels

of TGF- β , and Smad3 thus showcasing the protecting function that Salidroside exerts on chondrocytes.¹²

1.7 Transforming Growth Factor Beta (TGF- β)

The TGF- β family of growth factors is crucial for homeostasis as it is responsible of the regulation of many cellular mechanisms including differentiation, proliferation, migration and apoptosis along with the ECM production and breakdown.⁵² These growth factors were also found to promote cell proliferation and to halt the function of matrix metalloproteinases (MMP).⁵³ In fact, TGF- β is involved in conserving chondrocytes' phenotype during passaging, as well as it stimulates the synthesis of collagen type II.¹⁵ TGF- β targets a pair of transmembrane serine/threonine kinases that are referred to as the TGF- β type II (TBR2) and type I (TBR1) or activating receptor-like kinase 5 (ALK) receptors.⁵⁴ Therefore, TGF- β binds to TBR2, which leads to the phosphorylation of TBR1/ALK, thus activating the ALK5 kinase activity.⁴² Then, ALK5 phosphorylates Smad2 and Smad3, that stick to the same mediator Smad4, then move to the nucleus where there will be an interaction between them and many transcription factors, co-activators, and co-repressors in order to ensure the regulation of gene expression.⁴³ TGF- β also stimulates ALK1, another TGF- β type I receptor that plays a role in phosphorylating Smad1, Smad5 and Smad8.⁴⁴ In addition to the Smad signaling, TGF- β also promotes non-Smad mechanisms, like the MAPK kinase pathways, phosphatidylinositol-3-kinase (PI3K)/AKT pathways and Rho-like GTPase signaling pathways.⁴⁵ TGF- β is strongly regulated by the function of many extracellular and intracellular proteins that control the work of TGF- β in a temporal, spatial and cell-type dependent way. TGF- β works on the development, maintenance and growth of the

articular cartilage, as it leads to the stimulation of early events in the process of chondrogenesis, which involves chondrogenic condensation, the osteoblastic differentiation and ossification, along with the vascular invasion⁵⁹, thus resulting in the synthesis of articular cartilage at long bones' ends through the Smad-3-based pathway.⁶⁰ TGF- β not only stimulates the synthesis of ECM proteins like collagen type II and aggrecan, but it also works on blocking the degradation of the proteins of ECM through the increase of the synthesis of protease inhibitors.⁶¹ Besides, TGF- β is able to counteract the catabolic work of IL-1 and TNF- α on cartilage degradation.⁶² In addition, increasing evidence shows that TGF- β receptors' expression levels are changed in OA. As a matter of fact, TBR1 levels were found to be much lower in a patient with OA's cartilage than those in a normal patient's cartilage.⁶³ Both ALK5 and ALK1 expression also decrease in the case of OA, with ALK1 which inhibits collagen type II expression decreasing at a lesser rate than that of ALK5 that potentiates the expression of collagen type II.^{63,64}

CHAPTER 2

SCOPE OF THE THESIS

Osteoarthritis, mainly targeting the aging population, represents an important socioeconomic burden. Since cartilage is not able to heal naturally post-injury or when affected by a disease, medical intervention is required. Currently, there are no efficient disease-modifying drugs, and the available surgical procedures are not able to fully bring back the initial healthy state of the damaged articular cartilage. Even though some techniques such as ACT are promising, they face several limitations, essentially due to the limited number of chondrocytes available for transplantation, as well as the dedifferentiation of these cells upon passaging. The use of biomimetic materials to synthesize 2D substrates for cartilage tissue engineering could enhance results for ACT surgeries. The aim of this work is to prevent late passage chondrocytes dedifferentiation in a culture system combining alginate sulfate with Salidroside and TGF- β .

To assess the effect of Salidroside and TGF- β on the viability and proliferation of chondrocytes cultured on 2D biotinylated sulfated alginate substrates. Since sulfate groups represent a crucial part in the structure of the cartilage tissue, we hypothesized that sulfation of alginate which is believed to repair the phenotype of cartilage would lead to a more chondrogenic material. Thin films constructed using biotin-streptavidin interactions were used to enhance the cell-culturing factors and avoid the dedifferentiation of the cultured chondrocytes. In fact, the LbL technique was employed earlier to synthesize nanofilms from ECM macromolecules, but these studies often result in films that were either unstable or repellent to cells. Biotinylated sulfated alginate substrates (2D substrates) with various degrees of sulfation were immobilized using

biotin-streptavidin interactions to form those 2D films. Both passage 2 (P2) and passage 4 (P4) chondrocytes were seeded onto the prepared 2D films in the presence or absence of Salidroside and TGF- β to test for their viability and proliferation using Live/Dead and Trypan Blue assays for up to 7 days.

In order to assess the gene expression of late passage chondrocytes in presence of Salidroside and TGF- β , PCR was performed to check for the expression of genes that will signal the maintenance of late passage's chondrocytes' phenotype. Hence, the expression and synthesis of type II collagen, type I collagen, SOX9, RUNX2, SZP, MMP13, and aggrecan were assessed by quantitative real time PCR (qRT-PCR), for both P2 and P4 chondrocytes. The results of this study were interesting as the combination of Salidroside with TGF- β was found to best enhance the proliferation and gene expression of cartilage specific genes, thus protecting chondrocytes from dedifferentiation.

Western Blot was performed to detect protein secretion of P2 and P4 chondrocytes when cultured in presence and absence of Salidroside and TGF- β , on alginate sulfates of different degrees of sulfation. The results of this study showed that there could be a certain correlation between Salidroside and the downregulation of collagen type I secretion, however, it needs further investigation to be able to really assess that effect and its conditions.

The thesis ends with a summary of results and an overlook on challenges and future opportunities.

CHAPTER 3

MATERIALS AND METHODS

In this chapter, descriptions of the materials and methods used in this thesis are detailed.

3.1 Chondrocyte isolation

Chondrocytes were isolated from the knees of 6-month-old calves sourced from a local slaughterhouse, abiding by the established protocols. Cartilage shavings were sliced using a sterile blade and they were then incubated with 0.2% Pronase in DMEM that contains 1% antibiotic-antimycotic for 2 hours at 37°C and 7% CO₂ with mild stirring. After being digested by pronase, the tissue was washed three times for 2 minutes each time using DMEM containing 1% antibiotic-antimycotic and then incubated for 6 hours in 0.03% collagenase in DMEM supplemented with 1% antibiotic-antimycotic at 37°C and 7% CO₂ with gentle stirring. Cells were isolated from the digested matrix by filtering them through 100 µm and 40 µm cell strainers in sequences. Cell counting and viability evaluation were done using an automated cell counter (Countess™ Automated Cell Counter, Invitrogen AG, Basel, Switzerland), with viability surpassing 90% for all separations. The isolated cells were cultured at a density of 10,000 cells/cm² in DMEM along with 1% antibiotic-antimycotic, 10% FBS, and 50 µg/mL L-ascorbic acid. When cells reached 80-90% confluency, they were removed by adding trypsin/EDTA and then seeded again at a density of 5,000 cells/cm² for later passages.

3.2 Cell Culture

P1 and P3 Chondrocytes were seeded and expanded until passage 2 and passage 4 respectively, in T75 Flasks containing DMEM-F12 media supplemented with 10% FBS, 1% penicillin-streptomycin, and 50 µg/mL Ascorbic Acid. Cells were released with trypsin/EDTA and then seeded on the prepared 2D films.

3.3. 2D Films Build-up and Cell Seeding

200 µL of 0.1 mg/mL biotinylated Bovine serum (bBSA) was added in each well of the 48-wellsplate and incubated at 4°C overnight. After washing the plates with phosphate buffer saline (PBS), 200 µL of 25 µg/mL Streptavidin was added to each well of the plate and incubated for 2 hours at room temperature. Next, and after washing the plates with PBS, biotinylated alginate sulfate with different sulfation degrees (0.0, 1.0, 1.5, 2.0) was added for 1 hour at room temperature after which they were washed again with PBS. DMEM-F12 complete media (+10% Fetal Bovine Serum, +1% penicillin-streptomycin, +50 µg/ml ascorbic acid) will be added to the wells, and incubated for 1h. Wells without coating served as negative controls, wells containing heparin side-on and end-on will serve as positive controls for the alginate sulfate.

P2 and P4 chondrocytes were cultured on the formed layers in 48 well plates at a seeding density of 50×10^3 cells per well, in various conditions, on different substrates: (1) on Plastic (as negative control), (2) Heparin, (3) AlgSulf0.0, (4) AlgSulf1.0, (5) AlgSulf1.5, (6) AlgSulf2.0. Chondrocytes were subjected to each of these conditions in three groups as follows: (1) Chondrocytes + TGF-β (10 ng/ml) + Salidroside (1.33 µM), (2) Chondrocytes + Salidroside (1.33 µM), (3) Chondrocytes + TGF-β (10 ng/ml), to compare the effect of the combination of Salidroside and TGF-β, to that of each agent on

its own, on the proliferation of P2 and P4 chondrocytes. P2 and P4 chondrocytes were incubated in a 37 °C 5% CO₂ incubator (Figure 5).

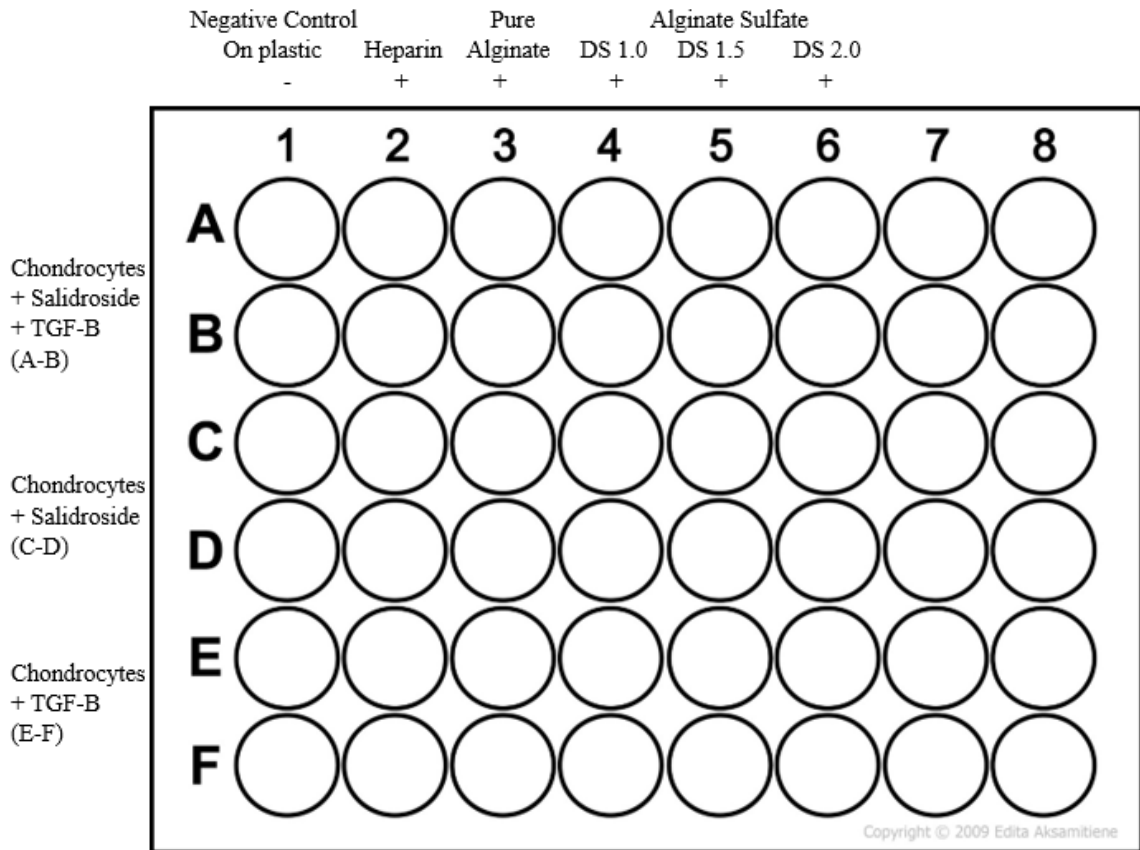


Figure 5: Conditions distribution on 48 well plates

3.4 Trypan Blue

Following the culture of P2 and P4 chondrocytes with the different conditions, Trypan Blue test was performed at 3 time points (days 1,4 and 7) to check for the cells proliferation. Chondrocytes were treated with trypsin-EDTA and collected after PBS wash. Then, cells were incubated with 0.4% w/v trypan blue solution for 2-3 min at room temperature. Cell viability was determined by using a hemocytometer. The percentage of cell survival for every treatment group was found by counting the live and dead cells. The

percentage of live cells was determined by dividing the number of living cells with the total number of cells in percentage.

3.5 Live/Dead Assay

The culture system was maintained until day 7 and Live/Dead assay was performed to detect for the cells viability and check for the toxicity of Salidroside. The cells are stained using 150 μL solution containing of 0.1 μM Ethidium homodimer and 1 μM calcein AM. Cells are then be incubated for 35 minutes, washed with PBS and then observed under a fluorescent microscope. Live cells are stained in green and dead cells in red.

3.6 Gene Expression

Cultured chondrocytes were first washed with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ and aspirated. 500 μL of TRIzol was then added to the cells which were scraped next. After that, the medium was transferred to a sterile 1ml Eppendorf tube, vortexed well for 30 seconds, and stored at -20°C . On the following day, 100 μL of chloroform was added to the Eppendorf tubes, which were homogenized thoroughly by vortexing. They are then incubated for 5 minutes on ice, vortexed again, and centrifuged at 12000 g for 15 minutes at 4°C . This has lead to the formation of three different phases: an organic phase (phenol/proteins), an interphase (DNA), and an aqueous phase (RNA). The aqueous phase ($\sim 200\mu\text{L}$) was collected, transferred to the new Eppendorf tubes, and the pellet was discarded. 200 μL of isopropanol was added and vortexed for 15 seconds. The samples were then incubated for 5 minutes on ice, vortexed, and centrifuged at 12000 g for 15 minutes at 4°C to observe the RNA pellet. After discarding the supernatant, 400 μL of 75% ethanol prepared RNase/DNase-free water, was added, and the sample got centrifuged at 7500g for 5

minutes at 4°C. After the ethanol wash was done twice, the supernatant was discarded and RNA was resuspended in 22µL of RNase-free water, and incubated in an oven at 60°C for 5 minutes. Next, RNA dosage was performed using a Nanodrop, reverse transcription (RT) using a thermocycler, and qPCR. For RNA dosage, 1µL of the sample was placed on the block (RNase-free water), samples were measured with cleaning between each. The RNA concentration were obtained in ng/µL. The purity ratios A260/280 (regarding cDNA) and A230/260 (regarding salts) were between 1.5-2.

3.7 Protein Extraction

To prepare RIPA buffer, 0.005 g of SDS (0.1%), 0.025 g sodium deoxycholate (0.5%), 2.5 ml NaCl (150 mM), 50 µl EDTA (1mM), 2.5 ml tris-HCl of pH 8 (50mM), and 71µl NP-40 (1%) were all combined together. Additionally, protease inhibitors were added, NaF as a phosphatase inhibitor, sodium orthovanadate as another phosphatase inhibitor. The final volume was adjusted with H₂O to reach 5 ml. Samples were next retrieved from the -80 °C freezer and placed on ice. An appropriate amount of lysis buffer was added to the cell pellets and they were kept on ice for 15 minutes. Next, samples were centrifuged at 4°C for 10 minutes at a high speed (13,000 rpm). The supernatant was then carefully transferred to a new tube. A Bradford Assay was performed to determine the protein concentration in the lysates. The protein samples were then stored at -20°C overnight.

3.8 Western Blot

A 1.5 mm thick thin and thick glass setup was assembled and checked for leaks with water. 10 ml resolving gel and 3 ml stacking gel were prepared and then poured with isopropanol to eliminate bubbles. After solidification, the stacking gel was poured and

the comb was inserted. Protein samples were centrifuged for 1 minute after adding Laemmli 4X and heating at 95°C for 10 minutes. The electrophoresis box was filled with migration buffer (200 ml 5X buffer mixed with 800 ml water) and placed on an agitator. 37-40 µl or 60 µl of protein samples were then loaded with 3.7 µl of the ladder mixed with 20µl of 1X Laemmli into the wells, adding 20 µl of 1X Laemmli to the last empty well. The gel was run at 80V until proteins passed the stacking gel, then at 100V. The transfer buffer was then prepared (100ml 10X buffer, 100ml water, 200ml methanol, 600ml water, and 1ml of 10% SDS) and stored at 4°C. Next, the gel was removed, and the transfer sandwich with 2 black sponges and blotting paper were assembled. The gel was placed on the black side, and the nitrocellulose membrane on top. The transfer box was inserted with the black side (-) on black and white side (+) on red, with an ice pack and ice in the bucket. The transfer at 350mA and 70V was run for 3 hours. The membrane was stained with Ponceau red for 1 minute, it got washed multiple times with 0.3% TCA, dried between two papers, and scanned using blue paper. It was then blocked with 5% milk (2.5g in 50ml) in TBS-T (200ml 10X TBS, 1800ml water, 2ml Tween 20) or 3% BSA for 1 hour at room temperature. Wash was performed 3 times for 10 minutes each in TBS-T at room temperature on a shaker. Incubation with the primary antibody in 1% BSA (0.5g in 50ml) in PBS or TBS-T in a plastic bag was done for 1 hour at room temperature on a shaker. Incubation with the secondary antibody (anti-mouse at 1:5000) in 5% milk or 1% BSA in TBS-T in a plastic bag for 1 hour at room temperature on a shaker. 1:1 ECL reagents were added and the blot was then observed on a chemidoc. For stripping, the membrane was incubated in stripping buffer for 20 minutes at room temperature on a shaker, washed twice for 5 minutes each with TBS-T, stripped again for 20 minutes, and washed twice more with TBS-T. The stripping buffer was prepared by

dissolving 3.75g glycine, 2.5ml 10% SDS, and 2.5ml Tween 20 in 250ml autoclaved water, adjusting pH to 2.2 with HCl, and stored at 4°C.

3.9 Statistical Analysis

Data were obtained from the samples and represented as the mean \pm standard deviation (SD). Statistical assessment was carried out by analysis of two-way ANOVA and post hoc Tukey's tests where P values of less than 0.05 were considered significant. Statistical analysis was performed by means of Prism software (GraphPad Software, La Jolla, CA, USA).

CHAPTER 4

RESULTS

4.1 Proliferation Assay

4.1.1 Proliferation Assay for P2 Chondrocytes

The proliferation assay for Passage 2 (P2) chondrocytes using the Trypan Blue technique was done in function of different factors: on plastic (negative control), on Heparin, on pure alginate, on alginate sulfate with degrees of sulfation of 1, 1.5, and 2, all in the presence of TGF- β , Salidroside and the combination of the two.

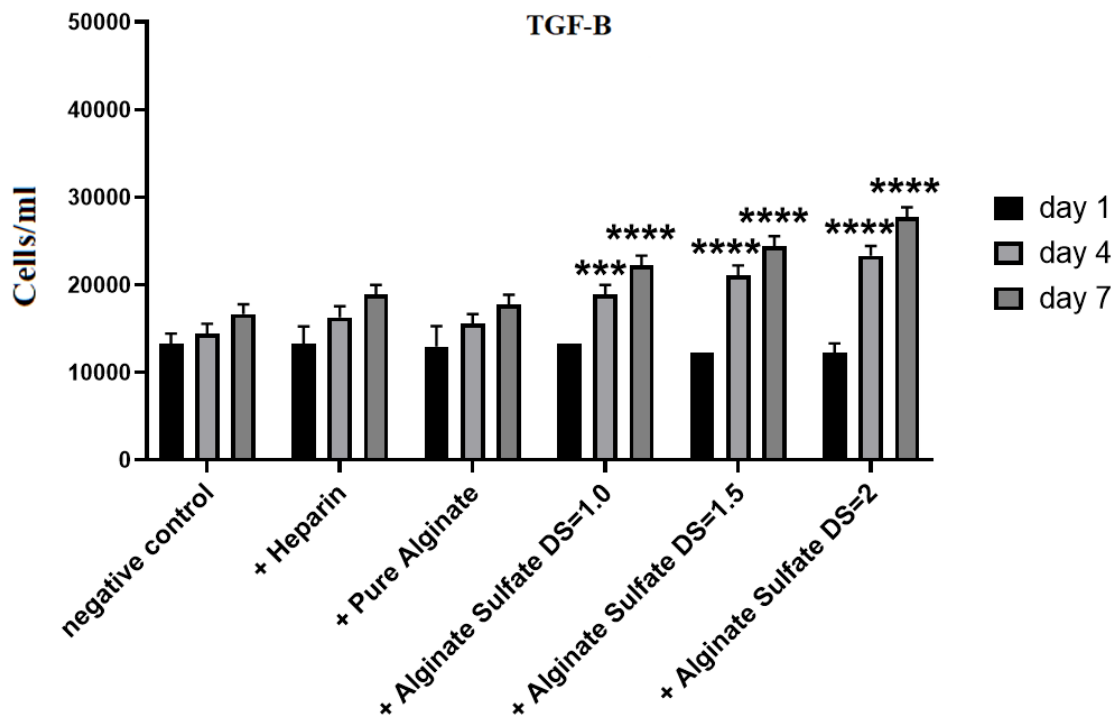


Figure 6: Proliferation assay for P2 chondrocytes subjected to TGF- β only, when grown on plastic (negative control), on heparin (+ Heparin), on pure alginate (+Pure Alginate), on alginate sulfate of degree of sulfation 1 (+ Alginate Sulfate DS=1.0), on alginate sulfate of degree of sulfation 1.5 (+ Alginate Sulfate DS = 1.5), and on alginate sulfate of degree of sulfation 2 (+ Alginate Sulfate DS = 2) on days 1, 4 and 7.

For P2 chondrocytes grown on plastic in presence of TGF- β only, the cells first exhibited a gradual increase in numbers, which eventually reached a plateau,

demonstrating a narrow proliferation potential in this case. As for cells cultured on Heparin, they showed a steady and significant surge in number during the experiment. When P2 chondrocytes were grown in presence of TGF- β only, on Alginate Sulfate with a degree of sulfation of 1, they displayed a significantly higher proliferation rate on day 4 ($p = 0.001$) and on day 7 ($p < 0.001$), when compared to the negative control. They were also found to have higher proliferation rates in contrast to the control and pure alginate. Likewise, P2 chondrocytes grown in presence of TGF- β only, on Alginate Sulfate with a degree of sulfation of 1.5 showed a significantly larger proliferation rate on day 4 ($p < 0.001$) and on day 7 ($p < 0.001$) when compared to the negative control. The proliferation trend was similar to that obtained with alginate sulfate of degree 1, but with slightly higher cell numbers, demonstrating improved conditions for the culture of chondrocytes. As for P2 chondrocytes grown on Alginate Sulfate with a degree of sulfation of 2, they were also found to have a significant increase in proliferation rate on day 4 ($p < 0.001$) and day 7 ($p < 0.001$), in presence of TGF- β only in the media. This condition displayed the highest increase in chondrocyte amount during the experiment, demonstrating that a higher degree of sulfation offers the most suitable medium for cell growth.

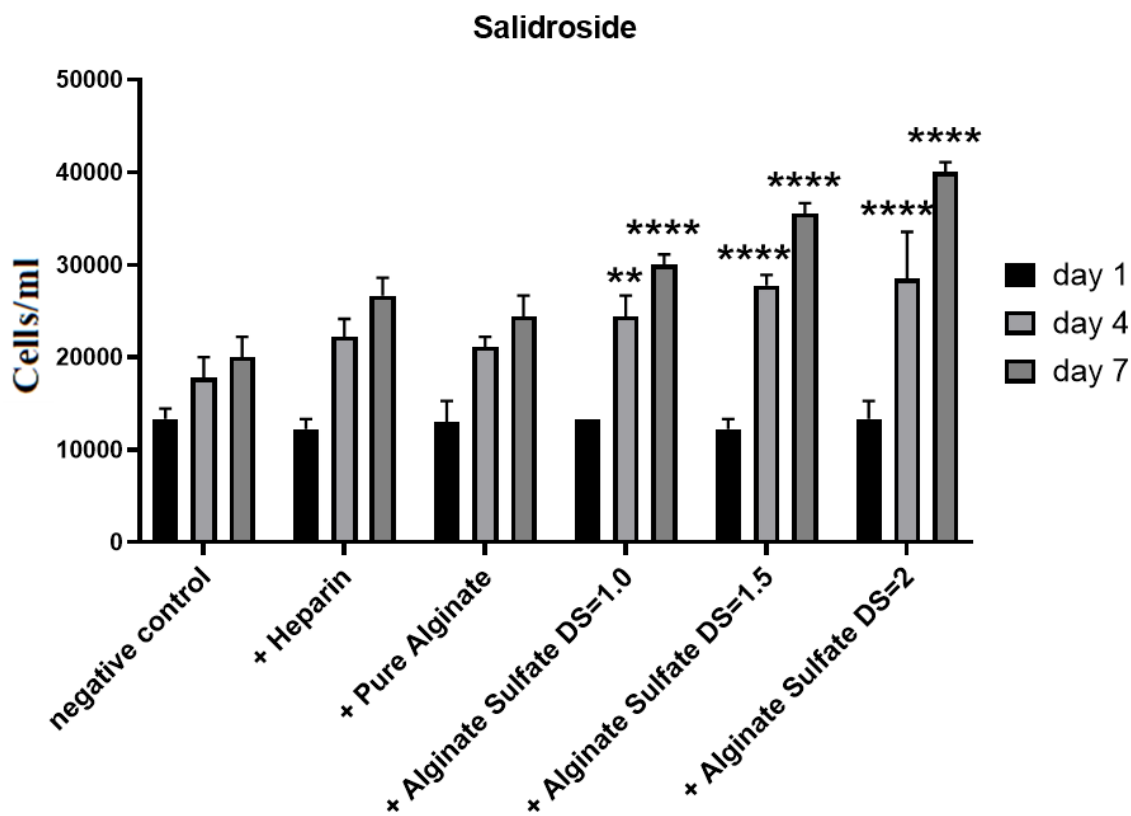


Figure 7: Proliferation assay of P2 chondrocytes subjected to Salidroside only, when grown on plastic (negative control), on heparin (+ Heparin), on pure alginate (+Pure Alginate), on alginate sulfate of degree of sulfation 1 (+ Alginate Sulfate DS=1.0), on alginate sulfate of degree of sulfation 1.5 (+ Alginate Sulfate DS = 1.5), and on alginate sulfate of degree of sulfation 2 (+ Alginate Sulfate DS = 2) on days 1, 4 and 7.

As for P2 chondrocytes grown in presence of Salidroside only on Alginate Sulfate with a degree of sulfation of 1, they displayed a significantly higher proliferation rate on day 4 ($p = 0.003$) and on day 7 ($p < 0.001$), when compared to the negative control. Similarly, P2 chondrocytes grown in presence of Salidroside only, on Alginate Sulfate with a degree of sulfation of 1.5 showed a significantly higher proliferation rate on day 4 ($p < 0.001$) and on day 7 ($p < 0.001$) when compared to the negative control. The same trend has been noticed with P2 chondrocytes grown on Alginate Sulfate with a degree of sulfation of 2, which were also found to have a significant increase in proliferation rate on day 4 ($p < 0.001$) and day 7 ($p < 0.001$), in presence of Salidroside only in the media.

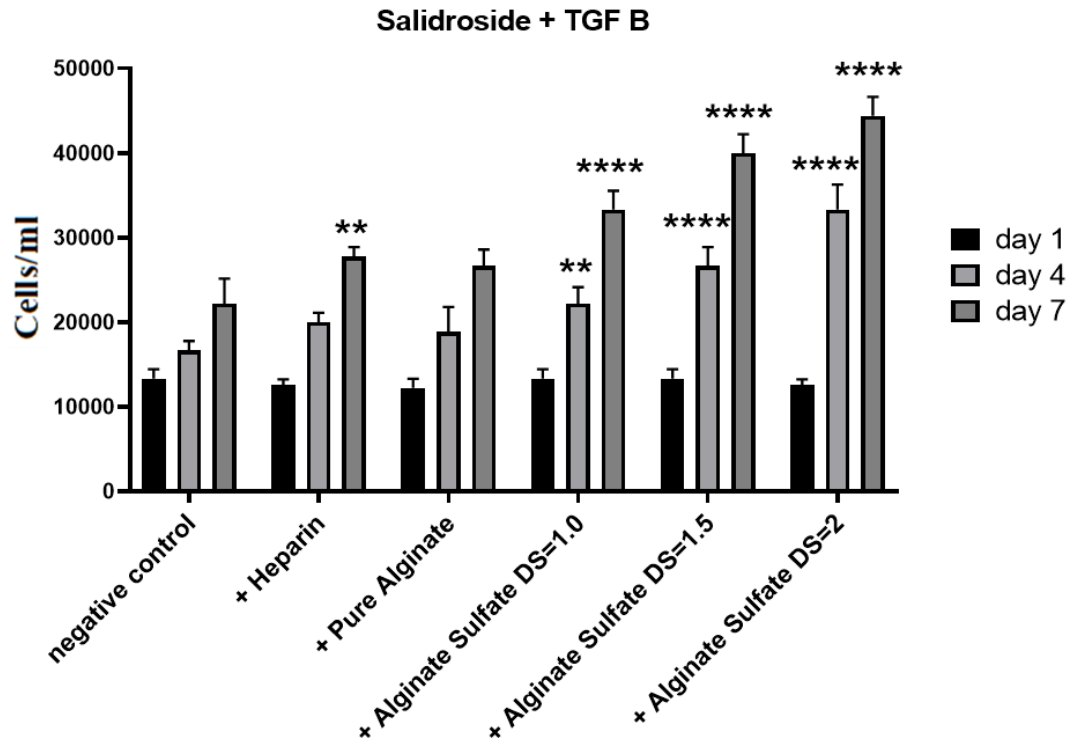


Figure 8: Proliferation assay of P2 chondrocytes subjected to Salidroside and TGF- β , when grown on plastic (negative control), on heparin (+ Heparin), on pure alginate (+Pure Alginate), on alginate sulfate of degree of sulfation 1 (+ Alginate Sulfate DS=1.0), on alginate sulfate of degree of sulfation 1.5 (+ Alginate Sulfate DS = 1.5), and on alginate sulfate of degree of sulfation 2 (+ Alginate Sulfate DS = 2) on days 1, 4 and 7.

In presence of both TGF- β and Salidroside in the media, when P2 chondrocytes were grown in on Alginate Sulfate with a degree of sulfation of 1, they displayed a significantly higher proliferation rate on day 4 ($p = 0.003$) and on day 7 ($p < 0.001$), when compared to the negative control. As for P2 chondrocytes grown in presence of both TGF- β and Salidroside, on Alginate Sulfate with a degree of sulfation of 1.5, they were found to have a significantly larger proliferation rate on day 4 ($p < 0.001$) and on day 7 ($p < 0.001$) when compared to the negative control. This variation was also observed when P2 chondrocytes were grown on Alginate Sulfate with a degree of sulfation of 2, which were also found to have a significant increase in proliferation rate on day 4 ($p < 0.001$) and day 7 ($p < 0.001$), in presence of both TGF- β and Salidroside in the media. P2

Chondrocytes grown on Heparin also displayed a significantly higher proliferation rate on day 7 only ($p = 0.003$) when compared to the negative control. The third graph displays the most important surge in chondrocyte proliferation. The number of cells highly increases, proving that the combination of Salidroside and TGF- β importantly promotes chondrocyte proliferation more efficiently than either treatment alone.

4.1.2 Proliferation Assay for P4 Chondrocytes

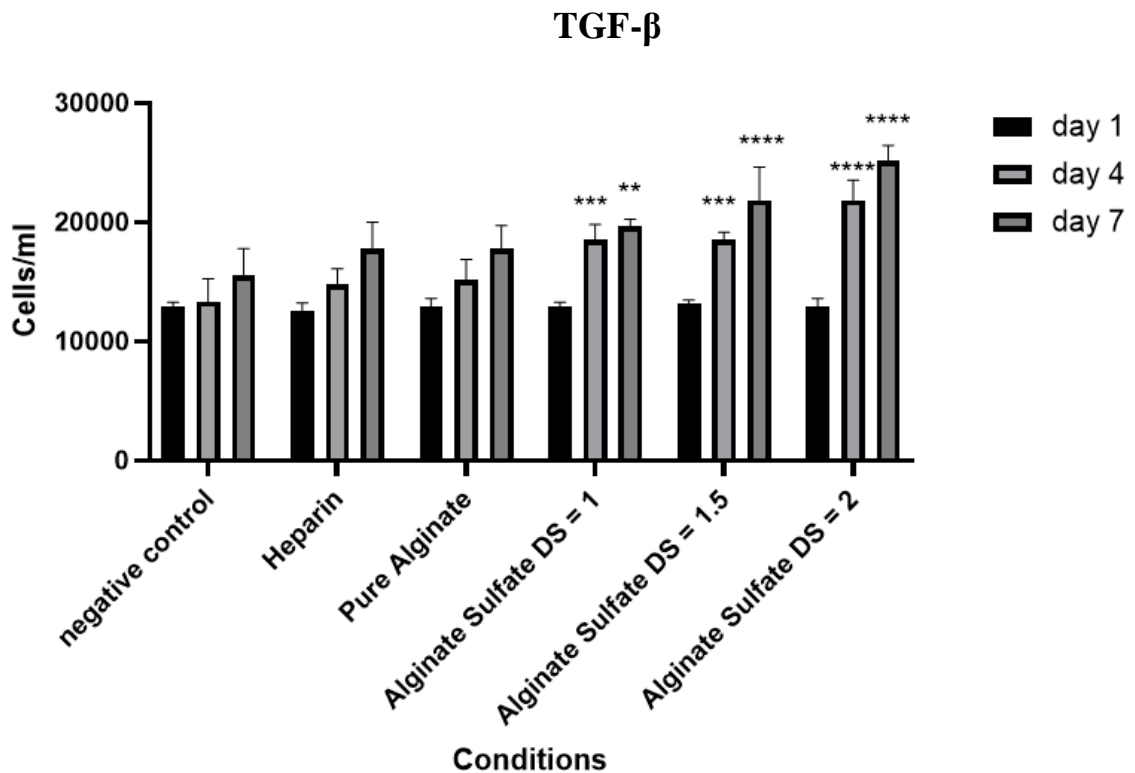


Figure 9: Proliferation assay of P4 chondrocytes subjected to TGF- β , when grown on plastic (negative control), on heparin (Heparin), on pure alginate (Pure Alginate), on alginate sulfate of degree of sulfation 1 (Alginate Sulfate DS=1), on alginate sulfate of degree of sulfation 1.5 (Alginate Sulfate DS = 1.5), and on alginate sulfate of degree of sulfation 2 (Alginate Sulfate DS = 2) on days 1, 4 and 7.

P4 chondrocytes were treated with TGF- β under multiple conditions for a period of seven days. The conditions tested consisted of a negative control, Heparin, Pure Alginate, and Alginate Sulfate with degrees of sulfation (DS) of 1, 1.5, and 2. The

numbers of cells per milliliter are registered on day 1, day 4, and day 7. In the case of the negative control and Heparin, they show almost stable and small amount of cells on the three days, displaying minimal chondrocytes growth. On Pure Alginate, the number of chondrocytes moderately increases over time, revealing some enhancement of cell proliferation on day 4 ($p=0.3989$) and on day 7 ($p=0.2393$) in contrast with the negative control. However, the most significant increases are recorded with Alginate Sulfate conditions. Specifically, Alginate Sulfate of DS = 1 condition results in a significant increase in chondrocytes number from Day 1 to Day 7 compared to the negative control ($p=0.0068$). This impact is further amplified in Alginate Sulfate of DS = 1.5 ($p<0.0001$) and Alginate Sulfate of DS = 2 ($p<0.0001$) conditions, with the condition involving Alginate Sulfate of DS = 2 displaying the highest proliferation rate, attaining the maximum cell count on Day 7.

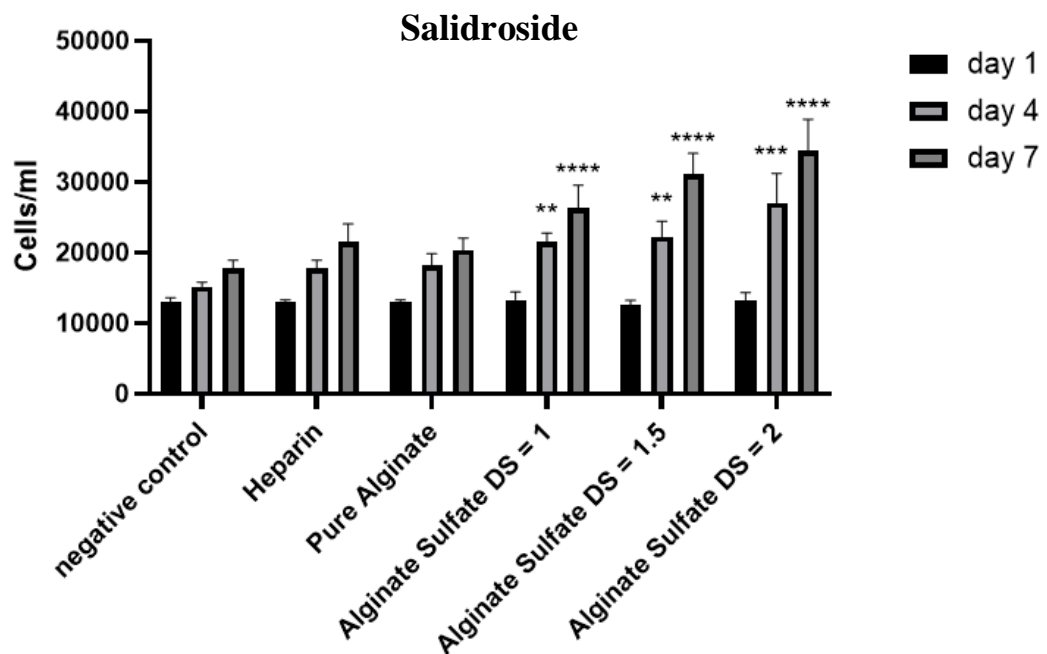


Figure 10: Proliferation assay of P4 chondrocytes subjected to salidroside, when grown on plastic (negative control), on heparin (Heparin), on pure alginate (Pure Alginate), on alginate sulfate of degree of sulfation 1 (Alginate Sulfate DS=1), on alginate sulfate of degree of sulfation 1.5 (Alginate Sulfate DS = 1.5), and on alginate sulfate of degree of sulfation 2 (Alginate Sulfate DS = 2) on days 1, 4 and 7.

The following graph displays the proliferation of P4 chondrocytes treated solely with Salidroside under the same type of conditions. The numbers of chondrocytes were also measured on day 1, day 4, and day 7. The Heparin condition again show a low and consistent cell amount, signaling a non-significant proliferation compared to the control on day 4 ($p=0.4417$) and on day 7 ($p=0.1469$). Treatment with Pure Alginate results in an increase in cell proliferation, though still non-significant compared to the control, on day 4 ($p=0.3184$) and day 7 ($p=0.4408$). Significant increases are recorded with Alginate Sulfate conditions. The Alginate Sulfate of DS = 1 condition reveals an important increase in cell proliferation from Day 1 to Day 7, in contrast with the control ($p=0.0012$ on day 4 and $p<0.0001$ on day 7). The Alginate Sulfate of DS = 1.5 condition displays even more important proliferation rates increases on day 4 ($p=0.0012$) and day 7 ($p<0.0001$), and the Alginate Sulfate of DS = 2 condition results in the highest cell proliferation in contrast to all tested conditions on day 4 ($p<0.0001$), with an optimal increase in cell numbers by Day 7 ($p<0.0001$).

The third graph illustrates the proliferation of P4 chondrocytes when subjected to a combination of Salidroside and TGF- β under the identical aforementioned conditions. Culture of chondrocytes on Heparin exhibits low and stable cell numbers, not significantly different than those of the control on day 4 ($p<0.0001$) and day 7 ($p<0.0001$). Pure Alginate treatment leads to moderate cell proliferation not significantly different than the control on neither day 4 ($p=0.9725$) nor day 7 ($p=0.5205$). However, significant increases are observed with Alginate Sulfate conditions. Particularly, the Alginate Sulfate of DS=1 condition exhibits a significant rise in cell proliferation on day 4 ($p=0.0101$) and day 7 ($p<0.0001$), which is further enhanced in the DS = 1.5 condition on day 4 ($p<0.0001$) and day 7 ($p<0.0001$). The Alginate Sulfate of DS=2 condition leads to the

highest proliferation rate, which is significantly higher than the control on day 4 ($p < 0.0001$) and day 7 ($p < 0.0001$), with a steep and stable increase in numbers of chondrocytes, and attaining the peak on Day 7.

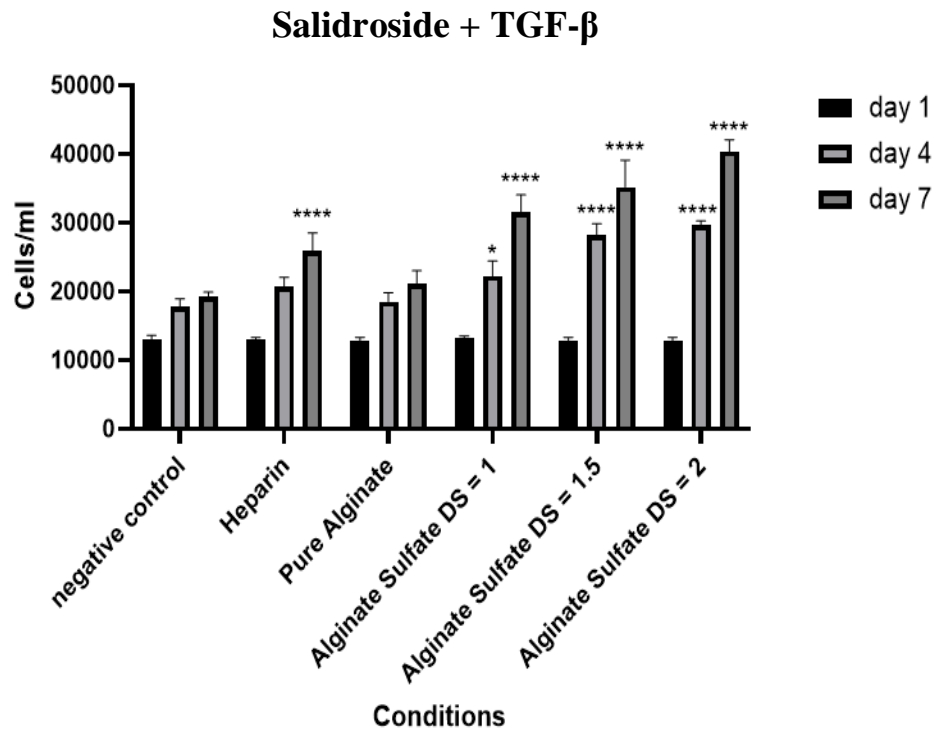


Figure 11: Proliferation Assay of P4 chondrocytes when subjected to Salidroside and TGF- β , when grown on plastic (negative control), on heparin (Heparin), on pure alginate (Pure Alginate), on alginate sulfate of degree of sulfation 1 (Alginate Sulfate DS=1), on alginate sulfate of degree of sulfation 1.5 (Alginate Sulfate DS = 1.5), and on alginate sulfate of degree of sulfation 2 (Alginate Sulfate DS = 2) on days 1, 4 and 7.

4.2 Live/Dead Assay

The obtained images from the live/dead assay using Ethidium Homodimer and Calcein AM display P2 and P4 chondrocytes under six different treatment conditions: with Salidroside and TGF- β (ST) on Alginate Sulfate of degree of Sulfation 2 (Alg2), and on Pure Alginate, with Salidroside alone on Alginate Sulfate of degree of Sulfation 2

(Alg2), and on Pure Alginate, and with TGF- β alone on Alginate Sulfate of degree of Sulfation 2 (Alg2), and on Pure Alginate. The assay distinguishes live cells (stained green with Calcein AM) and allows a clear visualization of their shape in function of the many treatments they are subject to.

4.2.1 Live/Dead Assay of P2 Chondrocytes

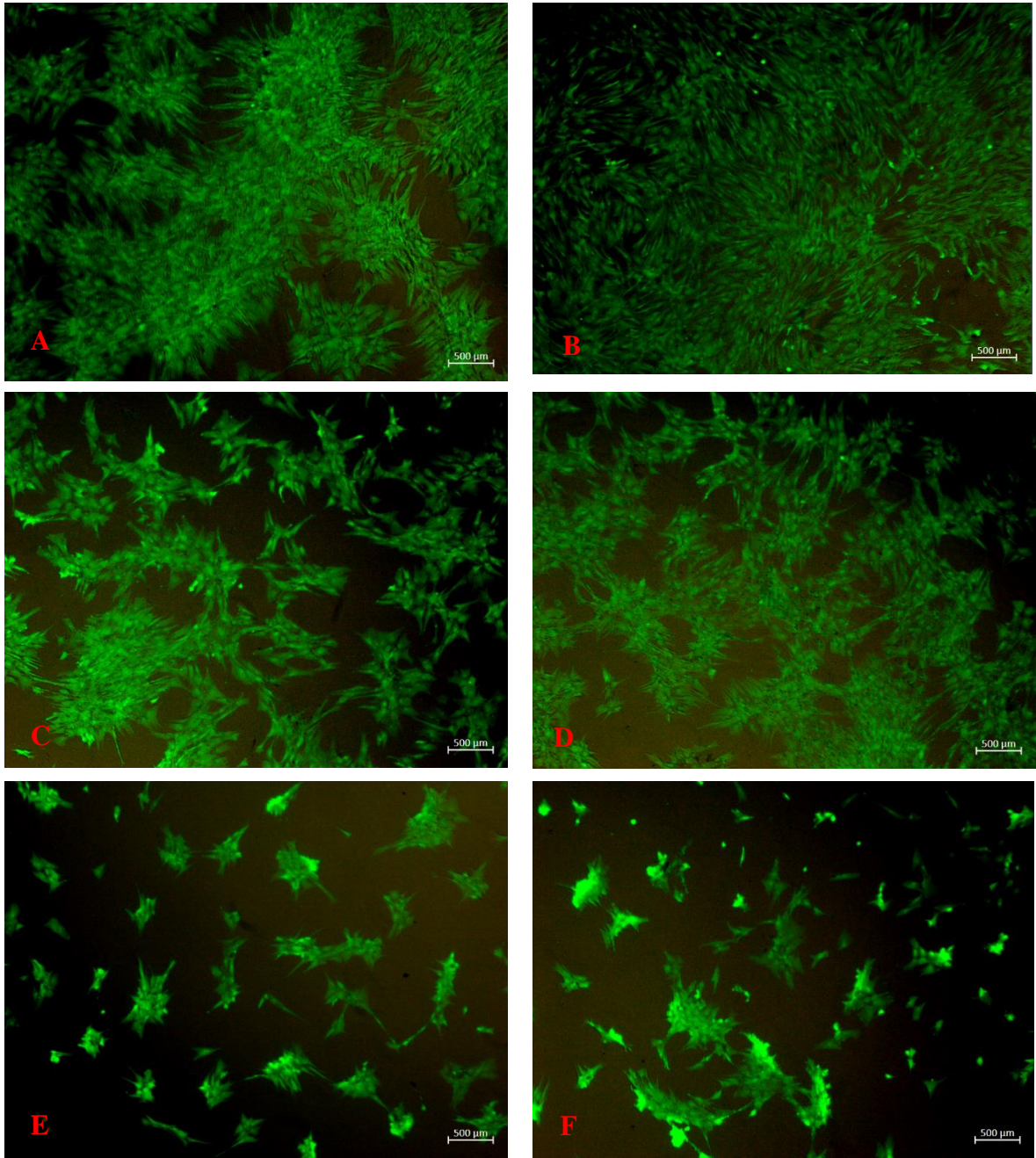


Figure 12: Live/dead assay of P2 Chondrocytes under various conditions: A: In presence of Salidroside and TGF- β on pure alginate. B: In presence of Salidroside and TGF- β on alginate sulfate of degree of sulfation 2. C: In presence of Salidroside on pure alginate. D: In presence of Salidroside on alginate sulfate of degree of sulfation 2. E: In presence of TGF- β on pure alginate. F: In presence of TGF- β on alginate sulfate of degree of sulfation 2.

P2 chondrocytes treated with Salidroside on pure alginate display a high density of green fluorescence, revealing high viability. The chondrocytes exhibit a rounded and polygonal shape, typical of healthy chondrocytes, showing that Salidroside alone can ensure cell viability and maintain the phenotype efficiently. When P2 chondrocytes were grown with Salidroside on alginate sulfate (degree of sulfation = 2.0), a larger density of green fluorescent cells was observed, compared to the pure alginate condition. The chondrocytes are uniformly dispersed and display a proper chondrocytic phenotype, which sheds a light on the impact of Salidroside on enhancing cell viability and protecting the phenotype of chondrocytes with the sulfated alginate matrix. As for the treatment of P2 chondrocytes with a combination of Salidroside and TGF- β on alginate sulfate (degree of sulfation = 2.0), it exhibited the largest density of green fluorescent cells, signaling the highest chondrocytes viability compared to other conditions. Chondrocytes displayed well-defined chondrocytic shape with a mixture of rounded and some elongated shapes, highlighting the optimal environment for chondrocyte protection and viability. When P2 chondrocytes were subjected to TGF- β on pure alginate, the cell density obtained was less in contrast to the conditions containing Salidroside. The chondrocytes treated displayed a fibroblastic morphology, signaling a shift towards dedifferentiation. P2 chondrocytes cultured with TGF- β on alginate sulfate (degree of sulfation = 2.0) were found to be slightly more numerous than chondrocytes treated on pure alginate, highlighting enhanced viability. However, chondrocytes still displayed a more fibroblastic shape in contrast to treatments containing Salidroside.

4.2.2 Live/Dead Assay of P4 Chondrocytes

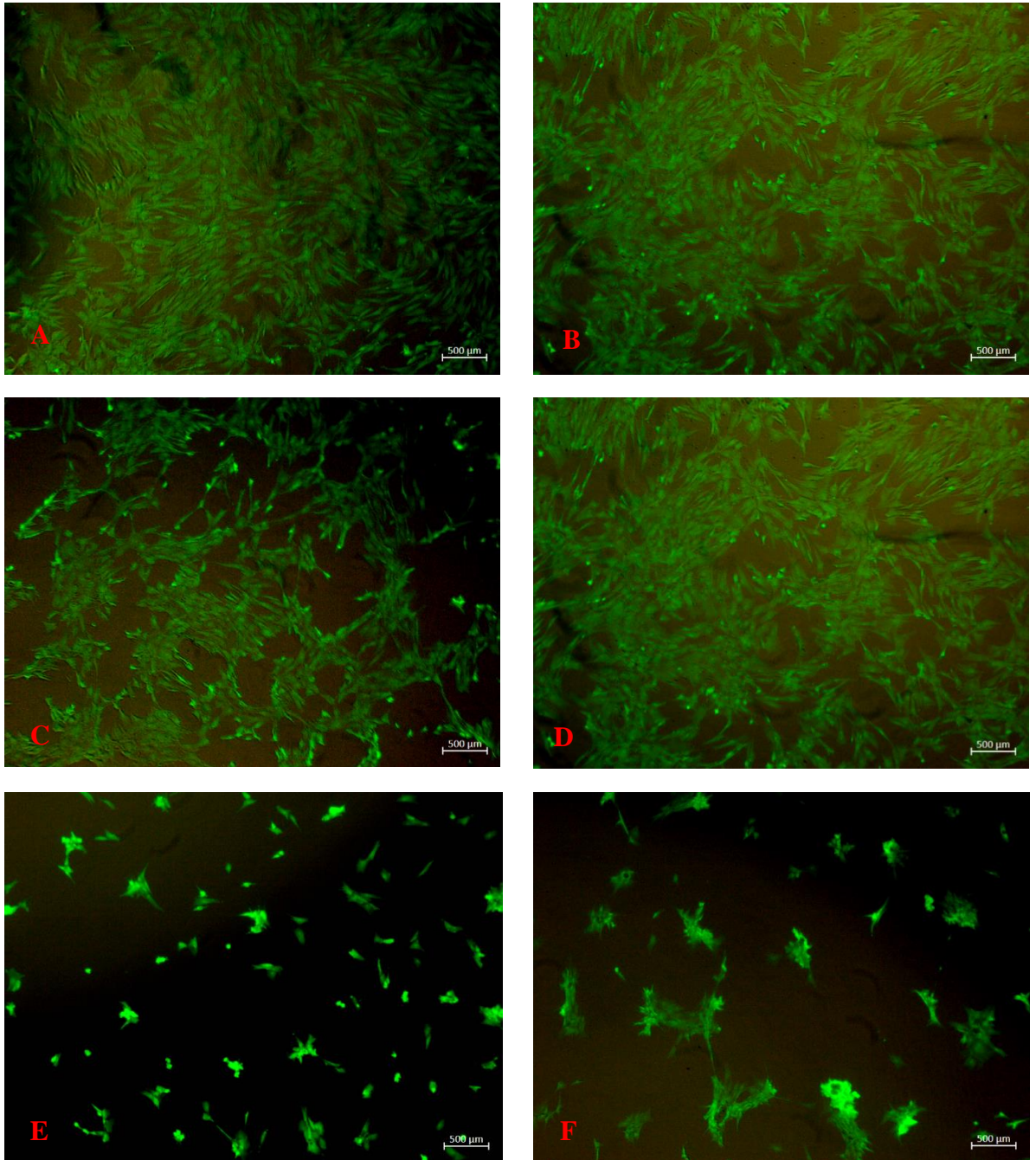


Figure 13: Live/dead assay of P4 Chondrocytes under various conditions: A: in presence of Salidroside and TGF-B on pure alginate. B: In presence of Salidroside and TGF-B on alginate sulfate of degree of sulfation 2. C: In presence of Salidroside on pure alginate. D: In presence of Salidroside on alginate sulfate of degree of sulfation 2. E: In presence of TGF- β on pure alginate. F: In presence of TGF-B on alginate sulfate of degree of sulfation 2.

P4 chondrocytes treated with Salidroside on pure alginate displayed a mix of chondrocytic and fibroblastic morphologies, displaying partial maintenance of the chondrocyte phenotype. When P4 chondrocytes were subjected to Salidroside and cultured on on alginate sulfate (degree of sulfation = 2.0), the cell density and green fluorescence intensity obtained were higher than that resulting from the pure alginate condition. Chondrocytes displayed principally chondrocytic shape, revealing a better protection of the chondrocyte phenotype and a more important level of viability. P4 chondrocytes treated with a both Salidroside and TGF- β together on pure alginate showed high green fluorescence and high cell density, indicating good cell viability. The chondrocytes exhibited a chondrocytic shape, highlighting an efficient protection of the chondrocytes' morphology. As for P4 chondrocytes cultured with TGF- β on pure alginate, their cell density and green fluorescence intensity were lower in contrast to the conditions containing Salidroside. Cells displayed more fibroblastic morphology, revealing a shift towards dedifferentiation and less cell viability. In the case of P4 chondrocytes cultured with TGF- β on alginate sulfate (degree of sulfation = 2.0), cell density obtained is slightly higher than the pure alginate treatment, showing an improved viability. However, as for morphology of the cells, they were found to display a mix of chondrocytic and fibroblastic shapes.

4.3 Gene Expression

4.2.1 Gene Expression in P2 Chondrocytes

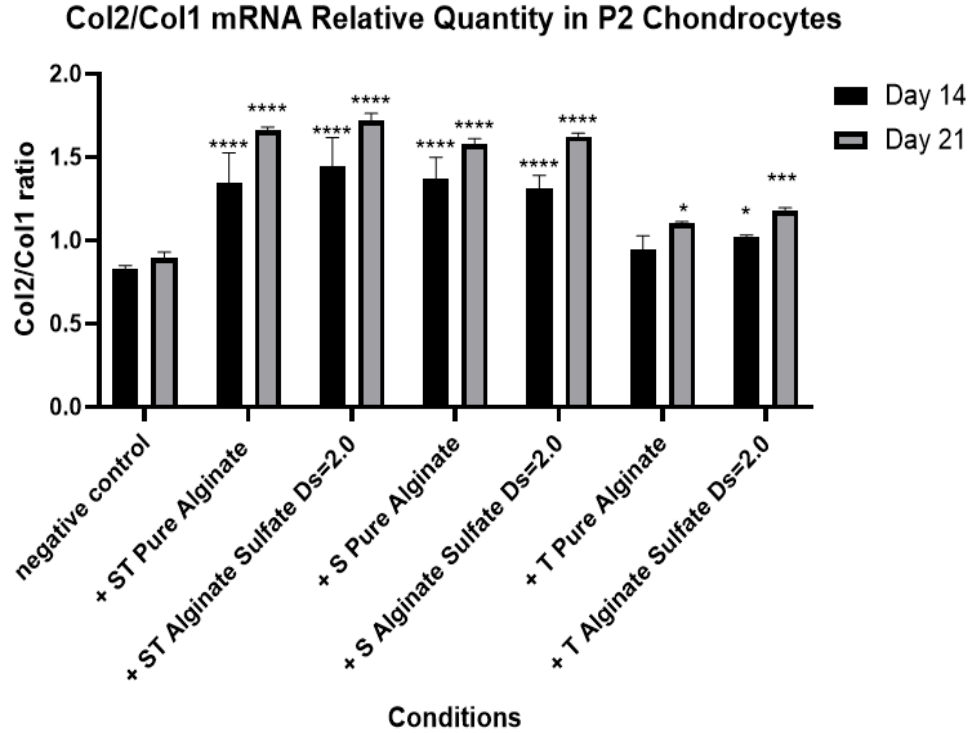


Figure 14: Col2/Col1 mRNA Relative Quantity in P2 Chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside and TGF- β (+ ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+ S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+ T Alginate Sulfate Ds=2.0), on days 14 and 21.

The graph shows the relative quantity of Col2 to Col1 mRNA in Passage 2 (P2) chondrocytes when subject to many conditions on days 14 and 21. The conditions of the experiment include a negative control, and treatments with pure alginate or alginate sulfate of a degree of sulfation of 2.0 in the presence of Salidroside (S), TGF- β (T), or a combination of Salidroside and TGF- β (ST) together.

In the case of the negative control, the Col2/Col1 ratio stayed less than 1.0 on both Day 14 and Day 21, showing that Col2 levels are low relatively to Col1. For chondrocytes cultured on pure alginate, the combination of Salidroside and TGF- β lead to an important increase in the Col2/Col1 ratio on Day 14 ($p < 0.0001$) and Day 21 ($p < 0.0001$), with values exceeding 1.5 on both time points. The treatment with Salidroside alone on Pure Alginate resulted as well, in a robust increase, as results surpassed 1.5 on Day 14 ($p < 0.0001$) and Day 21 ($p < 0.0001$). Nevertheless, in the case of TGF- β alone, chondrocytes cultured on Pure Alginate displayed ratios below 1.0 on Day 14 ($p = 0.3253$) and Day 21 ($p = 0.019$), which reveals a less efficient promotion of Col2 expression.

In the conditions involving alginate sulfate with a degree of sulfation of 2.0, the addition of both Salidroside and TGF- β together lead to the the highest Col2/Col1 ratio, reaching around 1.8 on both Day 14 ($p < 0.0001$) and Day 21 ($p < 0.0001$). The medium containing Salidroside alone, on Alginate Sulfate of a degree of sulfation of 2.0 also resulted in a significant increase, with ratios going beyond 1.5 at Day 14 ($p < 0.0001$) and Day 21 ($p < 0.0001$). Treatment with TGF- β alone on this same medium lead to a ratio exceeding 1.0, which was a significant difference compared to the negative control on Day 14 ($p < 0.03$) and Day 21 ($p = 0.0012$) but less than the other conditions.

SOX9/RUNX2 mRNA Relative Quantity in P2 Chondrocytes

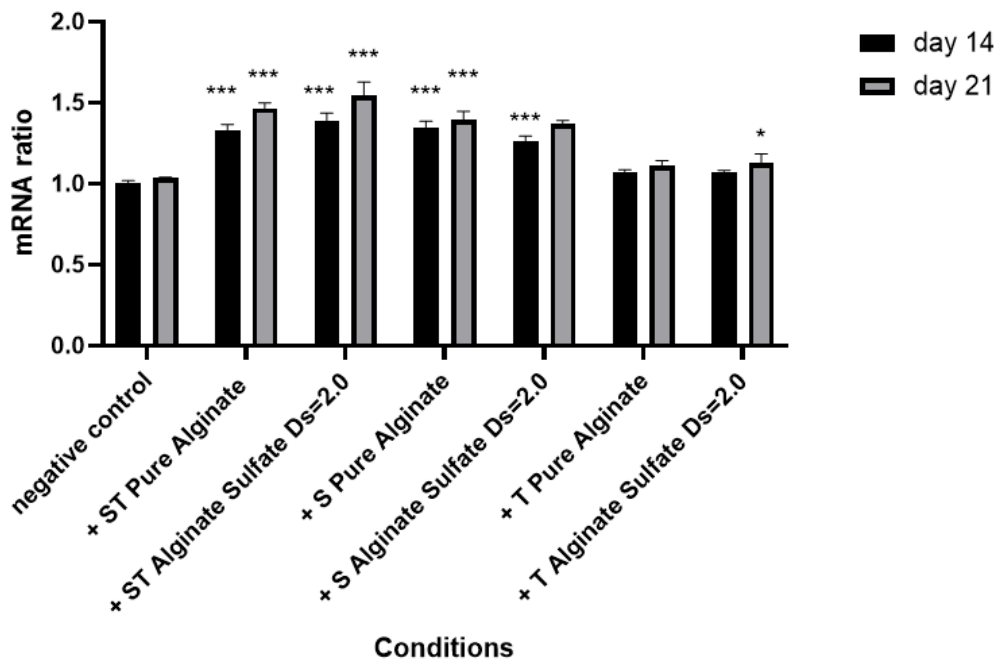


Figure 15: SOX9/RUNX2 mRNA relative quantity in P2 chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside and TGF- β (+ ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+ S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+ T Alginate Sulfate Ds=2.0), on days 14 and 21

The graph in figure 15 illustrates the SOX9/RUNX2 mRNA relative quantity in P2 chondrocytes under the same previously tested conditions at Day 14 and Day 21. In the case of the negative control, the SOX9/RUNX2 ratio stayed around 1.0 on both days, signaling stable expression of these transcription factors. On pure alginate treatments, in presence of both Salidroside and TGF- β , the levels of SOX9/RUNX2 ratio increased significantly on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$) to reach approximately 1.5 on day 21. In the presence of Salidroside alone and TGF- β alone the ratio increased significantly on day 14 ($p < 0.0001$, $p = 0.0024$) and day 21 ($p < 0.0001$, $p = 0.0075$) to attain

1.4 and 1.2 respectively. On alginate sulfate of degree of sulfation of 2.0, the combination of Salidroside and TGF- β lead to most significant increase in the SOX9/RUNX2 ratio on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$). When treated with Salidroside alone and TGF- β alone, results showed important increases in ratio on day 14 ($p = 0.0025$) and day 21 ($p = 0.0169$) to attain around 1.5 and 1.3, respectively on day 21.

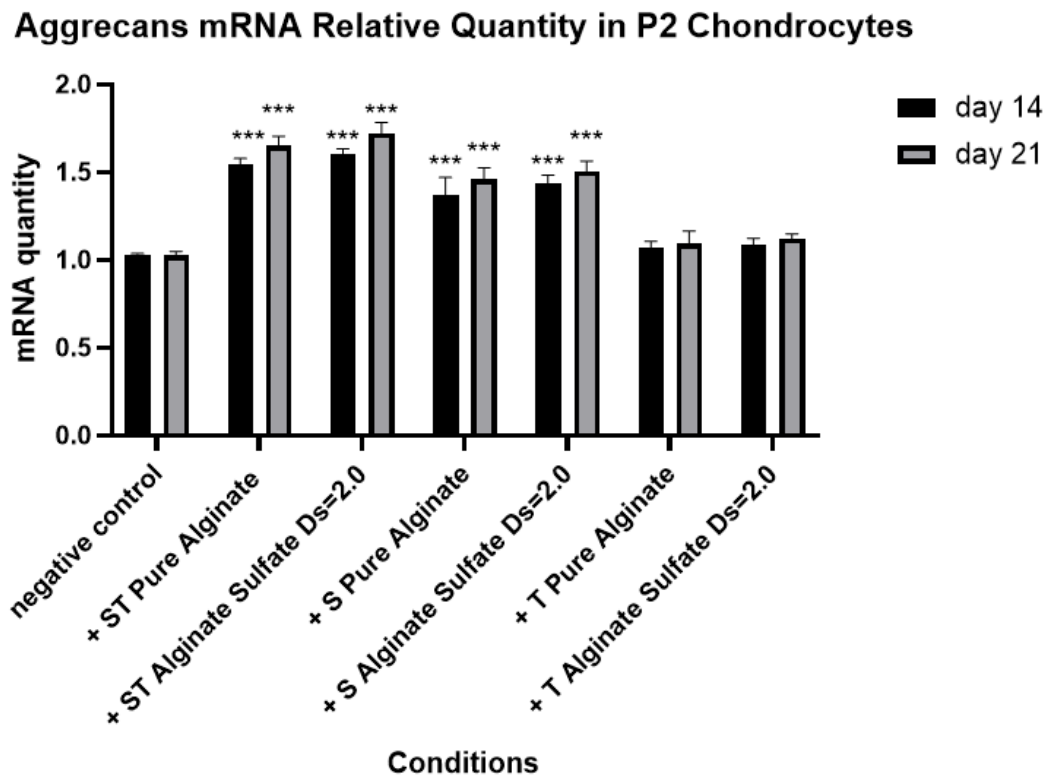


Figure 16: Aggrecans mRNA Relative Quantity in P2 Chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside and TGF- β (+ ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+ S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+ T Alginate Sulfate Ds=2.0), on days 14 and 21

The graph in figure 16 displays the relative amount of Aggrecans mRNA in P2 chondrocytes under the aforementioned treatments at day 14 and day 21. For conditions

including pure alginate, the combination of Salidroside and TGF- β lead to significantly higher Aggrecans mRNA quantity, on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$) when compared to the negative control. When treated on pure alginate with Salidroside alone and TGF- β alone, chondrocytes were found to express lower and non-significant levels compared to the control on day 14 ($p < 0.0001$, $p = 0.8768$) and on day 21 ($p < 0.0001$, $p = 0.4568$), with results approximately of 1.5 and 1.3, respectively.

In treatments that include alginate sulfate of degree of sulfation of 2.0 and the use of Salidroside combined to TGF- β , the largest increase in Aggrecans mRNA quantity was obtained, reaching approximately 1.8 on both day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$). Salidroside alone also lead to important increases, on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$), while the treatment with TGF- β alone lead to a smaller non-significant increase, with mRNA quantity around 1.4 on day 14 ($p = 0.5901$) and day 21 ($p = 0.132$).

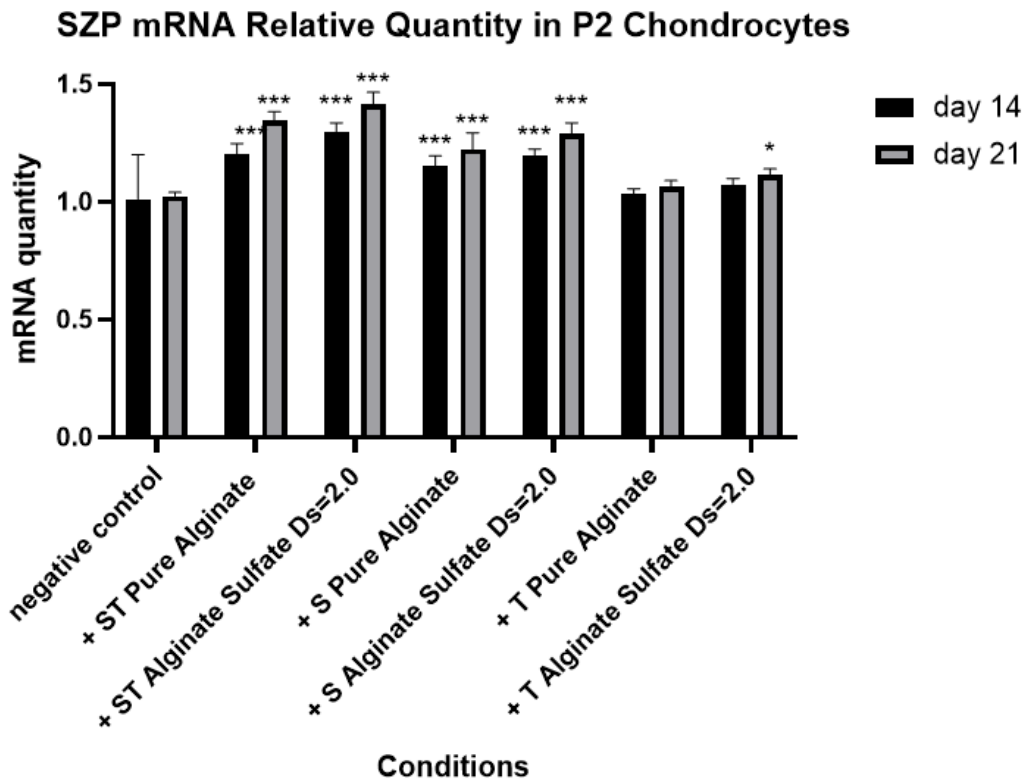


Figure 17: SZP mRNA Relative Quantity in P2 Chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside TGF- β (+ ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+ S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+ T Alginate Sulfate Ds=2.0), on days 14 and 21

The graph in figure 17 illustrates the relative quantity of SZP mRNA in P2 chondrocytes under the previously mentioned conditions at both day 14 and day 21. In pure alginate treatments, the combination of Salidroside and TGF- β significantly lead to a surge in SZP mRNA levels on day 14 ($p=0.005$) and day 21 ($p<0.0001$), to reach approximately 1.5. When Salidroside is added alone, results showed a non-significant increase on day 14 ($p=0.0528$), and a significant increase on day 21 ($p=0.0032$). When TGF- β is added alone, results displayed a non-significant increase of the ratio on day 14 ($p=0.9978$) and day 21 ($p=0.9226$).

In the condition of alginate sulfate of degree of sulfation of 2.0, the combination of Salidroside and TGF- β lead to the highest increase in SZP mRNA levels on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$), reaching approximately 1.6. Salidroside alone, added to Alginate Sulfate of degree of sulfation 2 lead to a significant surge in the ratio value on day 14 ($p = 0.0071$) and day 21 ($p = 0.0001$); TGF- β alone showed a non-significant increase of the ratio on day 14 ($p = 0.7272$) and day 21 ($p = 0.3538$), to around 1.4 and 1.3, respectively.

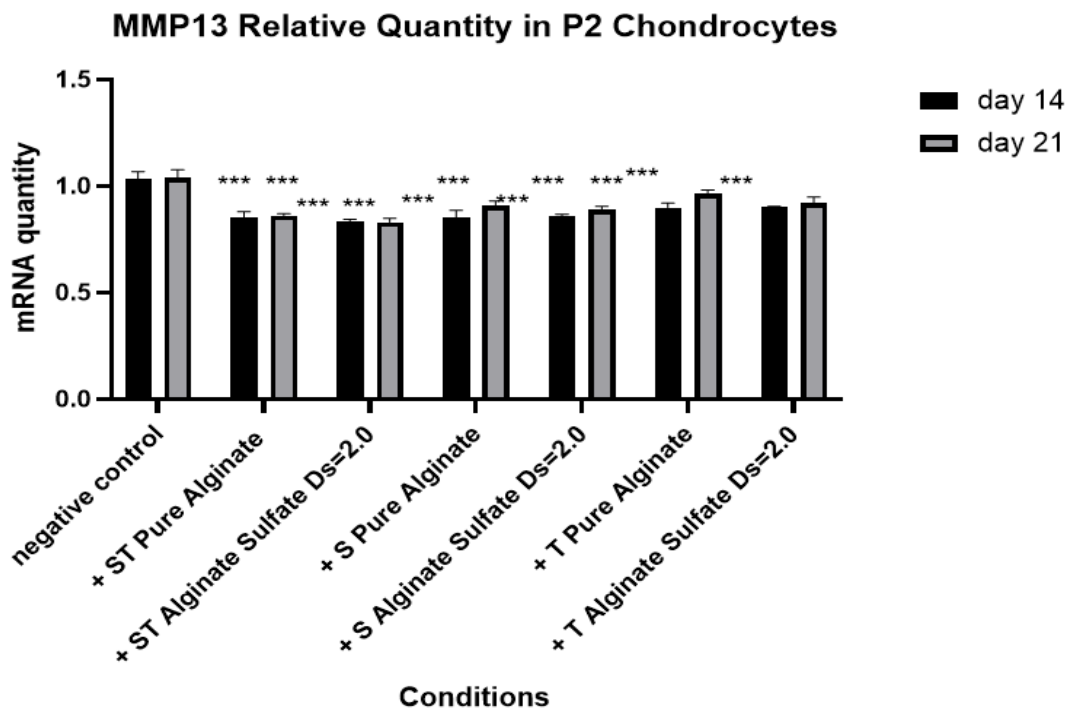


Figure 18: MMP13 Relative Quantity in P2 Chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside and TGF- β (+ ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+ S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+ T Alginate Sulfate Ds=2.0), on days 14 and 21.

The graph in figure 18 shows the relative amount of MMP13 mRNA in P2 chondrocytes under several conditions at day 14 and day 21. In pure alginate treatments, the combination of Salidroside and TGF- β resulted in a significant decrease in MMP13 mRNA quantity to around 0.7 on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$). Adding Salidroside to chondrocytes cultured on Pure Alginate also lead to a significant decrease of MMP13 values on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$). As for TGF- β alone, it also displayed drops in MMP13 quantity, significant on day 14 ($p < 0.0001$) and non significant on day 21 ($p = 0.0955$), with values reaching around 0.8 for Salidroside alone and 0.9 in the case of . TGF- β alone. For chondrocytes cultured in presence of alginate sulfate of degree of sulfation of 2.0, with the combination of Salidroside and TGF- β together, MMP13 mRNA quantity was subject to a significant drop on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$), reaching around 0.6. Salidroside alone displayed a significant decrease in MMP13 on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$). Besides, TGF- β alone showed significant reductions of this former level on day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$).

4.3.2 Gene Expression in P4 Chondrocytes

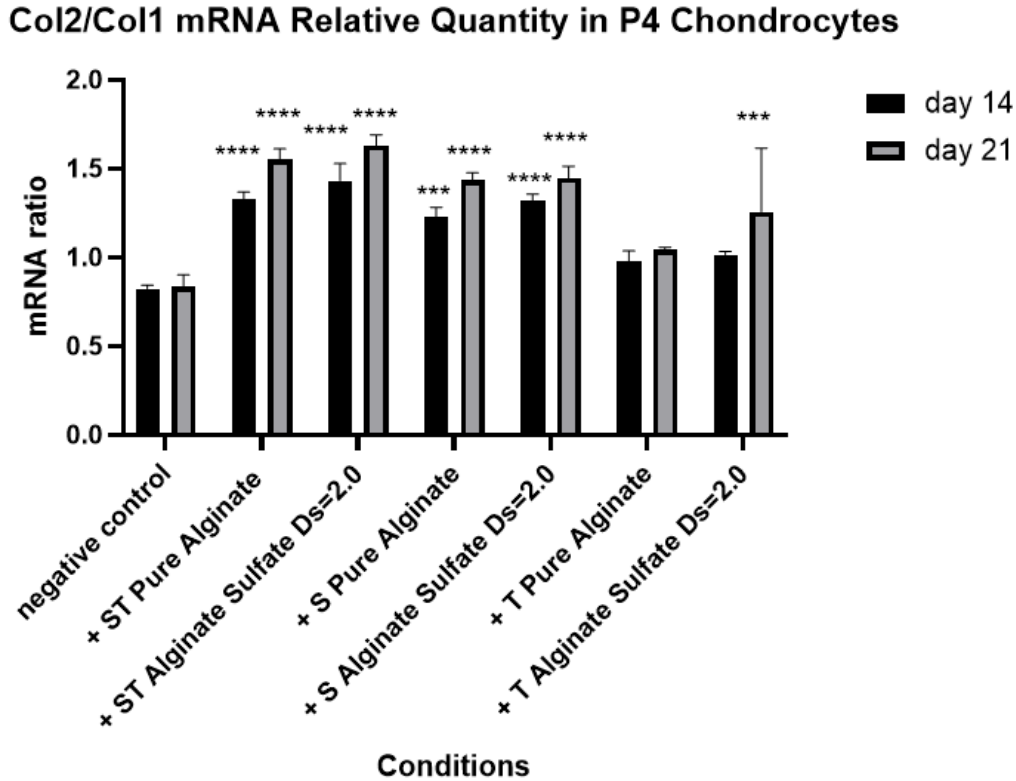


Figure 19: Col2/Col1 mRNA Relative Quantity in P4 Chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside and TGF- β (+ ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+ S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+ T Alginate Sulfate Ds=2.0), on days 14 and 21

The graph of figure 19 shows the relative quantity of Col2 to Col1 mRNA in P4 chondrocytes under several conditions at day 14 and day 21. Conditions consist of a negative control, and treatments with pure alginate or alginate sulfate (degree of sulfation = 2.0) in the presence of Salidroside (S), TGF- β (T), and a combination of Salidroside and TGF- β (ST).

Treatments with the combination of Salidroside and TGF- β on pure alginate resulted in a significant upsurge, with a ratio around 1.6 at Day 14 ($p < 0.0001$) and

reaching approximately 1.8 at Day 21 ($p < 0.0001$). Likewise, when the same combination was applied on alginate sulfate, it also led to a significant increase with a ratio of approximately 1.7 at Day 14 ($p < 0.0001$) and reaching approximately 1.8 at Day 21 ($p < 0.0001$). Treatment with Salidroside alone on pure alginate and alginate sulfate displayed significant upsurges in the ratio; for chondrocytes grown on pure alginate, ratio was significantly higher on day 14 when compared to negative control ($p = 0.0005$), and it increased to reach about 1.5 on day 21 ($p < 0.0001$) while chondrocytes cultured on Alginate Sulfate had a significantly higher ratio than that of the control ($p < 0.0001$), which increased to attain 1.4 on day 21 ($p < 0.0001$). TGF- β alone displayed the lowest increases, with ratios slightly exceeding 1.0, highlighting limited efficiency in promoting Col2 expression and inhibiting the expression of Col1; on pure alginate, the ratio wasn't significantly higher than that of the control on day 14 ($p = 0.3535$) and on day 21 ($p = 0.1238$). Likewise, on alginate sulfate, results weren't significantly different than that of the control on day 14 ($p = 0.1633$) and on day 21 ($p = 0.0003$).

SOX9/RUNX2 mRNA Relative Quantity in P4 Chondrocytes

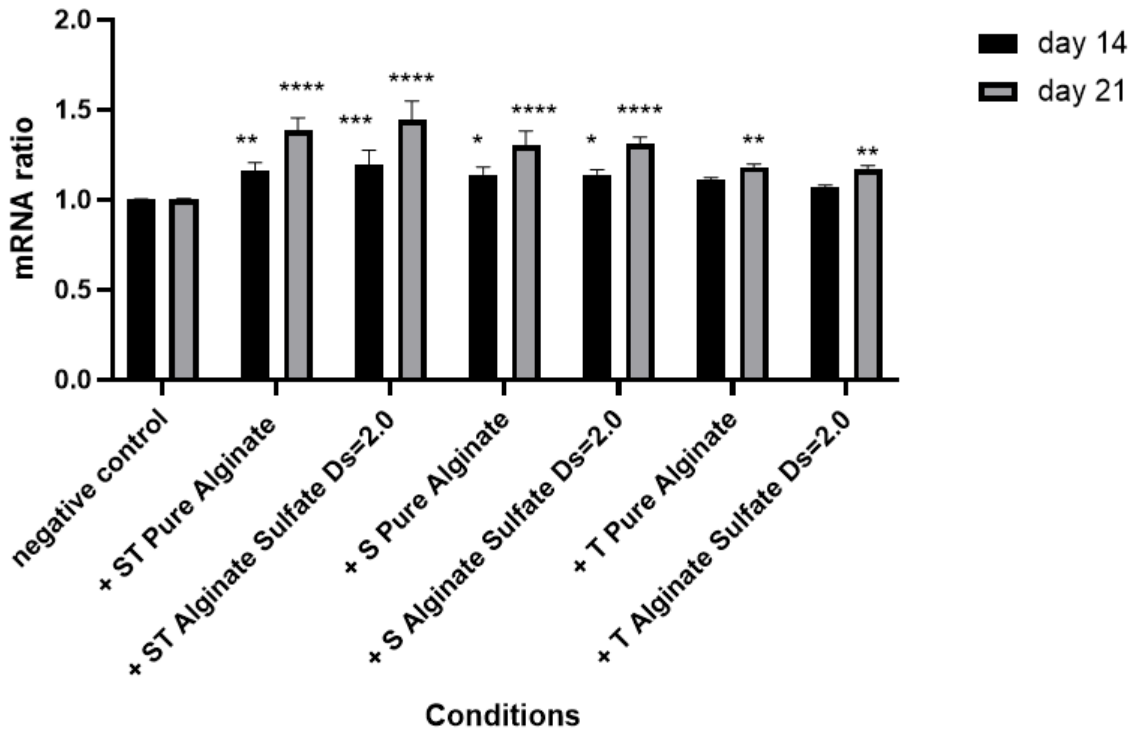


Figure 20: Sox9/Runx2 mRNA Relative Quantity in P4 Chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside and TGF- β (+ ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+ S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+ T Alginate Sulfate Ds=2.0), on days 14 and 21

The graph in figure 20 shows the relative quantity of SOX9 to RUNX2 mRNA in P4 chondrocytes under the previously mentioned conditions at both days 14 and 21. The combination of Salidroside and TGF- β on pure alginate and alginate sulfate demonstrated significant increases in the ratio on day 14 ($p=0.0033$, $p=0.0004$ respectively) and on day 21 ($p<0.0001$, $p<0.0001$ respectively). Salidroside alone was found to have a similar impact but with slightly lower increases; on pure alginate, in presence of Salidroside alone, the ratio was significantly higher than that of the control on day 14 ($p=0.0114$) and on day 21 ($p<0.0001$). Similarly, on alginate sulfate, ratios were significantly higher in

contrast with the negative control on day 14 ($p=0.0117$) and on day 21 ($p<0.0001$). TGF- β alone lead to low increases, non-significant on day 14 ($p=0.0674$ for pure alginate and $p=0.3722$ for alginate sulfate), significant on day 21 ($p=0.0011$ for pure alginate and $p=0.0026$ for alginate sulfate).

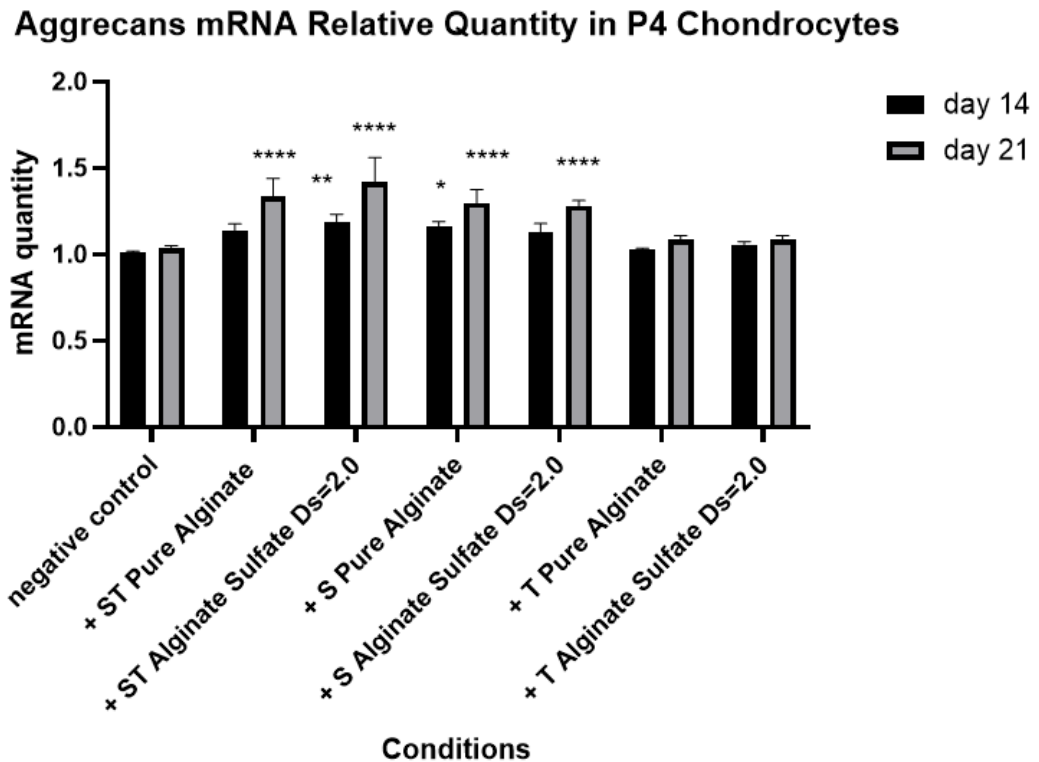


Figure 21: Aggrekans mRNA Relative Quantity in P4 Chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside and TGF- β (+ ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+ S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+ T Alginate Sulfate Ds=2.0), on days 14 and 21

The graph in figure 21 displays the relative quantity of Aggrekans mRNA in Passage 4 (P4) chondrocytes under the same discussed conditions at day 14 and day 21. The treatment with ST on pure alginate resulted in an upsurge in Aggrekans mRNA levels,

attaining approximately 1.5 at day 14 ($p=0.0665$) and 1.6 at day 21 ($p<0.0001$). The ST condition on alginate sulfate also showed a significant increase on day 14 ($p=0.0044$) and day 21 ($p<0.0001$). As for the treatment consisting of Salidroside alone on pure alginate and alginate sulfate, on day 14 it showed significant increases on pure alginate but not on alginate sulfate ($p=0.0218$ for pure alginate and $p=0.0873$ for alginate sulfate), and on day 21 both results were significant in contrast to the negative control ($p<0.0001$ for pure alginate and alginate sulfate), with mRNA levels reaching around 1.4 and 1.3 respectively. TGF- β alone in both media showed slight, non-significant increases, with values around 1.1 to 1.2, indicating a limited impact on Aggrecans expression (on day 14, $p=0.9989$ for pure alginate and $p=0.8612$ for alginate sulfate; on day 21, $p=0.7721$ for pure alginate and $p=0.7628$ for alginate sulfate).

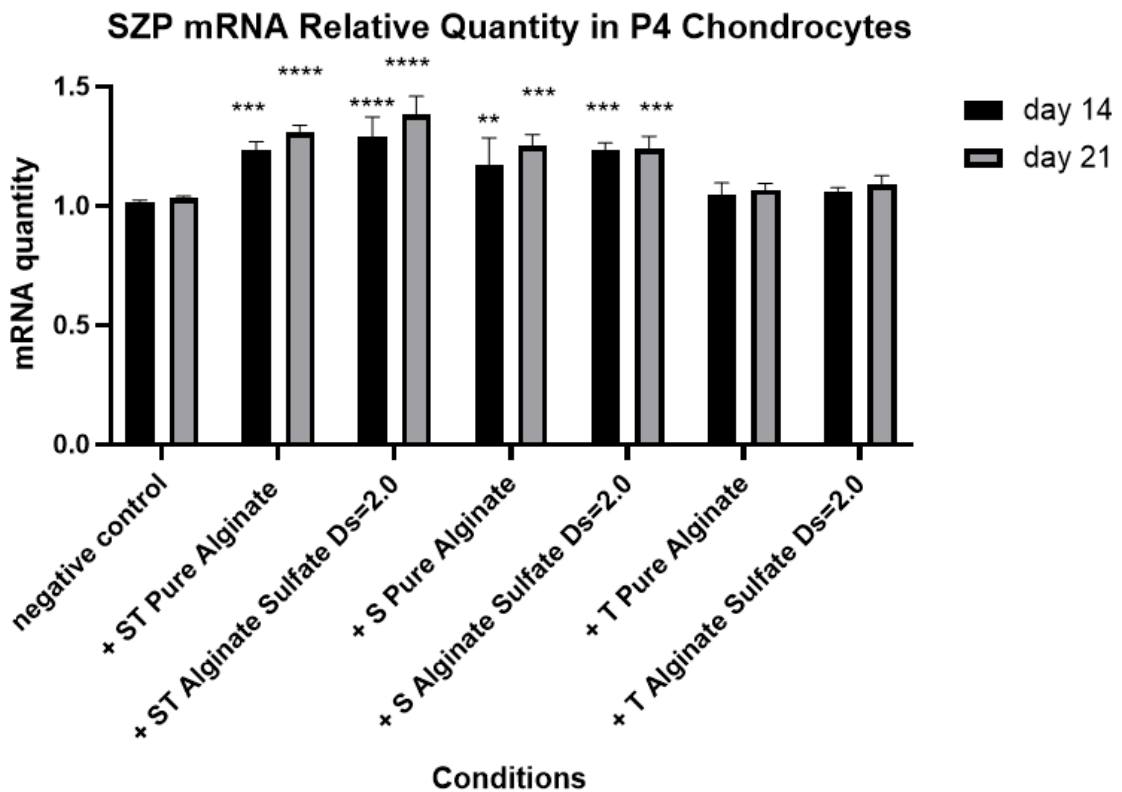


Figure 22: SZP mRNA Relative Quantity in P4 Chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside and TGF- β (+ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+T Alginate Sulfate Ds=2.0), on days 14 and 21

The graph in figure 22 presents the relative quantity of SZP mRNA in P4 chondrocytes under the aforementioned conditions on days 14 and 21. The ST combination on pure alginate displayed significant increases, reaching approximately 1.4 at day 14 ($p=0.0002$) and 1.5 at day 21 ($p<0.0001$). ST on alginate sulfate also exhibited significantly higher levels in contrast with the negative control, reaching around 1.4 ($p<0.0001$ on days 14 and 21). Salidroside alone displayed significant increases in SZP

mRNA on both matrices on day 14 ($p=0.0089$ on pure alginate and $p=0.0002$ on alginate sulfate) and on day 21 ($p<0.0001$ on pure alginate and on alginate sulfate). The treatment consisting of TGF- β alone showed non-significant impact, with levels around 1.1 to 1.2, on both day 14 ($p=0.9726$ on pure alginate and $p=0.8239$ on alginate sulfate) and day 21 ($p=0.9396$ on pure alginate and $p=0.6146$ on alginate sulfate).

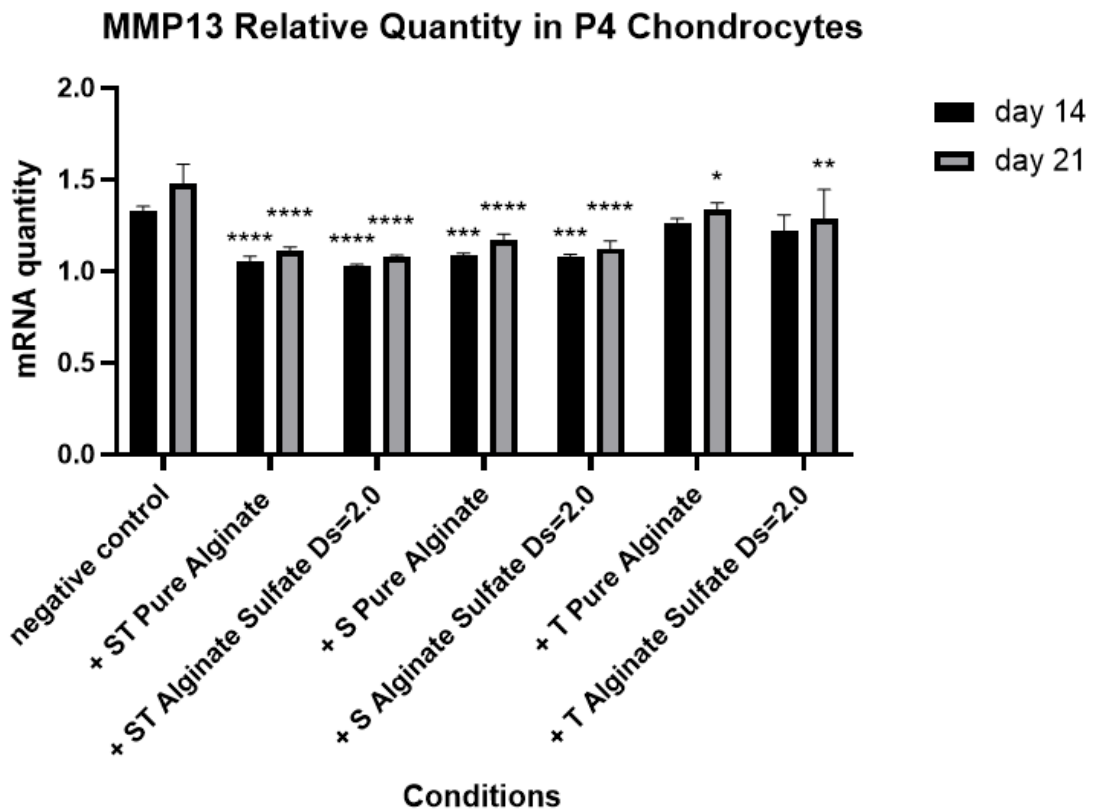


Figure 23: MMP13 Relative Quantity in P4 Chondrocytes, when grown on plastic (negative control), on pure alginate in the presence of Salidroside and TGF- β (+ST Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside and TGF- β (+ ST Alginate Sulfate Ds=2.0), on pure alginate in the presence of Salidroside only (+S Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of Salidroside only (+ S Alginate Sulfate Ds=2.0), on pure alginate in the presence of TGF- β only (+T Pure Alginate), on alginate sulfate of degree of sulfation 2 in the presence of TGF- β only (+ T Alginate Sulfate Ds=2.0), on days 14 and 21.

The graph in figure 23 shows the relative quantity of MMP13 mRNA in P4 chondrocytes under multiple conditions at days 14 and 21. ST treatments on both pure alginate and alginate sulfate showed the most significant reductions in MMP13 expression ($p < 0.0001$), with levels going below 0.8 to 0.9 at both time points. With the treatment consisting of Salidroside alone, results demonstrated a significant on day 14 ($p = 0.0003$ on pure alginate and $p = 0.0002$ on alginate sulfate) and on day 21 ($p < 0.0001$) in contrast to the negative control, with levels reaching around 1.0 to 1.1. When added alone, TGF- β lead to the least significant reduction, on day 14 ($p = 0.6685$ on pure alginate and $p = 0.1623$ on alginate sulfate) but significantly higher than the negative control on day 21 ($p = 0.0386$ on pure alginate and $p = 0.0040$ on alginate sulfate) keeping MMP13 levels around 1.2 to 1.3.

4.4 Protein Expression in P2 Chondrocytes

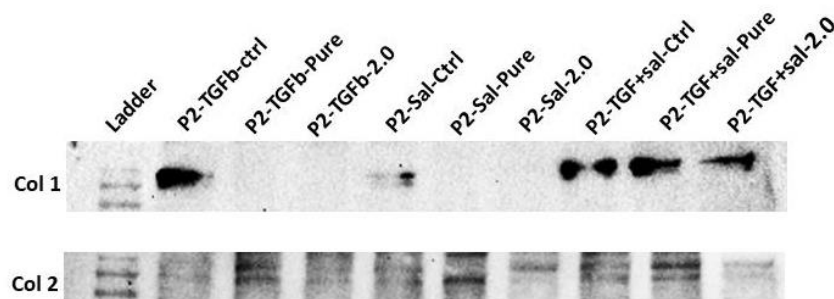


Figure 24: Western Blot results of P2 Chondrocytes under multiple treatments.

The Western blot experiment accurately charts the expression profiles of collagen type I (Col1) and type II (Col2) among various treatment conditions applied to chondrocytes at passage 2 (P2). The experimental factors evaluated consist of a negative control group grown on plastic, pure alginate scaffolds, and alginate sulfate scaffolds with a degree of sulfation of 2, as well as the three different treatments which the transforming

growth factor-beta (TGF- β), Salidroside, and the combination of the two. For P2 chondrocytes, the negative control condition showed a high expression of Col1 among the different treatments, and very low Col2, indicating a substantial dedifferentiation. In contrast, chondrocytes treated with TGF- β or Salidroside alone showed a reduction in Col1 expression and an increase in Col2, especially when these factors were combined with alginate sulfate scaffolds. This alginate sulfate group displayed the lowest levels of Col1 and highest levels of Col2 among all conditions, highlighting the most significant conservation of the phenotype of chondrocytes.

CHAPTER 5

DISCUSSION

5.1 Proliferation Assay

The results obtained out of the proliferation assay of both P2 and P4 chondrocytes prove that the medium on which chondrocytes are cultured has an important impact on their proliferation. Chondrocytes grown on plastic had the lowest proliferation. Those cultured on pure alginate displayed a slower proliferation rate when compared to sulfated alginate, which is highly related to insufficient cell-matrix interactions and nutrients accessibility. Chondrocytes grown on Heparin showed a more effective proliferation, which is most likely because of Heparin's ability to enhance cell adhesion and growth factor interactions. This shows that Heparin offers a more suitable medium for the growth of chondrocytes compared to the control.

The different alginate sulfate scaffolds demonstrated that a higher degree of sulfation can enhance and accelerate chondrocyte proliferation. Chondrocytes on alginate sulfate with higher degrees of sulfation lead to progressively higher growth rates, suggesting that the sulfated alginate matrices allow efficient cell-matrix interactions and growth factor holding, thus enabling a higher proliferation rate of chondrocytes.

These findings echo previous studies, which suggest that sulfated alginate scaffolds, especially those with higher degrees of sulfation, are able to significantly promote chondrocyte proliferation. This is highly important for treatments in cartilage tissue engineering, since the optimization of the ECM would result in enhanced cell growth and more efficient tissue regeneration. The combination of Salidroside and TGF- β provides the best treatment to enhance chondrocyte proliferation compared to adding Salidroside

or TGF- β alone. This effect can be explained by the different but complementary mechanisms of action of TGF- β and Salidroside.

Salidroside, known for its antioxidant and anti-inflammatory characteristics, enhances cell survival and proliferation by controlling multiple signaling pathways, such as the PI3K/Akt and MAPK/ERK pathways. These pathways are highly important for cell proliferation, differentiation, and survival. Salidroside is able to inhibit oxidative stress and inflammation and thus provides a more convenient environment for chondrocyte growth. As for TGF- β , it plays a crucial role in chondrocyte growth and differentiation, as it activates the Smad signaling pathway, which controls gene expression related to cell proliferation and matrix synthesis. Besides, TGF- β promotes the production of ECM components, like proteoglycans and collagen, which are crucial for the synthesis of cartilage tissue and its maintenance. The combination of Salidroside and TGF- β was found to significantly increase the proliferation of chondrocytes. Salidroside's antioxidant and anti-inflammatory functions may increase the responsiveness of chondrocytes to TGF- β by providing them with a more suitable medium. Moreover, TGF- β can intensify the proliferative signals stimulated by Salidroside with its efficient growth-promoting properties. This combination thus leads to a stronger proliferation of chondrocytes, as highlighted by the highest numbers of cells in the combination condition.

5.2 Live/Dead Assay

The live/dead assay results shed a light on the important role of both the ECM composition and the combined use of bioactive factors in enhancing chondrocyte viability and protecting the phenotype. Treatments including Salidroside, particularly when

combined with TGF- β , consistently lead to higher cell viability and a more enhanced protection of the chondrocyte phenotype. Adding TGF- β alone displays limited efficacy, especially in the absence of Salidroside.

Alginate sulfate (degree of sulfation = 2.0) optimizes the effects of Salidroside and TGF- β , as it offers a more supportive extracellular environment that effectively mimics the structure of the cartilage matrix. This combination results in a highly enhanced cell viability and protection of chondrocytes' phenotype, highlighting the important impact of the combination of bioactive factors with an enhanced matrix. Hence, these results portray the value of using a treatment that combines bioactive compounds with modified extracellular matrices for practices involving cartilage tissue engineering. Such treatment can enhance chondrocyte growth, survival, and protection of their morphology, which are essential for an effective cartilage repair and regeneration.

5.3 Gene Expression

The set of graphs presents an important assessment of the impact of different conditions on the mRNA expression levels of key markers in Passage 2 (P2) chondrocytes and Passage 4 (P4) chondrocytes. The conditions consist of a negative control and treatments with pure alginate or alginate sulfate (degree of sulfation = 2.0) in the presence of Salidroside (S), TGF- β (T), and a combination of Salidroside and TGF- β (ST). The markers studied are Col2/Col1, Aggrecans, MMP13, SZP, and SOX9/RUNX2.

The data show that the multiple treatments have a significant impact on the expression of Col2 relative to Col1 in P2 and P4 chondrocytes, highlighting the factors that best prevent chondrogenic dedifferentiation. In the negative control, the low levels of

Col2/Col1 ratio shows that P2 and P4 chondrocytes display a low level of chondrogenic differentiation. This initial result sheds a light on the necessity of growth factors and bioactive components to promote chondrogenesis.

In the pure alginate treatments, both treatment with Salidroside alone and the treatment in which Salidroside is combined with TGF- β lead to significant surges in the Col2/Col1 ratio, highlighting their efficient role in preventing chondrogenic dedifferentiation. Salidroside, known for its anti-inflammatory and antioxidant properties, likely enhances cell survival and matrix production, creating an environment conducive to chondrocyte differentiation. The lower efficiency of TGF- β alone in pure alginate conditions reveals that while TGF- β is crucial for chondrocyte proliferation, it needs supplementary support from compounds like Salidroside to maximize its impact.

The conditions including alginate sulfate with degree of sulfation of 2.0 lead to the highest Col2/Col1 ratios, specifically with the treatment by both Salidroside and TGF- β combined. For instance, the higher degree of sulfation in alginate sulfate improves the binding affinity for growth factors and provides the natural ECM environment, offering ideal settings for enhancing the proliferation and maintaining the phenotype chondrocytes. The combination condition seems to enhance both cell proliferation and prevents dedifferentiation through better cell-matrix interactions and growth factor binding.

The Col2/Col1 ratio is an essential indicator of chondrogenic dedifferentiation, since Col2 (type II collagen) is a major component of cartilage and Col1 (type I collagen) is mainly associated with fibrotic tissue. The results reveal that the combination of Salidroside and TGF- β , especially with alginate sulfate of degree of sulfation of 2.0,

significantly increases the Col2/Col1 ratio, suggesting better chondrogenic differentiation and lesser chondrogenic dedifferentiation. This condition efficiently showed the highest ratios, proving that the combined treatment is the best at enhancing the extracellular environment for the production of cartilage-specific markers.

Aggrecans are crucial proteoglycans present in cartilage, important for its structure and role. The combination of Salidroside and TGF- β in alginate sulfate also lead to the highest Aggrecans mRNA levels, reinforcing the role of this treatment in providing a suitable chondrogenic environment. Besides, pure alginate treatments with Salidroside alone or TGF- β alone showed higher Aggrecans expression but to a lesser extent, highlighting the important superior effect of the combined treatment in enhancing matrix synthesis.

Matrix metalloproteinase-13 (MMP13) is a catabolic enzyme that works on the breakdown of cartilage matrix components, hence its inhibition is required in the process of chondrogenic differentiation. The treatments that consisted of Salidroside alone, or its combination with TGF- β , both lead to important reduction in MMP13 mRNA levels, especially in alginate sulfate. This reveals a decrease in matrix degradation and an environment more favorable to the stability and protection of cartilage.

SZP (superficial zone protein) is important for the lubrication of cartilage lubrication and the functioning of joints. The highest SZP mRNA levels were obtained with the treatment consisting of Salidroside and TGF- β together in alginate sulfate, highlighting that this mixture is able to stimulate matrix synthesis and also optimize the production of proteins crucial for the functions of joint. Each of Salidroside and TGF- β alone also

increased the expression of SZP, but the combination treatment showed the highest impact.

The SOX9/RUNX2 ratio shows the equilibrium between chondrogenic and osteogenic differentiation, with SOX9 stimulating chondrogenesis and RUNX2 stimulating osteogenesis. A high SOX9/RUNX2 ratio is favorable for chondrogenic differentiation. Combining Salidroside and TGF- β , particularly with alginate sulfate, resulted in the highest ratios, proving that this treatment efficiently stimulates chondrogenic over osteogenic differentiation. This shows that treatment consisting of Salidroside, TGF- β and alginate sulfate, enhances the environment for cartilage-specific gene expression while inhibiting osteogenic mechanisms.

Therefore, the factors of this treatment promotes the expression of chondrogenic markers (Col2 and Aggrecans), inhibits catabolic activity (MMP13), and increases the synthesis of important proteins like SZP, while keeping a high SOX9/RUNX2 ratio which indicates efficient chondrogenic differentiation.

For instance, Salidroside has antioxidant and anti-inflammatory properties, which endow it to inhibit oxidative stress and inflammation. Hence, it creates a more adequate medium for chondrocyte growth and survival. By controlling signaling pathways such as PI3K/Akt and MAPK/ERK, Salidroside promotes cell growth and prevents dedifferentiation. As for TGF- β , it is a powerful growth factor that stimulates chondrogenesis by activating the Smad signaling pathway, which controls the expression of genes involved in cartilage synthesis. It enhances the production of ECM components and promotes chondrocyte growth while preventing their dedifferentiation. However, its full impact is attained when it is combined with other efficient compounds such as

Salidroside. Alginate sulfate, particularly with a high degree of sulfation, mimics the ECM composition and structure more specifically than pure alginate. Its higher binding affinity for growth factors like TGF- β offers a more stable and suitable environment for chondrocytes. This improved matrix interaction provides more efficient cell attachment, proliferation, and differentiation, resulting in improved results in cartilage tissue engineering.

5.4 Western Blot

The patterns in the expression of Col1 and Col2 display important insights into the state of chondrocytes under different culture treatments and factors. The consistent reduction in expression of Col1 when going from plastic to alginate and further to alginate sulfate highlights the efficiency of these substrates in preserving chondrocyte phenotype, essentially against dedifferentiation. Alginate sulfate, with its higher degree of sulfation, is expected to offer a more suitable microenvironment to chondrocytes in contrast to pure alginate. This is due to better imitation of the ECM, providing more enhanced cell-matrix interactions crucial for protecting the identity of chondrocytes. However, this wasn't detected clearly in the results of the current Western Blot performed and might require further runs to be better perceived.

The treatments with TGF- β alone and Salidroside alone, especially when combined with alginate sulfate, significantly lead to the reduction of Col1 expression and simultaneously increased the amount of Col2. This dual effect highlights the therapeutic potential of these conditions in not only stopping chondrocytes' dedifferentiation but also contributing to the expression of matrix components that are essential for the proper function of the cartilage. The efficiency of these combinations could be highly promising

for late passage chondrocytes which are highly prone to dedifferentiation because of their prolonged culturing. This experiment suggests that the combination of TGF- β and Salidroside with alginate sulfate could be an important tool in preventing or inhibiting the progressive loss of chondrocyte phenotype even in late passages.

Overall, the results confirm that the choice of the adequate scaffold and bioactive factors is crucial to enhance the stability and health of chondrocytes in culture. Alginate sulfate, especially in presence of TGF- β and Salidroside, offers a promising solution to maintain late passage chondrocytes phenotypes, by significantly decreasing the production of dedifferentiation markers and promoting the expression of essential cartilage proteins. This technique has potent implications at the level of regeneration and repair of cartilage, providing a hopeful opportunity for enhancing therapeutic strategies in osteoarthritis and other illnesses affecting the cartilage.

CHAPTER 6

CONCLUSION

The work of this thesis highlights the benefits of combining Salidroside and TGF- β together, in order to significantly promote the proliferation, viability, and prevent the chondrogenic dedifferentiation of late passage chondrocytes, especially when cultured on alginate sulfate matrices with a high degree of sulfation such as DS=2. The findings obtained from the various assays shed a light on Salidroside's antioxidative and anti-inflammatory characteristics, along with TGF- β 's potent chondrogenic potential, which provides an optimal environment for maintaining the phenotype of chondrocytes and preventing their dedifferentiation. This combination treatment, particularly on a alginate sulfate, leads to enhanced cell-matrix interactions, optimized retention of growth factors, and suitable expression of key chondrogenic markers such as Col2, Aggrecans, and SOX9, while reducing dedifferentiation markers such as Col1, and inhibiting the catabolic activity revealed by decreased MMP13 expression. These results have promising implications for approaches involving cartilage tissue engineering to treat osteoarthritis. In fact, these findings indicate that such efficient treatments are able to optimize chondrocyte performance and result in more efficient cartilage repair and regeneration techniques. Future work should delve deeper into the molecular processes of the synergistic effects of this combination treatment, in order to better refine and benefit from their potential in tissue engineering procedures.

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