**FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO**


# MSCs chondrogenesis in 3D scaffolds

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# *Abstract*

Articular cartilage has a very limited intrinsic capacity for repair after injury. To date, no effective treatment exists for full thickness articular cartilage defects. Numerous Tissues Engineering works have focused in chondrogenic differentiation, aiming to improve the wellbeing of patients that suffer from cartilage degeneration.

Cartilage is comprised basically of matrix and chondrocytes. Multipotent Mesenchymal Stromal Cells (MSCs) can differentiate into chondrocytes and thus are a source of cells for cartilage repair. For *in vitro* chondrogenesis, MSCs must be cultured in a 3D scaffold, enhancing interactions between cells. Chitosan has a high potential for construction of 3D scaffolds for cartilage regeneration due to its versatility of processing, antimicrobial properties, enzymatic biodegradability and low immunogenicity. *In vivo,* cartilage tissue has around 1%-5% of oxygen pressure, much lower than the atmospheric oxygen tension (20%). In this context, the main objective of this work was studying the effect of oxygen pressure ($pO_2$) on differentiation of hMSC into chondrocytes in 3D chitosan scaffolds.

Bone marrow human (h)MSCs were seeded at 800 000 cells/20µl in chitosan scaffolds and incubated in 20% or 5% $pO_2$ for up to 28 days. Cells were cultured in medium without or with factors that induce chondrogenesis, dexamethasone and TGF-$\beta_3$ (basal and chondrogenic medium). Samples were analyzed by Scanning Electron Microscopy, Live/Dead assay, hematoxylin/eosin (H&E) and toluidine blue staining, detection of collagen *type II* and GAGs / DNA quantification.

Upon H&E staining, cells and matrix were visualized within the scaffolds. In chondrogenic samples at 5%$pO_2$ cells were organized in clusters. Upon toluidine blue staining, samples should appear pink or blue, depending on the existence or not of GAGs. Although distinction between these two colors was difficult, due to the presence of tightly packed clusters, it was possible to visualize pink coloring in chondrogenic samples at 5%$pO_2$. In these conditions, collagen *type II* could be detected by immunohistochemistry. Importantly, quantification of GAGs by alcian blue indicates that more proteoglycans were produced in hypoxia than in 20% of oxygen pressure, with GAGs being produced even in basal conditions, if cells are cultured at 5%$pO_2$.

This work suggests that hMSCs can differentiate in chondrocytes and that hypoxic conditions (5% $pO_2$) lead to efficient chondrogenesis within chitosan scaffolds.

# *Resumo*

A cartilagem articular tem uma capacidade limitada de reparação após trauma. Até hoje, não se encontraram tratamentos efetivos para preenchimento dos defeitos cartilagíneos. Inúmeros trabalhos de Engenharia de Tecidos têm como foco a diferenciação condrogénica, com o objectivo de melhorar o bem-estar dos pacientes que sofrem desse tipo de degeneração.

A cartilagem é essencialmente composta por matriz e condrócitos. As Células Mesenquimais do Estroma (MSC) podem diferenciar-se em condrócitos e assim serem uma fonte celular para a reparação cartilagínea. Para a condrogénese *in vitro*, as MSC devem ser cultivadas em estruturas 3D, que permitem uma melhor interação celular. O quitosano tem um elevado potencial para ser usado como estruturas para regeneração cartilagínea, devido à sua versatilidade no processamento, propriedades microbianas, biodegradação enzimática e baixa imunogenicidade. *In vivo*, o tecido cartilagíneo encontra-se num ambiente de 1%-5% de pressão de oxigénio, pressão muito menor que a atmosférica (20%). Neste contexto, o principal objectivo deste trabalho foi estudar o efeito da pressão de oxigénio ($pO_2$) na diferenciação das hMSC em condrócitos em estruturas 3D de quitosano.

MSC de médula óssea humana (h) foram implantadas a uma densidade de 800 000 células/20µl em esponjas de quitosano, que posteriormente foram incubadas em 20% e 5% $pO_2$ durante 28 dias. As células foram mantidas em meios sem e com factores de indução da condrogénese, dexametasona e TGF-$\beta_3$ (designados como meios basais e condrogénicos, respectivamente). As amostras foram analizadas por Microscopia Electrónica de Varrimento, Live/Dead, colorações de hematoxilina/eosina (H&E) e toluidine blue, deteção de colagénio *tipo II* e quantificação de GAGs/ADN.

Após a coloração da H&E as células e matriz foram vizualizadas no interior das estuturas de quitosano. Nas amostras condrogénicas a 5%$pO_2$ as células aparecem organizadas em aglomerados. Na coloração de Toluidine blue as amostras aparecem de cor rosada ou azul, dependendo da presença ou não de GAGs. Embora a distinção entre as cores tenha sido difícil, devido à presença de aglomerados celulares, foi possível detectar cor rosa nas amostras condrogénicas a 5%$pO_2$. Nessas mesmas amostras o colagénio *tipo II* pode ser também detectado por imunohistoquímica. A quantificação dos GAGs por Alcian Blue indicou a presença de uma maior quantidade de proteoglicanos em hipoxia do que nas condições normais de oxigénio. Mesmo em condições basais a 5%$pO_2$ houve um aumento na quantidade de GAGs quando comparado com as amostras condrogénicas a 20%$pO_2$.

Este trabalho sugere que hMSCs podem diferenciar-se em condrócitos e que as condições de hipoxia (5%$pO_2$) favorecem a condrogénse em estruturas de quitosano.

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# *ABBREVIATIONS*

**AG:** anionic glycoconjugate

**ATP:** adenosine triphosphate

**BSA:** bovine serum albumin

**CsC:** chondroitin sulfate

**Col2a1:** collagen type II

**DA:** degree of acetylation

**DNA:** deoxyribonucleic acid

**dsDNA:** double-stranded DNA

**DMEM:** Dulbecco®'s Modified Eagle Medium

**ECM:** extracellular matrix

**EDTA:** diaminoethanetetraacetic acid

**EthD-1:** ethidium homodimer-1

**FBS:** Fetal bovine serum

**GAG:** glycosaminoglycan

**G-CSF**: granulocyte colony stimulating factor

**GM-CSF:** granulocyte-macrophage colony stimulating factor

**HA:** hyaluronic acid

**HCl:** hydrochloric acid

**IDO**: indoleamine 2,3-dioxygenase enzyme

**IFN-δ:** gamma- interferon

**IGF:** insulin-like growth factor

**ILs:** Interleukins

**ITS:** Insulin-transferrin-selenium

**YIGSR:** tyrosine-isoleucine-glycine-serine-arginine

**LIF:** leukemia inhibitory factor

**M-CSF**: macrophage colony stimulating factor

**MSC:** mesenchymal stem cell

**Ncad:** N-cadherin

**Ncam:** neural cell adhesion molecule

**NO:** nitric oxide

**$pO_2$:** oxygen pressure

**poly(HEMA):** poly-2-hydroxyethyl methacrylate

**PGA:** polyglycolic acid

**PG:** proteoglycans

**PGs:** prostaglandins

**RFU:** relative fluorescence units

**RGD:** arginine-glycine-aspartic acid

**RNA:** ribonucleic acid

**RT:** room temperature

**RT-PCR:** Reverse transcription polymerase chain reaction

**SEM:** scanning electron microscopy

**SOX:** Sry-type high mobility group box

**SRY-SOX9:** sex determining region Y - Sry-type high mobility group box 9

**SCF:** stem cell factor

**$H_2SO_4$:** sulfuric acid

**TGF-$\beta_s$:** transforming growth factors - β

**3D:** three dimensional

**TNC:** tenascin

**TNFs:** alpha tumor necrosis factor

**VEGF:** vascular endothelial growth factor

**W/V:** weight per volume

# *CHAPTER I*

# *Introduction*

## 1. Tissue Engineering

Tissue engineering is a recent field of research that involves the application of engineering science and technology to problems arising in Medicine and Biology. In 1993, it was defined as "… *an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function*" (Langer R., 1993).

The development of novel biomaterials is an interactive process that involves the creation of increasingly safer and more physiologically appropriate replacements for damaged or diseased human tissues. Biomaterial investigation has been stimulated by evolutions in Molecular Biology, Material Science, Engineering and mainly by understanding the interactions of materials with the physiological environment. One of the goals of this science was the creation of materials that exhibited inert behavior when placed in the body, i.e. were *bioinert*. These materials should also have: appropriate mechanical properties; corrosion resistance; and an absence of injurious effects such as toxicity, inflammation and allergy. The development of more efficient biomaterials requires improved rates of degradation and a combination of strength, flexibility and a combination of chemical molecules with the biomaterial (Griffith, 2000) (Hubbell A. et al., 1999) (Langer R. et al., 1999). These biomaterials are in development to promote or inhibit specific cell activities and then, such materials should provide the foundation for the molecular design of scaffolds that can be seeded with cells *in vitro* and subsequently implanted *in vivo.* Scaffolds, composed of synthetic polymer or natural materials, are designed in the nanoscale to mimic the natural extracellular environment and to help cell proliferation and differentiation (Hoerstrup S. et al., 2004) (Narayan R. J., 2010). A typical scaffold is a bioresorbable polymer in a porous configuration and desired geometry for a specific cell population.

In Tissue Engineering, biological and engineering challenges are focused on the components, cells, scaffolds and their control. One goal is to replace the lost tissue function by stimulating an interaction between materials (scaffolds), cells and growth factors, to generate a temporary structure that will deliver healthy cells into a scaffold in an injury local. Therefore, the first phase to promote regeneration of a functional tissue is the *in vitro* production of a tissue construct by chosen cells and scaffolds that provide an appropriate support environment for cells to proliferate and elaborate extracellular matrix. In the second phase, tissue constructs are implanted, *in vivo*, in the place of injury to recapitulate the normal functional architecture of the tissue (Fig.1). These steps constitute the general paradigm of tissue engineering (Hoerstrup S. et al., 2004).

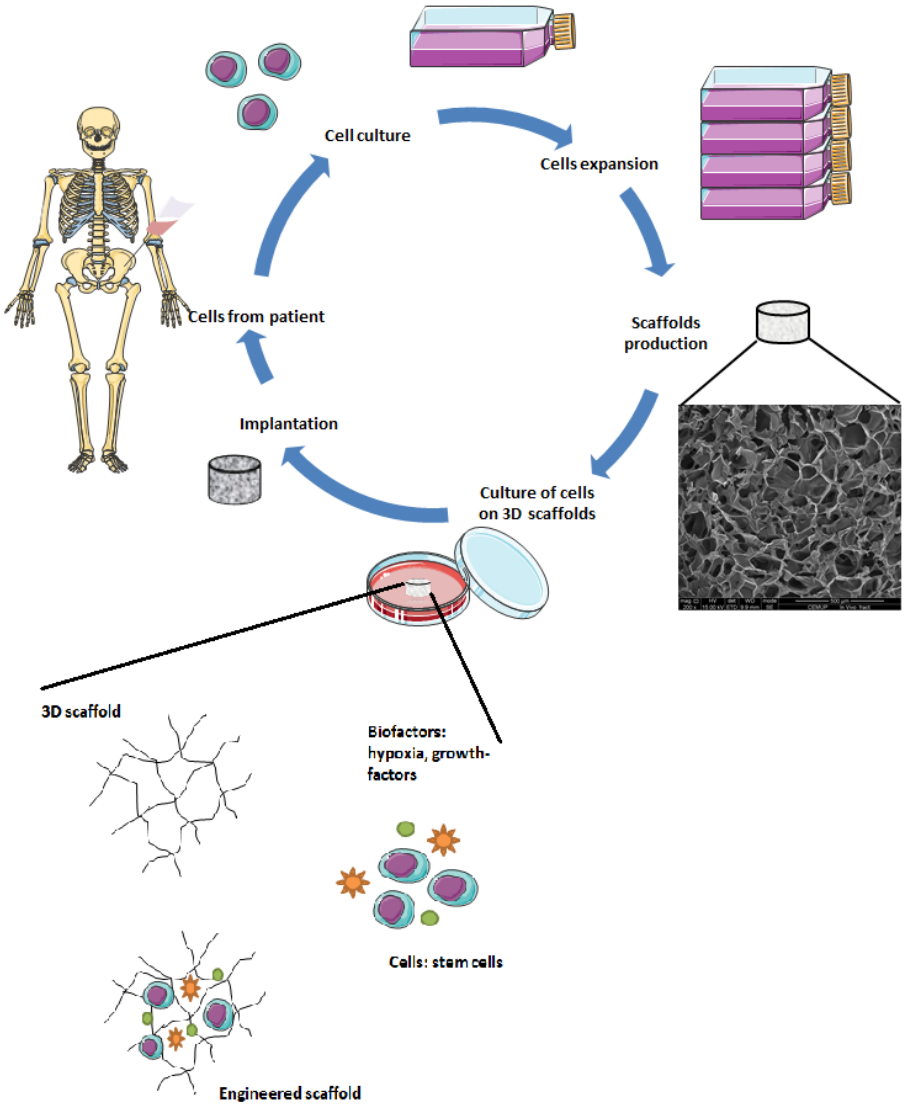


**Figure 1:** The cycle of Tissue Engineering. First, cells are removed from patients; second, these cells are cultured *in vitro*; third, cells are expanded; fourth, 3D scaffolds are produced; fifth, stem cells are cultured into scaffolds and stimulated to differentiate with biofactors. In these 3D scaffolds, cells and biofactors contact more easily between them than in 2D cultures. And finally, scaffolds with cells are implanted in the patient.

## 1.1. Three-dimensional scaffolds, a support to cells

Although 2D structures allow a good control of the components that cells find in their niche, the need to recreate the environment of cells in the human body has prompted investigation for new methods in tissue engineering.

The first aim in scaffold design is to achieve some level of mechanical support and regulate diffusion of nutrients and waste products. Biomaterials such as ceramics, metals and polymer composites, have indeed been successful in replacing the mechanical function of tissues, but have a limited ability to modulate repair and regeneration of host tissues (Balasundaram G. et al., 2007). A point to recognize as important in regulating behavior of cells is differences in the size of microstructures of materials. Cell migration or cell contraction can reduce the size of pores in these structures. This decrease can lead to a reduction in nutrients exchange between cells in the center and periphery of the structure. (Subramanian A. et al., 2005). The chemical composition and structure of scaffolds can also affect cell behavior and determine the performance of scaffolds, particularly affecting cell adhesion, spreading and phenotype of cells due to the change that scaffolds cause in glycoproteins involved in cell-cell adhesion (Amaral I.F. et al., 2005). For these reasons, the physical characteristics (porosity, pore geometry, interconnectivity, and architecture) and the chemical characteristics (surface content) of scaffolds influence cell function, shape, alignment, organization and apoptosis (Chan G. et al., 2008) (Mao J. et al., 2004). To promote the adhesion of extracellular matrix (ECM) proteins to scaffold and to increase the intracellular signaling by interaction of surface integrin receptor of cells with the substrates, biomaterials can be modified with peptide fragments such as, arginine-glycine-aspartic acid (RGD) and tyrosine-isoleucine-glycine-serine-arginine (YIGSR) (Chan G. et al., 2008).

To avoid potential problems for cells embedded in a 3D environment such as a lack of nutrients or other macromolecules, due to its difficulty in getting deep into a scaffold, these should have pores with sizes adequate for cellular development (Lutolf M. P. et al., 2009). In this context, an ideal scaffold should have a highly porous architecture with many interconnected pores and exhibit a high surface area-to-volume ratio, to support high rates of mass transfer, cell growth and vascularization (Mata A. et al., 2009). Also, scaffolds must include chemical signs for interaction with or mimicry of extracellular matrix components, growth factors and cells surface receptors (Sundararajan V. et al., 1999).

# 2. Endochondral Regeneration

Some cartilage tissues such as the nasal septum and articular cartilage persist throughout life, while others undergo changes leading to cartilage calcification and replacement by endochondral bone. The development of the skeleton through endochondral bone formation is one of the most complex processes in Biology. Creation of bone from cartilage, or endochondral ossification, involves the destruction and removal of cartilage and production of bone in the space formally occupied by cartilage, a mechanism that is crucial for longitudinal bone growth. It begins with migration of undifferentiated mesenchymal cells to sites destined to become bone. These cells undergo a condensation step and start expressing various cell adhesion molecules such as: all splice variants of type II collagen, N-cadherin, N-cam and tenascin C, and also expressing an important transcription factor for chondrogenesis and chondrocyte differentiation, SRY-box9 or SOX-9 (Zuscik M. J. et al., 2008). During the differentiation of mesenchymal stem cells in chondrocytes, there is production of an extracellular matrix rich in collagen *type II* and aggrecan. During this process, the region of resting chondrocytes differentiates into a zone of proliferative chondrocytes, called hypertrophic chondrocytes. The changes associated with maturation of chondrocytes include a switch from *type II* and type IX collagen to *type X* collagen synthesis, the induction of a high level of alkaline phosphatase activity and the expression of several matrix proteins (e.g. osteopontin and osteocalcin) (Leboy P. S. et al., 1997) (Gerber H.P. et al., 1999) (Sekiya I. et al., 2002). Oliveira *et al*. (Oliveira S. et al., 2008) investigated chondrocyte maturation *in vitro* and *in vivo* in a chicken embryo, and to analyze the maturation of chondrocytes, these were characterized by the presence of *type X* collagen in extracellular matrix and by increased alkaline phosphatase activity. In this study, hypertrophic chondrocytes were capable of promoting the last phase of endochondral bone formation.

Hypertrophy is the final stage in the life of chondrocytes. Upon its apoptosis, hypertrophic chondrocytes release angiogenic growth factors that diffuse into nearby tissues and trigger a cascade of events that includes: (I) invasion of endothelial cells into the hypertrophic zone, (II) vascularization of the zone, (III) attraction and attachment of osteoprogenitor cells to the hypertrophic matrix, (IV) proliferation and differentiation of the cells into mature bone cells, (V) destruction of the hypertrophic matrix coupled with creation of new bone matrix, (VI) mineralization of the bone matrix, (VII) bone remodeling associated with the proliferation and differentiation of hematopoietic stem cells. The end result is the replacement of hypertrophic cartilage by a calcified bone complete with functional marrow (Talts J. T. et al., 2006). During endochondral ossification distinct zones of cells are morphologically and molecularly identifiable by specify markers as shown in figure 2.

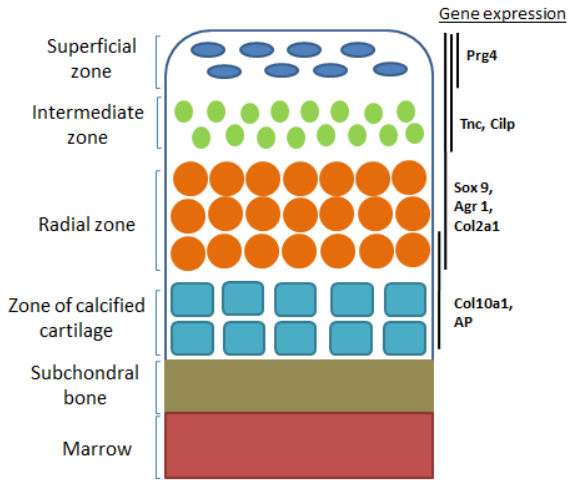


**Figure 2:** During articular cartilage development in endochondral ossification four cellular areas are visible: the superficial, intermediate, radial and calcified cartilage zones. Cells in the superficial zone express proteoglycan 4 (Prg4), Sox 9, Col2a1, Agc1, tenascin C (Tnc) and low levels of cartilage intermediate layer protein (Clip). Cells in the intermediate zone express the same molecules as in the superficial zone but with low levels of Prg4 and high levels of Clip. Chondrocytes in the radial zone are spherical and express markers of chondrocyte differentiation such as, Col10a1 and alkaline phosphatase (AP). [Adapted from: Zuscik M. et al.2008]

## 3. Cartilage, an avascular tissue

Articular cartilage is an avascular tissue that covers the surface of all the diarthrodial joints in the human body. Articular cartilage is a highly specialized tissue that provides low friction and allows efficient load bearing and its distribution, thus being critical in the movement of one bone against another. Its major constituents are specialized cells, chondrocytes. Its nourishment is supplied by synovial fluid produced by synovial vessels and synoviocytes (Imhof H. et al., 1997). The chondrocytes are embedded in a hyperhydrated extracellular matrix (water content ranges from 66% to 80%), with 20%-34% of solid components, 5%-6% of which are inorganic components (hydroxyapatite). Among the remaining organic contents, 48%-62% is composed of collagen *type II* and 22%-38% is composed of proteoglycans (McDevitt C. et al., 1994). Proteoglycans exert osmotic pressure to draw water into cartilage, with mechanical pressures forcing water out and producing an expansion pressure that is resisted by the tension in collagen fibers. These collagen fibrils are oriented in response to pressure, traction and shear forces (Imhof H. et al., 1997). The rigidity and elasticity of

the tissue are a consequence of the relative decompressibility of the proteoglycan molecules. The thick bundle of collagen fibers paralleled to the articular surface serves, not only as a restrictive layer, but also as a distributor of compression strengths (Brandt K. et al., 1993) (Neame P. J. et al., 1990). This mixture of fluids provides viscoelastic and mechanical properties for efficient function of cartilage (Vinatier C. et al., 2009).

As referred, extracellular material consists primarily of large hydrated proteoglycan aggregates, entrapped within a matrix of collagen fibrils. The entrapped proteoglycans are composed of a core protein that forms a backbone to which many glycosaminoglycan (GAG) chains covalently attached (Fig. 3). The chains extend perpendicularly from the backbone of the protein allowing trapping of large amounts of water. The molecular structure of GAGs consists of an unbranched polysaccharide made of a repeating disaccharide unit, which contains an acetylated amino sugar moiety (e.g. *N*-acetylglucosamine) and a sugar acid moiety (e.g. glucuronic acid). The amino sugar moieties in most GAGs are sulfated, hence increasing their anionic character.

The three major GAGs found in cartilage in order of abundance are chondroitin sulfate, keratan sulfate and hyaluronan. Hyaluronan (hyaluronic acid or HA) is unique amongst GAGs in that it does not contain any sulfate and is not involved in covalent attachment to any core protein. Numerous chains of chondroitin sulfate and keratan sulfate are covalently linked to a core protein which corresponds to the abundant cartilage proteoglycan, the aggrecan monomer.

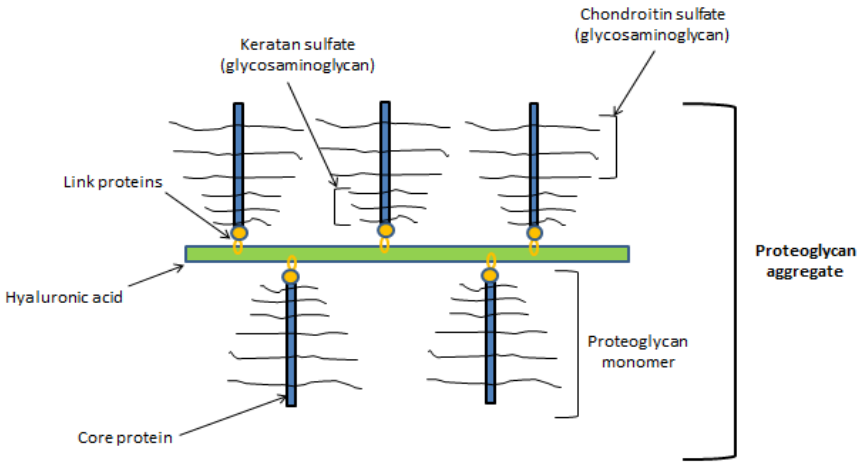


**Figure 3:** Diagram of part of a proteoglycan aggregate. The predominant proteoglycan monomer is aggrecan. The extracellular matrix of cartilage is composed of proteoglycans attached to a backbone of hyaluronic acid. Proteoglycan monomers have both chondroitin sulfate and keratin sulfate rich regions, and link proteins facilitate binding of monomers to hyaluronic acid.

In the normal metabolism of cartilage, chondrocytes guide the recycling of matrix components to answer the internal needs of remodeling. In the normal adult,

homeostasis of the matrix avoids either losses or gains of tissue. These processes are controlled by a variety of proteins named growth factors and inflammatory cytokines (Pelletier J. et al., 1993). Growth factors, such as insulin-like growth factor-1 (IGF-1) and transforming growth factor beta (TGF-β) stimulate aggrecan and collagen synthesis. These growth factors also modulate the anabolic and catabolic pathways of chondrocytes metabolism. Inflammatory cytokines, such as interleukins-1 (IL-1), interleukins-6 (IL-6) and alpha tumor necrosis factor (TNFs) stimulate matrix degradation (Majumdar M.et al., 2001) (Ehrlich M. et al., 2005).

The development of chondrocytes is the dynamic cellular process that leads to the establishment of various types of cartilage: hyaline, fibrous and elastic cartilage. Hyaline cartilage occurs in the trachea, the larynx, and also at the ends of bone, where it forms joints. It is responsible for the longitudinal growth of bone and reduces friction at joints. Temporary cartilage in mammalian embryos also consists of hyaline cartilage. Fibrous cartilage is found in discs between the vertebrae, in between the pubic bones and around the edges of the articular cavities such as the cavity in the shoulder joint. Cartilage tissue absorbs shocks that would otherwise be damaging. Elastic cartilage is similar to hyaline cartilage, but in addition to the collagenous fibers, the matrix of elastic cartilage also contains an abundant network of branched yellow elastic fibers. Elastic cartilage helps to maintain the shape and flexibility of joints in bones and strengthens and supports these structures (Zuscik M. J. et al., 2008).

Damaged cartilage has limited spontaneous repair response. The resident cartilage cells, chondrocytes, fail to mount an effective repair process and the cartilage appears unable to recruit local sources of progenitor cells to the articular surface. Cartilage injuries are often very common in patients with sports injuries, joint dysfunction and osteoarthritis (Fig. 4). Osteoarthritis is the most common joint disease that affects articular cartilage in older individuals and whose treatment options are limited. This degenerative process is initiated with the loss of proteoglycans, disruption of collagenous fibrillar network leading to cells apoptosis and deterioration of tissue (Nesic D. et al., 2006). Age is an important factor that causes changes in cartilage tissues such as, structural and biochemical matrix reorganization, surface fibrillation, alteration in proteoglycans composition, increase in collagen linking and decreased tensile strength and stiffness (Buckwalter J.A. et al., 1994). Chondrocytes also decrease their functionality by synthesis of smaller and irregular aggrecan with decrease in synthesis of proteoglycans, increased expression of senescence-associated β-galactosidase activity, telomere erosion and decreased response to insulin-like growth factor (IGF) (Martin J.A. et al., 2004). Programmed cell death (apoptosis) in chondrocytes occurs after injurious impact, leading to release of cytokines and nitric oxide (NO) and also being related to aging.

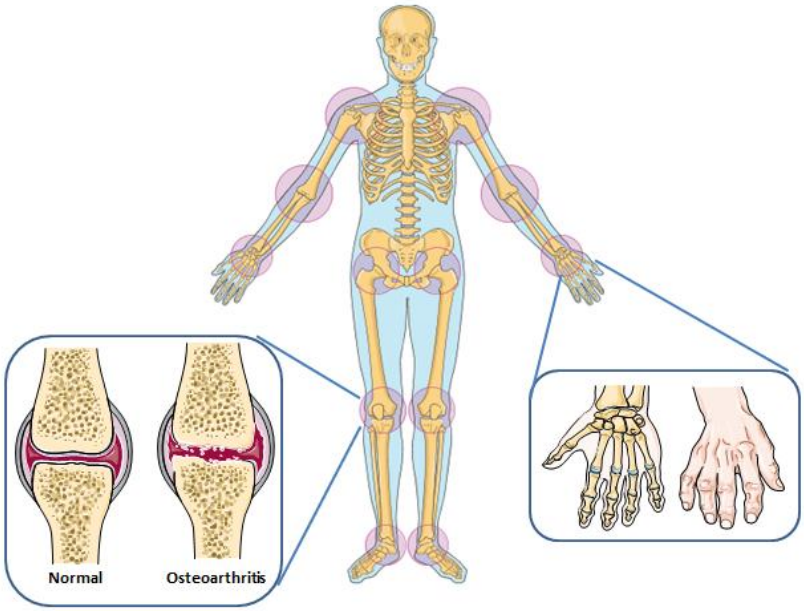


**Figure 4:** Osteoarthritis can appear in points with cartilage tissue. Osteoarthritis is most common in hands and in knees.

The development of new treatments for more efficient repair is very important because current clinical strategies (e.g. abrasion arthoroplasty, chondrocytes implantation or the use of perichondreal autografts) result in development of mechanically inferior fibrocartilage (Diao H. et al., 2009) (Nakagawa T. et al., 2009).

# 4. Chondrogenesis

Cartilage is an avascular tissue with an intercellular protein matrix reinforced by a 3D network of collagen fibrils (Aigner T. et al., 2003). Age or development of traumas in cartilage can be the beginning of extensive pain. Cartilage tissue has a limited self-repair capacity to repair due to the sparse distribution of highly differentiated, non-dividing chondrocytes, slow matrix turnover and low supply of progenitor cells and lack of vascular supply. At the moment, treatment methods for cartilage damage are often not good enough to restore normal function of tissue (Tuan R. et al., 2003).

## 4.1. Induction of chondrogenesis

Chondrogenesis is a process that is important for creation of chondrocytes both during embryogenesis and adult life. The analysis of cells and biomaterials that promote chondrogenesis has a primordial importance.

### 4.1.1. Human Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSCs) are adult multipotential cells with a capacity to differentiate into a variety of connective tissue lineages, including bone (osteoblasts), cartilage (chondrocytes) and fat (adipocytes) (Pittenger F. et al., 1999). Beside these lineages, MSCs can differentiate into myocytes, tendinocytes, cardiomyocytes, hepatocytes, renal cells, endothelial cells and neuronal cells (Djouad F. et al., 2009). The differentiation and self-replication capacities of MSCs open therapeutic opportunities for the repair or regeneration of damaged cartilage and musculoskeletal tissue (Gun-Il Im M. et al., 2006) (Devine S. et al., 2003). Cells with mesenchymal stem cell characteristics have been reported in many tissues including the bone marrow, adipose tissue and muscular tissue (Crisan M. et al., 2008) (Lutolf P.et al., 2009). In culture, MSCs exhibit fibroblastoid morphology, adhesion to plastic, capacity of autorenewal and differentiation. They can be expanded by more than 40 generations keeping the multipotent capacity. However, MSCs should not be used in high passages, because there is a reduction of mitosis rates and there is a high probability of accumulation of mutations (Deans R. et al., 2000) (Pittenger F. et al., 1999) (Reger R. et al., 2008).

MSCs express a large number of bioactive molecules including adhesion molecules, extracellular matrix proteins, cytokines and receptors for growth factors, which allow interactions with other cells (Huss R.et al., 2000). They also produce vascular endothelial growth factor (VEGF), stem cell factor (SCF-1), leukemia inhibitory factor (LIF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukins (IL-1, -6, -7, -8, -11, -14, and -15) and stromal cell-derived factor (SDF-1) (Pittenger M. et al., 2008). MSCs have immunosuppressive and healing capacities, by improving angiogenesis and preventing fibrosis (Djouad F. et al., 2009).

Many cells of the body present a series of surface markers that characterize their biological uniqueness. However, MSCs do not have specific immunophenotypic markers, and their characterization is established by identifying a profile of surface markers (Meirelles L. et al., 2006). MSCs express on the surface CD105 (designated as endoglin), CD73 (designated as ecto 5' nucleotidase) and CD90 (also known as Thy-1). On the other hand, MSCs must lack expression of CD45 (a pan-leukocyte marker), CD34 (primitive hematopoietic progenitors and endothelial cell marker), CD14 or CD11b (both expressed on monocytes and macrophages), CD79α or CD19 (markers of B cells) and HLA class II. To distinguish MSCs (Fig. 5) we must follow three criteria: cells must be adherent to plastic; cells must express on their surface the marker proteins mentioned above; and cells must have the ability to differentiate in mesenchymal lineages under controlled *in vitro* conditions (Dominici M. et al., 2006).

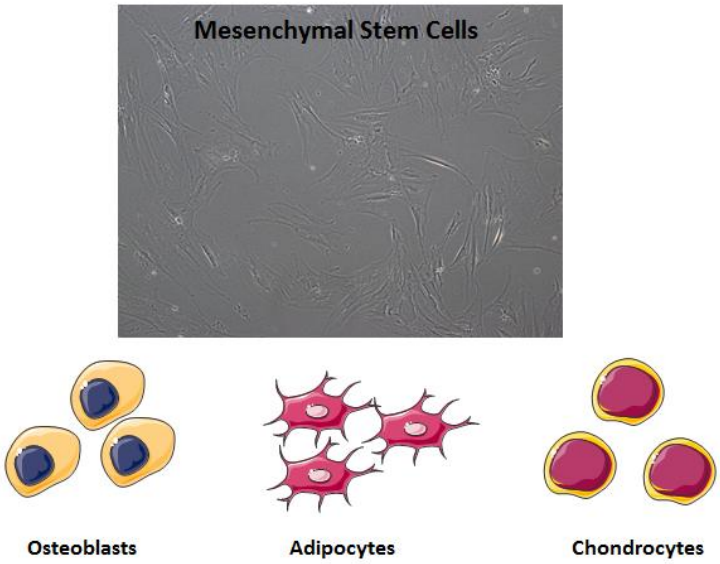


**Figure 5:** Culture-expanded human mesenchymal stem cells (hMSC) exhibit elongated morphology and adherence to plastic. MSCs have multipotential capacity to differentiate into osteoblasts, chondrocytes and adipocytes.

MSCs became the focus of therapeutic strategies due to its multipotent capacity and also to its potential to modulate immune responses with anti-proliferative and anti-inflammatory capacities. By direct contact of MSCs with a tissue (allogeneic or autologous) or through connection with gamma-interferon (IFN-γ), produced by the body's immune cells, MSCs are triggered to release several factors such as, prostaglandins (PGs), interleukins (ILs), transforming growth factors (TGFs), nitric oxide (NO) and indoleamine 2,3-dioxygenase enzyme (IDO), that will act on the cells of the immune system. (Djouad F. et al., 2009).

### 4.1.2. Stimuli for chondrogenesis

Multipotent MSCs may differentiate *in vitro* into chondrocytes, depending on the medium in which they are cultured, in a process called chondrogenesis. (Shyam P. et al., 2008). Chondrogenesis *in vitro* from bone marrow-derived MSCs requires cells to be cultured at high density in defined serum-free medium in a 3D environment that favors cell condensation and cell-cell interactions, analogous to what occurs during embryonic skeletal development (Johnstone B. et al., 1998) (DeLise A. et al., 2000).

Different formulations of growth media can be used to differentiate MSCs in chondrocytes. Critical factors for chondrogenesis *in vitro* are the necessity to expose

cells to signals from glucocorticoids and members of the transforming growth factor (TGF)-β family. Sekiya *et al*. induced *in vitro* chondrogenesis from MSCs by centrifuging cells and adding TGF-$\beta_3$, dexamethasone and Bone Morphogenetic Protein (BMP)–6 to the medium. The isoforms of TGF-β, TGF-$\beta_2$ and TGF-$\beta_3$ have been shown to be more effective in promoting chondrogenesis compared with others isoforms, such as TGF-$\beta_1$, causing a two-fold greater accumulation of glycosaminoglycans (GAGs) (Gun-Il Im M. et al., 2006). In Murdoch *et al*. differentiation in chondrocytes was achieved in the same conditions but without addition of members of BMPs family. In fact, for chondrocyte differentiation in humans, it is not necessary the use of BMPs (Pittenger M. et al., 2008). Dexamethasone, a synthetic glucocorticoid, has an ability to induce apoptosis and consequently, its receptors are found in the majority of developing organs. For this reason, dexamethasone promotes the maturation of chondrocytes and thus induces chondrogenesis in culture (Cristea B. et al., 1992), (Kawashima H. et al., 2003).

For *in vitro* chondrogenesis, it is necessary to remove the fetal bovine serum (FBS). The use of serum in culture medium has disadvantages, including physiological variability and consistency, shelf life, possible contamination, cost and potential effects of growth inhibitors (Sahnghoon L. et al., 2009). Thus, serum-free media has major positive benefits. One advantage is the ability to make a medium selective for a particular cell type, for example, a serum-free medium in the absence of growth inhibitors, or with chalones and glucocorticoids. The reduction of batch-to-batch variation, lower possibility of bacterial, viral and mycoplasma contamination and low protein levels resulting in fewer problems in downstream processing are others advantages in this type of medium (Keenan J. et al., 1998) (Freshney R., 2000). A defined medium to induce chondrogenesis consists of high glucose DMEM supplemented with antibiotics, ITS culture supplement, ascorbic acid, L-proline, sodium pyruvate, TGF-βs and dexamethasone (Djouad F. et al., 2009) (Sekiya I. et al., 2002) (Pittenger M.et al., 2008). All these compounds have an essential role in culture of chondrocytes. The addition of glucose and ITS in serum-free medium are necessary to provide enough nutrients for differentiation of MSCs in chondrocytes. These chondrocytes need a high level of glucose, unlike other cells such as osteoblasts. ITS culture supplement is a combination of insulin, transferrin, selenium, linoleic acid and bovine serum albumin (BSA). Insulin is a hormone supplement; transferrin serves as a binding protein for hormones and nutrients. Selenium is a cofactor for glutathione peroxidase and it is necessary for cell proliferation; linoleic acid is a precursor of prostaglandins and it is necessary for culture of non-transformed cells. BSA is a carrier for linoleic acid and other lipids, cofactors and hormones. For all these reasons ITS is an important supplement in culture of cells in serum reduced conditions. The amino acid L-proline is necessary to stabilize the collagen α-helice conformation, because proline ring structure helps in this stabilization. Therefore, the existence of L-proline in medium for chondrogenic differentiation contributes to collagen formation. Ascorbic acid stimulates collagen production and consequently affects differentiation in chondrocytes. In addition, ascorbic acid is an antioxidant and it can protect chondrocytes against toxic hydroxyl radicals and peroxides (Haart M. et al., 1999), (Leboy S et al., 1997). Pyruvate is an important chemical compound for metabolic pathways of cells. It is one of the initial promoters of the Krebs cycle thus being important in providing energy to cells (Berg J. et al., 2002).

*In vivo*, chondrocytes are exposed to a decrease of oxygen concentration and still maintain the capacity of proliferation and differentiation (Haselgrove J. et al., 1993) (Brighton C., 1984). Thus, it is important to understand how hypoxia, affects chondrogenesis. Cartilage has no blood supply, deriving nutrients from the surrounding synovial fluid (Malda P. et al., 2003). Khan *et al*. showed that a critical factor for chondrogenic differentiation *in vitro* is the oxygen tension. *In vitro*, cells are normally cultured in an atmosphere containing 20% of oxygen, but MSCs have been reported to proliferate more rapidly in lower oxygen concentrations. Khan W. *et al.* observed that cells cultured in air with 5% oxygen pressure proliferate more rapidly than in normal oxygen conditions (Khan W. et al., 2009). This may reflect the fact that, *in vivo*, articular cartilage and bone marrow are reported to exist within a range of 1% to 7% in oxygen concentration (Wang D. et al., 2005). Also, Khan *et al*. have shown that MSCs have a better chondrogenic response in cell aggregate culture and in lower oxygen tension (Khan W.S. et al., 2009). Studies have demonstrated that hypoxic conditions can increase proliferation rates and enhance differentiation along the different mesenchymal lineages (Das R. et al., 2010). Thus, hypoxia appears to be an important regulatory factor for proliferation, differentiation and for matrix production by chondrocytes with an increase in expression of chondrogenic markers (Das R. et al., 2010) (Khan W. et al., 2009). However, this parameter alone is not sufficient to induce chondrogenic differentiation (Das R. et al., 2010).

### 4.1.3. Chitosan as a Biomaterial for chondrogenesis

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked *D*-glucosamine (deacetylated unit) and *N*-acetyl-*D*-glucosamine (acetylated unit). Chitosan is produced commercially by partial deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) (Kuo Y. et al., 2010). Commercial preparations have degrees of acetylation (DA) ranging from 50% to 90%. This acetylation degree is defined by the molar fraction of *N*-acetyl-*D*-glucosamine units (Fig. 6) in such a way that, if there is a greater quantity of these units there is a greater degree of acetylation. Depending on the source and preparation procedure, their molecular weight may range from 300kD to over 1000kD (Tomihata K. et al., 1997). The DA of chitosan influences the solubility, crystallinity and enzymatic degradation, where this degradation is faster with higher DA (Amaral I. et al., 2005). Chitosan is a crystalline polysaccharide that is insoluble in aqueous solutions above pH 7, but in dilute acids with a pH inferior to 6, the free amino groups are protonated and the molecules becomes soluble (V. Sundarajan et al., 1999) (Kim S. et al., 2003). Chitosan has properties that make it a good candidate for use in humans and animals, such as: low toxicity, low inflammatory response after implantation and antimicrobial properties (Mao J. et al., 2004). Also, chitosan can have an important contribution in promoting wound healing, due to secretion of growth factors and cytokines which, in turn, enhance fibroblast activity and fibrous tissue synthesis. This ability is also promoted by the tendency to form polyelectrolyte complex with heparin (anticoagulant).

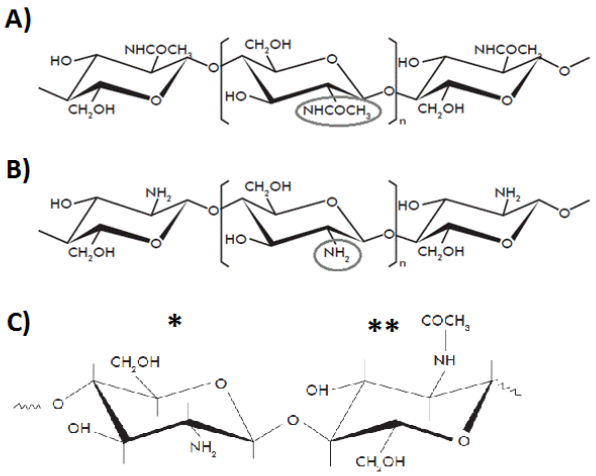


**Figure 6:** Structural representation of A) fully acetylated chitosan B) fully deacetylated chitosan and C) connected units of *N*-acetyl-*D*-glucosamine (*) and D-glucosamine (**) - chitosan chain. [Adapted from: (Ramírez A. et al., 2010) and (Peniche C. et al., 2010)]

In addition to these properties, chitosan has some chemical peculiarities that make it a good biomaterial for the induction and development of chondrogenesis. The *N*-acetyl-*D*-glucosamine units in chitosan have a structure similar to *N*-acetyl-glucosamine from glycosaminoglycans (GAG), naturally present in the extracellular matrix of cartilage (used to link the core proteins that form proteoglycans) (Subramanian A. et al., 2005). The cationic nature of chitosan allows electrostatic interactions with GAGs and other negatively charged species. These interactions help retaining these molecules within a tissue scaffold during the process of degradation of a scaffold and formation of a tissue (Sundararajan V. et al., 1999).

## 4.2. Detection of chondrogenesis

During chondrogenesis, MSCs start expressing various extracellular matrix and cell adhesion molecules such as, collagen *type II* [Col2a1(IIa)] (Sandell L. J. et al., 1991), N-cadherin (Ncad) (Oberlender S. A. et al., 1994), N-cam (Ncam1) (Widelitz R. B. et al., 1993), tenascin C (Tnc) (Mackie E. J. et al., 1987) and mainly express an essential transcription factor, SRY-Sox 9 (SOX 9). During and after condensation of MSCs, cartilage-specific genes are switched on and their products include collagen *type II*, *IX* and *XI* and the link protein aggrecan.

The transcription factors of the SOX family (*Sry*-type high mobility group box) have an important role in chondrogenesis. (Foster J. W. et al., 1994) (Tsuda M. et al.,

2003). SOX-9 protein is an important regulator of the chondrocytes phenotype and controls the expression of the genes Col2a1, for collagen *type II* production (Lefebvre V. et al., 1997), Col9a1 for collagen type *IX*, Col11a2, for collagen *type XI* (Zhang P. et al., 2003) and aggrecan (Sekiya I. et al., 2000).

The expression of extracellular matrix molecules by chondrocytes must be controlled at the level of synthesis and degradation to ensure that the matrix is properly constructed and maintained. Part of this control is due to the Col2a1 gene, which gives rise to the main fibrillar collagen in the cartilage matrix. However, expression of Col2a1 gene is controlled through transcription factors that interact with both, the promoter and the chondrocyte-specific enhancer (Zhang P. et al., 2003). *Type II* and *type XI* collagen are also critical components of cartilage collagen fibrils (Eyre D., 2002). Interestingly, in rabbit studies of chondrogenesis, cells in chondrogenic matrix express collagen type *II* as opposed to no expression of collagen type *I* (Benya P. et al., 1977). For these reasons, the detection of genes involved in chondrogenesis such as collagen *type II*, SOX 9 and aggrecan, allows detection of cartilaginous matrix.

An essential point in differentiation of chondrocytes and consequently for normal skeleton development are changes in physiological levels of glycosaminoglycans (GAGs), hyaluronic acid and chondroitin sulfate (CsC) (Shambaugh J. et al., 1980). Gene expression of Col2a1 and ColXa1 increase during chondrogenesis, while there is also increased glycosaminoglycans accumulation and histological evidences of this increase can be detected. Thus, to characterize chondrogenesis, histological analyses are a rapid technique for its visualization. Hematoxylin/ Eosin staining is a staining method that works well with a variety of fixatives and displays a broad range of cytoplasmic, nuclear, and extracellular matrix features. Hematoxylin is an acid dye which carries a negative charge on the portion of the molecule. This dye reacts with cationic component of cells and tissues, which include chromatin, nucleolus and ribosomal RNA and staining structures in blue. Basic dyes such as, eosin stain structures in red or pink. This dye reacts with anionic components of cells and tissues such as phosphate groups, nucleic acids, sulfate groups of glycosaminoglycans and carboxyl groups of proteins. When these dyes are used together in chondrogenic tissues, hematoxylin stains the nucleus in blue and eosin stains GAGs in pink. (Fischer A. et al., 2008). This staining also allows visualizing of the morphology and distribution of cells within scaffolds. Observation of cells morphology can be a factor to help identifying differentiation in chondrocytes, which *in vivo* display a spherical morphology (Lahiji A. et al., 2000).

Toluidine blue is a polychromatic dye which exhibits different colors depending on the nature of its chemical binding with the components of the tissue. It is used to stain proteoglycans and glycosaminoglycans in tissues such as cartilage. The strongly acidic macromolecular carbohydrates of cartilage are colored red or dark pink by the blue dye (Terry D. et al., 2000). Alcian blue and toluidine blue dyes both form complexes with anionic glycoconjugates (AG) such as proteoglycans and glycosaminoglycans. But in contrast to cationic dyes, alcian blue binds to GAGs with high ionic strength forming insoluble complexes (Whiteman P. et al., 1973). Alcian blue has a hydrophobic core with positive charges attached. This positive charge will bind strongly to negatively charged polymers such as GAGs, but not to other components such as nucleic acids. The variety of negative charges in GAGs is a consequence of

more or less sulfated chains. The ionic strength formed between positive charges and negative charges of GAGs is proportional to the number of GAGs chains present in the sample (Bjornsson, 2001).

# 5. Chondrogenesis in different scaffolds

To reproduce chondrogenesis *in vitro*, MSCs must be cultured at a high density in defined serum-free medium in a 3D environment. Conventionally, chondrogenesis is promoted by culturing cells in pellets. However, its applications for clinical use are in doubt, mainly due to limited size and mechanical force of pellets (Tare R. et al., 2005). The use of 3D scaffolds to support and maintain contact between cells and factors for differentiation is currently being pursued.

Yoo M. *et al* studied the properties of mesenchymal stem cells in presence of factors for differentiation in chondrocytes such as dexamethasone and TGF-$\beta_1$. This differentiation was promoted by culturing cells in pellets, to identify some of the main molecular mechanisms involved in chondrogenesis and to identify markers of cartilage formation and regeneration. And in this study, it was verified an increase in collagen *type II* and *X* and the greater presence of glycosaminoglycans in cartilage tissue (Yoo M. et al., 1998).

Markway B. *et al*. studied chondrogenesis of human mesenchymal stem cells, in pellets, with dexamethasone and TGF-$\beta_1$ and above 2% of oxygen pressure. It was shown that there is an upregulation of collage type *II*, Sox-9 and proteoglycans deposition in pellets and an increase of the aggregate size in chondrogenic conditions at 2% of oxygen (Markway B. et al., 2010).

Griffon *et al*. compared chondrocytes proliferation and function within chitosan sponges and polyglycolic acid (PGA*) in vitro*. With the determination of water content, DNA, GAGs and collagen *type II* it was possible to conclude that, although the composition of PGA constructs is more similar to natural cartilage, chondrocytes in chitosan scaffolds have an improved proliferation and metabolic activity (Griffon D. et.al., 2006).

Ragetly G. *et al*. studied the effects of chitosan fibrous scaffolds and chitosan macroporous sponges in chondrogenesis of mesenchymal stem cells. After 21 days of culture, stem cells maintained their viability above 90% on all chitosan scaffolds. However, there was a higher increase in GAGs and collagen *type II* in chitosan fibers than in macroporous sponges (Ragetly G. et al., 2009).

Wang Y. *et al*. studied induction of chondrogenesis from human mesenchymal stem cells in 3D aqueous-derived silk scaffolds under the stimuli, dexamethasone and TGF- $\beta_3$. After three weeks of differentiation, the majority of cells with stimulus

appeared with a phenotype similar to native cartilage tissue, with spherical shapes. In addition, in these samples there was an increase in expression of collagen type *II*, type *X*, aggrecan and Sox-9 (Wang et al., 2005).

In these studies, factors for differentiation, dexamethasone and TGF-βs, were present when culturing cells in pellets or in scaffolds. However, the effect of oxygen tension in the differentiation of mesenchymal stem cells implanted into a 3D scaffolds remains unclear.

# *OBJECTIVE*

The aim of this study is to analyze the effects of an environmental stimulus – the oxygen tension - in chondrogenesis of human Mesenchymal Stem Cells in 3D chitosan scaffolds.

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# CHAPTER II

# Abstract

Articular cartilage has a very limited intrinsic capacity for repair after injury. *In vivo,* cartilage tissue is exposed to a hypoxic environment, with around 1%-7% of oxygen pressure ($pO_2$). Human Mesenchymal Stem Cells (hMSCs) are a main cell source for chondrogenesis *in vitro.* Cells are cultured in 3D scaffolds for enhanced interactions between cells and chitosan has a high potential for 3D constructions, mainly due to its similar structures with glycosaminoglycans (GAGs). In this context, the main objective of this work was to study the effect of oxygen pressure on differentiation of hMSC into chondrocytes in 3D chitosan scaffolds.

The behavior of cells seeded in chitosan scaffolds and cultured in medium without or with factors to induce chondrogenesis, dexamethasone and TGF-$\beta_3$, during 28 days under 20% or 5% of oxygen pressure was analyzed. It was found that in chondrogenic samples at 5%$pO_2$, cells were organized in clusters similar with what is observed *in vivo*. Furthermore, toluidine blue staining and immunohistochemistry of collagen *type II* indicate that GAGs and collagen *type II* seems to be present at 5%$pO_2$. Upon quantification of GAGs, it was found a higher amount in samples with chondrogenic chemical stimulus at 5%$pO_2$. And most importantly, the amount of GAGs per cell is high even in basal conditions at 5%$pO_2$. This study suggests that hMSCs embedded in chitosan 3D scaffolds can differentiate in chondrocytes and that hypoxia conditions (5% $pO_2$) leads to efficient chondrogenesis, even in the absence of TGF-$\beta_3$ and dexamethasone.

# 1. Introduction

Cartilage, due to its absence of vasculature, has a limited capacity to repair after injury which can lead, in the long term, to a joint arthrosplasty [12]. Articular cartilage has as major components, chondrocytes embedded in an extracellular matrix (ECM) rich in collagen fibers and proteoglycans. Approximately 95% of the collagen present in ECM is collagen *type II* [7] and in minor quantities, collagen *type VI*, *IX* and *XI* [4]. The predominant proteoglycan in articular cartilage is aggrecan, which consists of a protein core with glycosaminoglycans (GAGs) attached. Articular chondrocytes are naturally in a low oxygen tension, between 1% to 7% of oxygen concentration. This

environment of hypoxia can lead to an increase in expression of collagen *type II* and aggrecan [8] and consequently it is an important factor for chondrogenesis.

Human Mesenchymal Stem Cells (hMSCs) have multipotential ability for differentiation in adipocytes, osteoblasts and chondrocytes [14]. MSCs can also be easily isolated and have a high capacity for expansion *in vitro*. These capacities make hMSCs an attractive source of cells for cartilage regeneration. However, chemical and environmental factors for differentiation are essential. Sekiya *et al*. [17] induced *in vitro* chondrogenesis from MSC by adding TGF-$\beta_3$, dexamethasone and Bone Morphogenetic Protein (BMP)-6 to the cell culture medium and Murdoch *et al*. [11] has used the same supplements but without BMPs members. In fact, for chondrogenesis in human cells it is not necessary the use of stimuli of the BMP family [13].

Chondrogenesis requires a high density culture to favor cell-cell contacts and mimick the environment that occurs in skeletal development. Markway *et al*. studied the effects of TGF-$\beta_1$ and dexamethasone in chondrogenesis from hMSC in pellets under 2% $pO_2$. The increase of SOX-9 expression and collagen *type II* and proteoglycans production were verified [10]. Current protocols grow cells in pellets, but pellet cultures show limitations in their weak mechanical properties, uncontrolled morphology and size of pellets. Thus, these structures are inadequate for cartilage repair [19]. The chemical composition and structure of scaffolds to implant hMSC for chondrogenesis can influence the differentiation and affect the behavior of these cells. The biocompatibility and similarity of chitosan to GAGs present in cartilaginous tissue make this biomaterial a good candidate for construction of scaffolds to support and maintain hMSCs in differentiation in chondrocytes [9]. Griffon *et al*. compared proliferation and function of chondrocytes in sponges of chitosan and polyglycolic acid (PGA). It was concluded that, although the composition of PGA is more similar to natural cartilage, chondrocytes in chitosan scaffolds show improved proliferation and metabolic activity [5]. Ragetly *et al*. studied the effect of chitosan fibrils and macroporous scaffolds on chondrogenesis of MSCs. After 21 days of culture, stem cells maintained their viability above 90% on all chitosan scaffolds. However, production of GAGs and collagen *type II* was higher in chitosan fibers than in macroporous sponges [15]. Wang Y. *et al*. studied induction of chondrogenesis from hMSCs in 3D aqueous-derived silk scaffolds under the stimuli dexamethasone and TGF- $\beta_3$. After three weeks of differentiation, the majority of cells with stimulus appeared with a phenotype similar to native cartilage tissue, with spherical shapes. In addition, in these samples there was an increase in expression of collagen type *II*, type *X*, aggrecan and Sox-9 [19]. In these studies, factors for differentiation, dexamethasone and TGF-βs, were present when culturing cells in

pellets or in scaffolds. However, the effect of oxygen tension in differentiation of MSCs implanted in 3D scaffolds remains unclear.

The objective of our study was to study the effect of a hypoxic environment (5% of oxygen pressure) in chondrogenesis from hMSC in 3D chitosan scaffolds. We hypothesized that even without chemical stimulus, chondrogenesis can be stimulated in 5% of oxygen pressure, which mimicks the natural environment of chondrocytes in the human body.

# 2. Materials and methods

## 2.1. Production of scaffolds

3D scaffolds were prepared by freeze-drying. Firstly, chitosan (France-Chitine) was purified as described in Antunes J. *et al*. [2]. Briefly, chitosan was dried, hydrated and dissolved in hydrochloric acid fuming 37% (Merck). Chitosan solution was filtered through 100, 41 and 20 µm pore size Millipore filters with the help of a vacuum pump. After filtration, chitosan was precipitated with 0.1 M potassium hydroxide (Merck) solution. Chitosan was washed with MilliQ water and was placed in the freezer overnight at -80°C. Chitosan was then freeze-dried (Freezone 2.5, Labconco) and finally, milled (IKA mill) to obtain a fine powder.

For scaffolds production, a 2% solution of purified chitosan (degree of acetylation (DA): 12.00± 2.35%, molecular weight (MW): 324±27x$10^3$) [2] was prepared by adding acetic acid (Panreac) under strong vortex agitation. After a 24h incubation at 4°C, the chitosan solution was centrifuged at 4695G for 5 min and then casted 800µL per well in a 48-well plates. Plates were placed at -80°C and freeze-dried at -80°C for 48 h. Scaffolds were removed from the plate and cut in a parallelepiped shape with 5x5x2 $mm^3$ (Fig. 1).

For neutralization, scaffolds were impregnated, under vacuum, in successive ethanol solutions at 99.9%, 70%, 50% and 25% by incubating for 10 min or 20 min in solution of 70%. Finally, PBS and DMEM (GIBCO, Invitrogen) were added to the scaffolds, which were maintained at 4°C protected from light, until use.

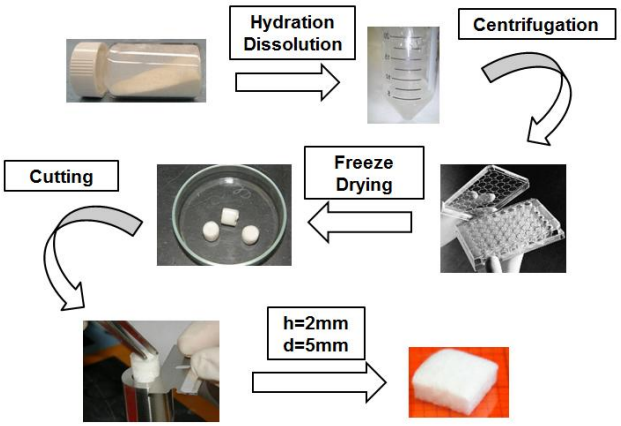


**Figure 1:** Production of chitosan scaffolds, starting with purified chitosan, its hydration and dissolution. After centrifugation to remove air bubbles, chitosan solution was cast carefully and without bubbles in 48 wells plate and frozen at -80°C in a horizontal plan. Chitosan was then freeze-dried and finally scaffolds were cut in parallelepiped shapes.

## 2.2. Scaffold characterization

Scaffolds were analyzed by Scanning Electron Microscopy (SEM) and Mercury Porosimetry. Visualization in SEM (FEI Quanta 400FEG ESEM / EDAX Genesis X4M, Schottky) was done with whole scaffolds and fractured scaffolds in liquid nitrogen with the aim to analyze the morphology of pores in the surface and deeper in the scaffolds.

To measure the percentage of pores in scaffolds, mercury porosimetry analysis was performed. Scaffolds were weighted, placed in a mercury porosimetry cell and the density was determined by mercury porosimetry (Poremaster 60, Quantachrome) - theoretical density. Next, others samples of scaffolds were weighted and immersed in a beaker with a known volume of mercury 99.99% (Sigma), the new volume was read. Then, with the differences between these two volumes and with the weight of scaffolds real density was calculated. With the difference between theoretical and real density the percentage of scaffolds porosity was obtained. To complement the mercury analyses, the average of porous diameters were calculated by the relation, as mentioned in Amaral *et al.* [1]. To obtain these values, scaffolds were broken in liquid nitrogen and analyzed in SEM. Results were the average of 75 measurements.

**d=** $\sqrt{\mathbf{l.h}}$ , l and h are the maximum and minimum pore lengths

## 2.3. Live / Dead assay

Samples were washed with PBS, followed by DMEM without phenol red (GIBCO, Invitrogen). Calcein AM (Invitrogen) solution at 2 μM of concentration in DMEM without phenol red was added. Plate was incubated for 45 minutes at 37°C. 10μL of ethidium homodimer-1 (EthD-1) (Invitrogen) at 5mM was added to samples, which were then visualized by confocal microscopy (Leica SP2 AOBS).

## 2.4. Cell culture and seeding into scaffolds

Human MSC previously isolated from bone marrow and stored frozen in liquid nitrogen were thawed as quickly as possible. Approximately 1 million of cells were seeded in T150 culture flasks with DMEM low glucose with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) (all from GIBCO, Invitrogen) at 37°C/5% CO2 in an atmosphere with oxygen. For cell expansion, medium was replaced twice a week. When cells reached 80% of confluence, they were detached from culture flasks and passed to new flasks, as follows. All culture medium was removed from the flasks, which were then washed twice with PBS (Sigma). Trypsin/1mM EDTA (GIBCO, Invitrogen) were added and flasks were incubated at 37°C until cells become detached. Thereafter, growth medium were added to neutralize trypsin. Cells were collected; centrifuged at 300g at 23°C for 10 minutes; and supernatant was discarded. Cells were then ressuspended in growth medium and platted in new tissue culture flasks at a density of approximately 3000 cells/$cm^2$. In these studies, MSCs were used in passage 9.

In the day prior to harvesting of cells, scaffolds were incubated in normal conditions of oxygen (20% $pO_2$) or in hypoxic conditions (5% $pO_2$). 800 000 cells concentrated in 20μL of medium without serum were added to each scaffold. Firstly, 400 000 cells in 10μL were put into each side of a scaffold and scaffolds with cells were incubated in 20% or 5% of oxygen pressure for 4 hours. Then, basal or chondrogenic media were added to each condition. Both media had high glucose (Sigma) (4,5 g/L) DMEM (GIBCO, Invitrogen) supplemented with 50μg/mL ascorbic acid (Sigma-Aldrich), 40μg/mL L-proline (Sigma), 100μg/mL sodium pyruvate (Sigma), 100μg/mL ITS culture supplement (BD Biosciences) and 100μg/mL antibiotics (penicillin and streptomycin) (GIBCO, Invitrogen). This medium was named basal medium. For chondrogenic medium we added 0.1μM dexamethasone (Sigma) and 10ng/mL TGF-$\beta_3$ (Immuno-

Tools). All media were prepared freshly and filtered with a filter of 0.2µm (Orange Scientific). Cells seeded were maintained for up to 28 days at 37°C in chondrogenic medium [5], with medium being changed twice per week.

## 2.5. Histological analysis

After 28 days of culture, scaffolds with cells were fixed in neutral buffered formalin (Surgipath) for 30 minutes at room temperature (RT). Samples were then dehydrated in an ethanol series for 1 hour and embedded in paraffin, cut in 5 µm sections onto slides and stained with either hematoxylin/ eosin or 1% toluidine blue/ 1% sodium borate.

### 2.5.1. Hematoxylin / Eosin staining

Sections were desparaffinized in xilol and hydrated in descending grades of ethanol (100%, 90%, 80%, 50%) for 2 min and for 3 min in water. Samples were incubated in Gill's hematoxylin for 3 min, in distillated water for 5 min, in ethanol 95% for 2 min, eosin for 3 min, and finally washed in distillated water twice. Next, samples were hydrated in ascending grades of ethanol (80%, 90%, 100%) incubating twice for 2 min in each solution and finally, sections were diafanized in xilol and covered with coverslips using a histofluid solution (Marienfeld). Samples were visualized in an Inverted Fluorescence Microscope (Zeiss).

### 2.5.2. 1% Toluidine Blue / 1% sodium borate

Solution of 1% toluidine blue/ 1% sodium borate was prepared as follows. A 1% sodium borate solution was dissolved and 1% toluidine blue was added. The solution was dissolved completely and filtered with Whatman paper. Sections were desparafinized in xilol at RT and then hydrated for 2 min in 100% and 95% ethanol. Samples were washed in distilled water for 2 min and incubated in the toluidine blue solution for 5 min at RT. Sections were then rinsed in tap water followed by ethanol 95% and 100% during 2 min each and diafanized in xilol for 5 min. Finally, samples were fixed in histofluid solution (Marienfeld) with coverslip. Samples were visualized in Microscope Inverted Fluorescence (Zeiss).

## 2.6. Immunohistochemistry analysis

An immunohistochemistry assay was performed to detect collagen *type II.* Firstly, sections were desparafinized, hydrated in a series of ethanol solutions and washed in PBS. Antigenic recovery was done with proteinase K (Sigma) at 37°C during 30 minutes. Next, hydrogen peroxide 3% (Prolabo) was added and washed with PBS followed by protein block (Dakocytomation). The anti-collagen *type II* primary antibody (clone II-II6B3, DSHB) diluted 1:100 in primary antibody dilution buffer (1% BSA (Sigma), 0.5% Triton X-100 (Sigma), 0.05% sodium azide (Sigma) and 0.01M PBS) was added and samples incubated for 12 hours at 4°C. Samples were washed with PBS and incubated with a M.O.M. biotinylated anti-mouse IgG antibody (1:100, in secondary antibody dilution buffer – 0.05% Tween (Sigma) and 0.01M PBS) (Vector Laboratories) for 1hour at RT, followed by Streptavidin-Biotin HRP solution (1:100 in secondary antibody dilution buffer) (VectaStain Elite ABC Reagent) for 30 minutes. Again, samples were washed with PBS and developed with chromogenic DAB (Dakocytomation) for 10 minutes. Finally, cells were contrasted with Gill's Hematoxylin. Samples were fixed in histofluid solution (Marienfeld) and visualized with an Inverted Fluorescence Microscope (Zeiss). A murine bone with a growth plate of chondrocytes was used as a positive control. An isotype-matched antibody (Mouse IgG1, Immunotools) was used as a negative control.

## 2.7. GAGs and DNA quantification

For glycosaminoglycans (GAGs) quantification scaffolds were enzymatically degraded with chitosanase (EMD Biosciences) [18]. Chitosanase was dissolved in acetate solution to a final concentration of 0.1U/mL and the scaffolds were submerged in this chitosanase solution in a proportion of 1:100 w/v and incubated for 12h at 37°C under agitation. The levels of GAGs were determined with Alcian Blue as in Karlsson M. *et al*. [6].

For normalization of the amount of GAGs per DNA, half of the each sample obtained from the enzymatic degradation was used for DNA quantification. After dissolution of scaffolds, 10% of triton X-100 (Sigma) was added to each sample and maintained at 4°C under agitation. To reduce the effects of triton X-100 (Sigma) in DNA quantification, the solutions were diluted in PBS for a final concentration of 0.1% of

triton X-100. Then, DNA was quantified with Quant-iT™ PicoGreen dsDNA Reagent and Kits (Invitrogen), according to the manufacturer's protocol.

# 3. Results

In our studies, the capacity of hMSCs to differentiate in chondrocytes when in chitosan scaffolds was characterized. The main aim of this study was to analyze the influence of oxygen tension in chondrogenesis on a 3D chitosan scaffold. For this, scaffolds were produced and characterized prior to hMSCs seeding. The effect of hypoxia on chondrogenesis of hMSCs embedded in chitosan scaffolds was measured by visualizing cells stained with hematoxylin/eosin and toluidine blue, by detection of collagen *type II* and by quantification of GAGs.

## 3.1. Characterization of chitosan scaffolds

Chitosan scaffolds were prepared by freeze–drying. The structure of these scaffolds was analyzed by SEM (Scanning Electron Microscopy) and by Mercury Porosimetry. Figure 2 [A] shows a highly porous microstructure with a heterogeneous pore distribution and with interconnected pores of different diameters.

With mercury porosimetry analysis, the porosity of the scaffolds, volume and surface area were determined. The percentage of porosity of these scaffolds was 97%, indicating that scaffolds are mostly constituted by pores. The volume determined by mercury porosimetry was $9.859 \times 10^{-01}$ cc/g and the surface area $4.121 \times 10^{-01}$ $m^2$/g.

In line with Amaral *et al*. [1] 75 pores sectioned longitudinally were measured upon SEM analysis. The average diameter of pores was 87.182 μm.

To analyze the behavior of hMSCs seeded in chitosan scaffolds, we performed an analysis by SEM and a Live / Dead assay. hMSCs located on the surface of scaffolds showed a different shape from the typical morphology adopted in 2D, and formed clusters (Fig. 2 [B-F]). Also, it is clear that cells are connected with each other, sometimes with long tethers (Fig.2 [B-E]). Cells found deeper in the scaffold are more isolated and stay bound to the walls of the chitosan scaffold (Fig.2 [C-F]).

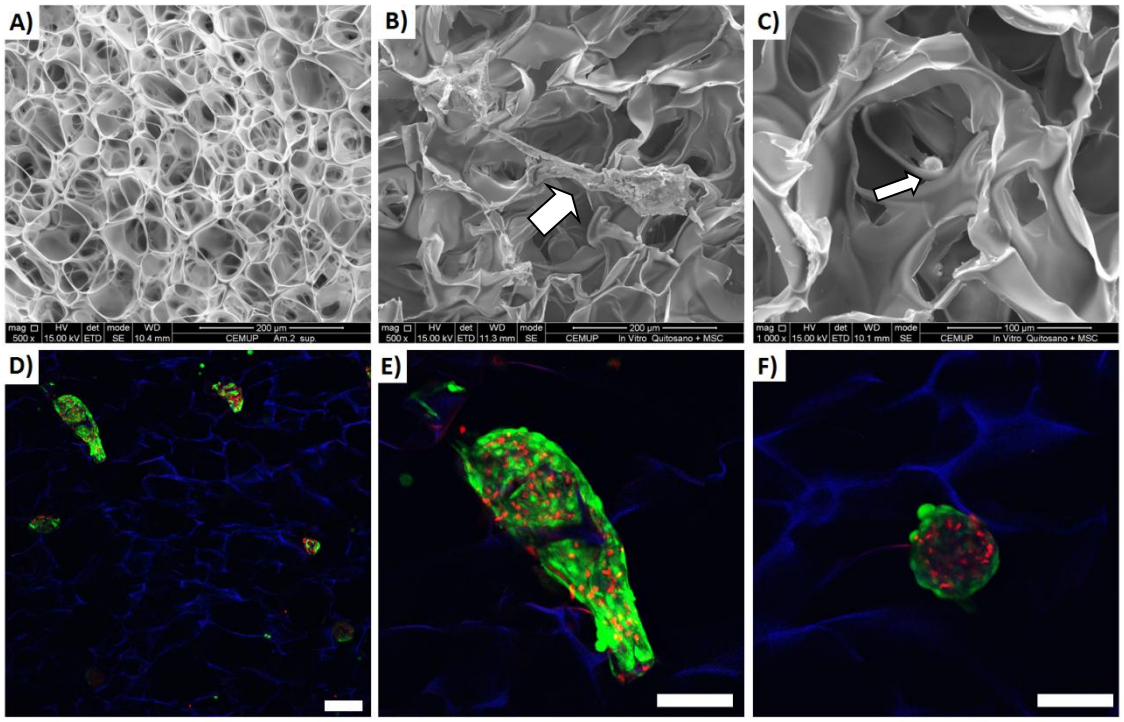


**Figure 2:** Morphology of hMSCs on chitosan scaffolds was analyzed by SEM [A-C] and Live / Dead assay [D-F]. **(A)** An heterogeneous porous matrix, containing pores with different diameters is evident. **(B)** Chitosan scaffolds with hMSCs, incubated for 4 days in medium with serum and without any further stimuli. Cells tend to form clusters and make bridges between them (demonstrated by the arrow). **(C)** In deeper zones of scaffolds isolated hMSCs could be detected (arrow). **(D)** The presence of cell clusters was also found when analyzing with Live/dead assay. Calcein (green) marked the live cells and ethidium homodimer-1 (red) marked the dead cells. Chitosan scaffold appears in blue. **(E)** A detail of a cell cluster forming a bridge between chitosan walls. **(F)** A smaller cluster was found deeper in scaffolds. [Scale bar = 100µm]

## 3.2. Histological analysis: Different morphology and GAGs detection of hMSC differentiating in hypoxia

Culturing cells in a 3D scaffold favors cell attachment, proliferation, differentiation and eventually leads to a better functionality. In this type of structures, cells can more easily obtain the nutrients and growth factors. Sections stained with hematoxylin/eosin show the distribution of hMSCs in scaffolds, cultured in different conditions of oxygen pressure and with or without chemical stimuli for differentiation in chondrocytes (TGF-$\beta_3$ and dexamethasone). Images show a different distribution of cells in the periphery or center of scaffolds (Fig. 3). This difference is biggest in chondrogenic samples at 20% $pO_2$, where there is a layer of cells in the periphery and the presence of cells in the center is insignificant (Fig. 3 [D]). However, in the center of this sample there is a fibrous matrix that is not visible in basal conditions at 20%$pO_2$ (Fig.3 [B]). In hypoxia, differences between cells in the periphery and the center of the scaffold are not as evident. In chondrogenic conditions at 5%$pO_2$ (Fig. 3 [C])

differences between center and periphery are not visible, and in basal conditions (Fig. 3 [A]) the difference is small even though the periphery has more cells than the center of the scaffold. In general, the amounts of cells in scaffolds are also different between 5% $pO_2$ and 20% $pO_2$. There are more cells in the periphery in conditions at 20% $pO_2$ than in 5% $pO_2$. On the other hand, in the center of the scaffolds, there are more clusters of cells at 5% $pO_2$, specially in chondrogenic conditions.

Another important visualization is the difference in the shape of cells at 5%$pO_2$ and 20% $pO_2$. In atmospheric concentrations of oxygen, cells appear in an elongated network. In contrast, in hypoxia, cells organize in clusters which have a more spherical shape when compared with cells at normal condition of oxygen. This round shape is more pronounced in chondrogenic samples at hypoxia (Fig. 3 [C]).

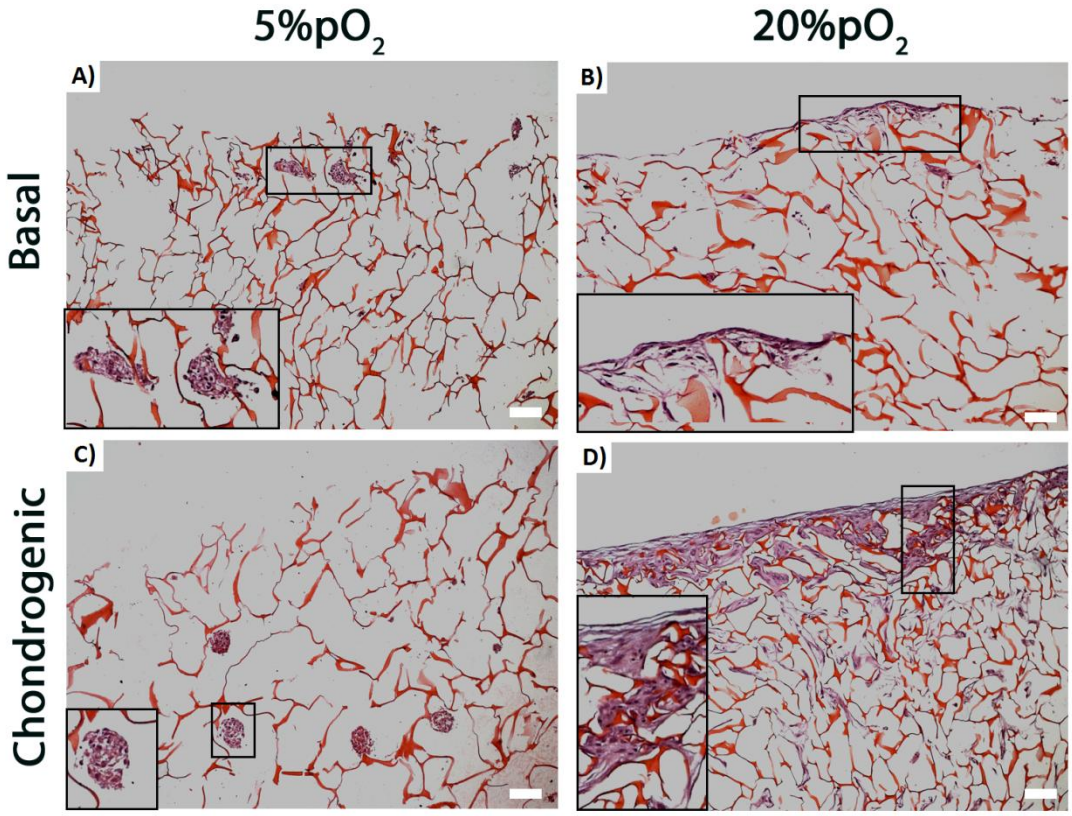


**Figure 3:** Hematoxylin/eosin staining of scaffolds seeded with hMSCs upon 28 days of culture under hypoxia [A,C] or normal [B,D] atmospheric conditions, with [C,D] or without [A,B] added factors for differentiation in chondrocytes. Circular shapes of cell clusters in hypoxia, mostly in (C) are evident when compared with cells under normal conditions of oxygen. These images are representative of three experiments. [Scale bar =100 µm]

Toluidine blue staining was also performed in basal or chondrogenic conditions, for cells cultured in 5%$pO_2$ or 20%$pO_2$ (Fig. 4). Toluidine blue links with polymers in such a way that GAGs stain by pink color. The variety of blue color is proportional to the number of GAG chains present in samples [6]. Weak pink coloring was visible in chondrogenic samples, with chondrogenic samples under hypoxia (Fig. 4 [C]) showing a slightly stronger pink staining and a lighter blue than under normal conditions of oxygen (Fig.4 [D]). An interesting observation was the presence of pink staining and a lighter blue in basal samples at 5%$pO_2$ (Fig. 4 [A]) than in chondrogenic samples at 20%$pO_2$ (Fig. 4 [D]) where blue staining is darker.

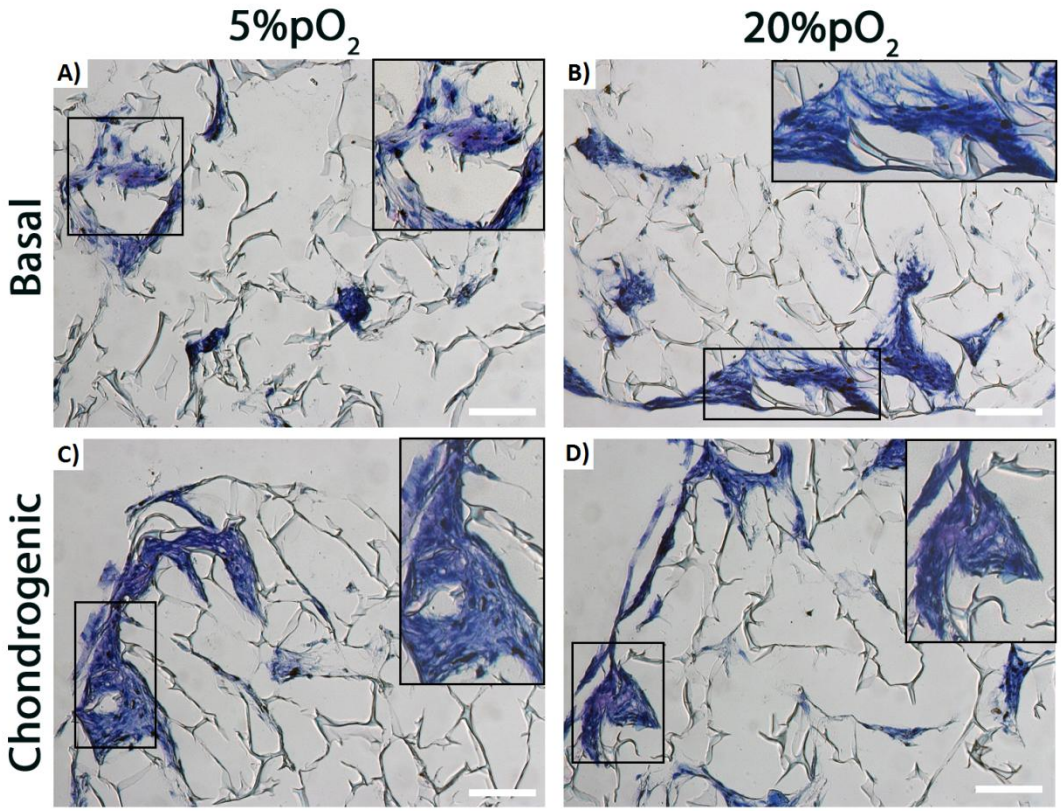


**Figure 4:** Toluidine blue staining of scaffolds seeded with hMSCs upon 28 days of culture under hypoxia [A,C] or normal [B,D] atmospheric conditions, with [C,D] or without [A,B] added factors for differentiation in chondrocytes. The presence of GAGs is detected by a pink staining, mostly present in samples in hypoxia condition. Images are representative of three experiments. [Scale bar =100 µm]

## 3.3. Detection of collagen *type II* by immunohistochemistry

Collagen *type II* is one of the major constituents of cartilage matrix and its detection helps in detecting cartilaginous tissue. For this, collagen *type II* was detected by immunohistochemistry. In the negative controls, with the isotype control IgG1 and without primary antibody in samples cultured with stimuli for chondrogenesis at 5%$pO_2$ , there was some unspecific staining of the chitosan staining, but fibers remained pink, not being detected collagen *type II* (Fig. 5 [A]). In the positive control, a section of a bone with a growth plate of chondrocytes, there was strong staining. Scaffold sections under normal or hypoxia condition showed evidences of chondrogenesis, with detection of collagen *type II* (Fig. 5 [B]). The matrix in basal condition at 20%$pO_2$ shows fibers with a weaker staining for collagen than in basal conditions at hypoxia. Matrices in chondrogenic samples have a stronger staining in hypoxic samples than in normal conditions of oxygen.

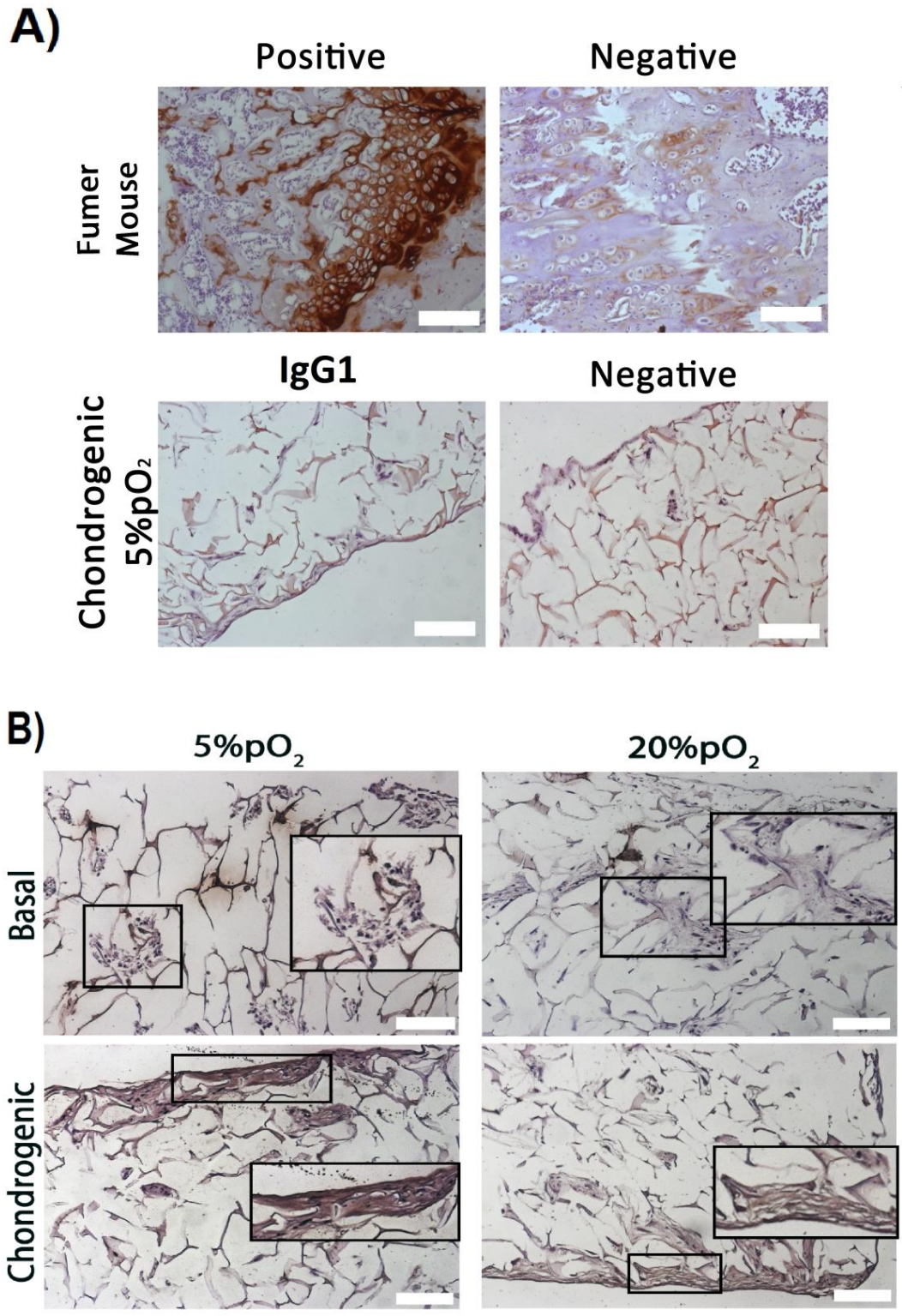


**Figure 5: A)** Positive and negative controls with IgG1 and without primary antibody for collagen *type II* detection by immunohistochemistry. **B)** Immunohistochemistry of collagen *type II* of scaffolds seeded with hMSCs upon 28 days of culture under hypoxia or normal atmospheric conditions, with or without added factors for differentiation in chondrocytes. The presence of collagen *type II* is detected by brown color. Collagen type II is mostly present in samples under chondrogenic stimuli, with a higher staining in samples in hypoxic conditions. Images are representative of three experiments. [Scale bar =100 µm Inserts show a zoomed section of the region indicated by a black box].

## 3.4. GAGs and DNA Quantification

The content in GAGs reflects proteoglycans biosynthesis and accumulation in a matrix. The toluidine blue staining indicated the presence of GAGs, but for a better comparison it is necessary to quantify the amount of GAGs produced in each condition. Figure 6 shows the concentration of GAGs in different conditions. There was a slight increase in the amount of GAGs per scaffolds with chondrogenic samples at 5%$pO_2$, when compared with the other conditions. Chondrogenic samples (with stimulus) have higher amounts of GAGs than basal samples (without stimulus), especially after 28 days of differentiation. There is, on average, an increase in GAGs content after 28 days of culturing, with a more increase in samples with stimulus at hypoxic conditions.

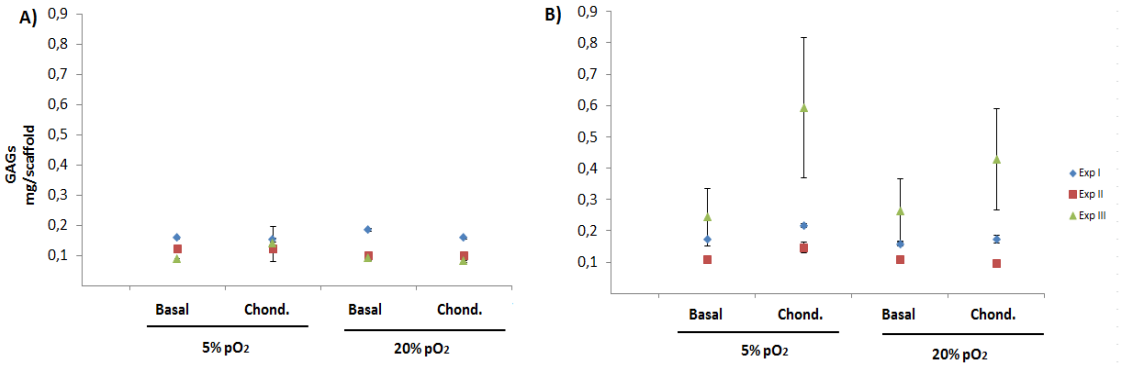


**Figure 6:** The amount of GAGs per scaffold with hMSCs after 14 (A) and 28 days (B) of incubation under hypoxia or normal oxygen conditions and with or without added factors for chondrogenic differentiation. There is an increase in GAGs content after 28 days, especially in hypoxia conditions. This figure shows data of three experiments (different symbols) with three replicas each.

To know the amount of GAGs produced per cell, data can be normalized with the amount of DNA. For that, DNA was quantified in the same samples analyzed for GAGs quantification. It was found an average decrease in DNA content from 14 days to 28 days of incubation, with the exception of chondrogenic samples in atmospheric conditions of oxygen (Figure 7). This decrease is greater with 5%$pO_2$ than 20% $pO_2$.

DNA quantification results were different from data obtained with a resazurin test (data no show). There was an increase in metabolic activity along the 28 days, as opposed to a decrease in DNA amount, indicating that cells remain active in differentiation for up to 28 days.

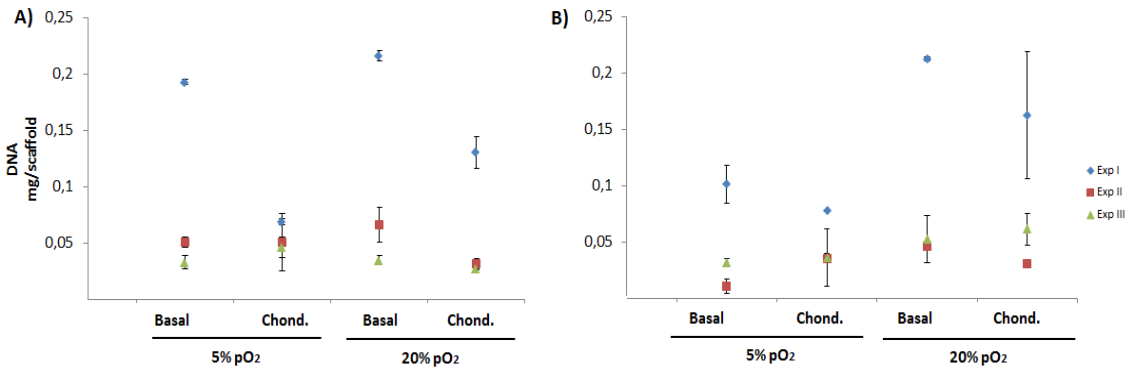


**Figure 7:** The amount of DNA in scaffolds with hMSCs after 14 (A) and 28 (B) days of incubation in hypoxia or normal oxygen conditions and with or without added factors for chondrogenic differentiation. Overall, on average, there was a small decrease in the amount of DNA from 14 to 28 days, more evident in samples at 5%$pO_2$. This figure shows data of three experiments (different symbols) with three replicas each.

Figure 8 shows the normalized amount of GAGs per DNA. There was a large increase in GAGs content per cell, in samples at 5%$pO_2$ compared with samples at 20%$pO_2$. Importantly, this increase is also evident when basal samples at 5%$pO_2$ are compared with chondrogenic samples at 20%$pO_2$. Between chondrogenic samples in hypoxia and chondrogenic samples in normal conditions of oxygen the amount of GAGs/DNA is more than double.

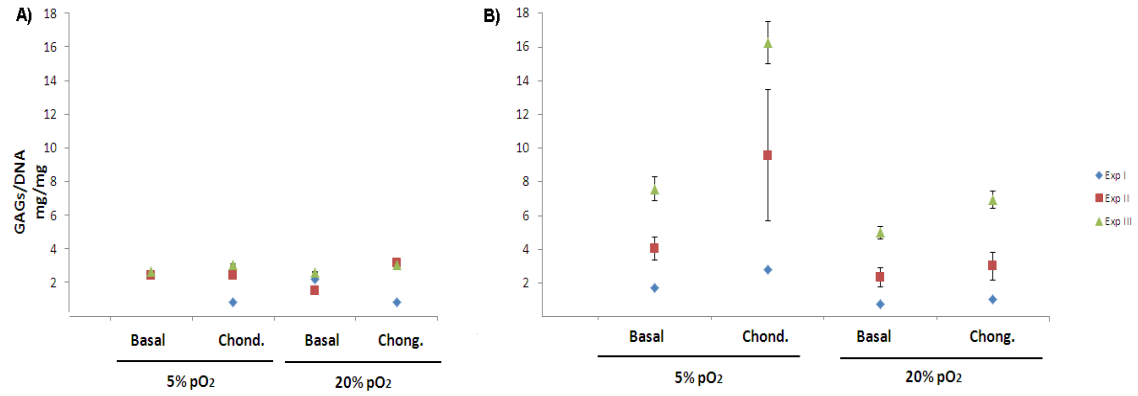


**Figure 8:** Amount of GAGs/DNA in scaffolds with hMSCs after 14 (A) and 28 (B) days of incubation in hypoxia or normal oxygen conditions and with or without added factors for chondrogenic differentiation. This figure shows data of three experiments (different symbols) with three replicas each.

The increase in GAGs content in samples cultured in hypoxia indicate that oxygen pressure may be a stimuli for chondrogenesis even in the absence of further chemical stimuli.

# 4. Discussion

The main aim of this study was to analyze the effects of hypoxia (5%$pO_2$) in the differentiation of hMSCs into chondrocytes in 3D chitosan scaffolds. The use of chitosan as a biomaterial for chondrogenesis has been investigated, particularly due to its similarity to various GAGs found in articular cartilage [9]. In our study, chitosan scaffolds were analyzed in terms of porosity, pore diameter and interconnectivity. 3D scaffolds were produced from a chitosan acid solution which was frozen at -80°C to form ice crystals, and freeze-dried for sublimation of the ice, thus forming a macroporous structure. The structure of chitosan scaffolds consisted of interconnected macropores, as indicated by the values of porosity: 97% of a scaffold is constituted by pores. When pore diameters were determined by analyzing SEM photographs (based on Amaral *et al*. [1]) the average pore diameter was found to be 87.182μm. This value is similar to the one reported in Amaral *et al*., 2005 (≈ 85μm), where the DA of chitosan was similar to the DA in our study. Pores have an average diameter of ≈87μm, and can be considered as macropores; however, it is important to mention the presence of micropores deep in the scaffolds, which cannot be detected by SEM but are detected by mercury porosimetry [9]. This macroporosity associated with microporosity can favor the production of chondrogenic matrix into scaffolds.

Successful repair of cartilage defects requires a cell source for differentiation into chondrocytes. MSCs derived from adult bone marrow are a good candidate to this cell source, which can differentiate in chondrocytes when in presence of appropriate stimuli [20]. Morphology of MSCs seeded in chitosan scaffolds was analyzed by SEM and with a Live/Dead assay (Fig. 2). It was possible to observe the presence of clusters of cells and connections / bridges between cells adhering to different walls of the scaffolds. In deeper zones of scaffolds it was possible to visualize spherical small clusters of cells adhering to the walls of the scaffold. These spherical cell clusters can be important for hMSCs differentiation in chondrocytes since some studies suggest a

relationship between morphology of cells and cell-specific functions [3] and also because the spherical morphology is more similar to chondrocytes *in vivo* [9].

The influence of hypoxia in chondrogenesis is a topic of great interest, because chondrocytes grow in a low-oxygen environment *in vivo* [16]. However, there are no studies describing the effect of hypoxia on MSC seeded in 3D chitosan scaffolds. The importance of tissue environmental factors in induction of MSC differentiation is known. Considering nongenetic and environmental factors, one of the most important factors is oxygen tension, particularly for chondrogenic differentiation, because *in vivo*, chondrocytes grow in a low-oxygen tension (1%-7%) - hypoxia [16]. In our studies, we analyzed hMSC differentiation in scaffolds of chitosan at 5% of oxygen tension and compared with atmospheric concentrations of oxygen (20% $pO_2$). Chondrogenic differentiation was induced with the chemical factors TGF-$\beta_3$ and dexamethasone. When hematoxylin/eosin stained sections were analyzed, it was observed a big difference in the cells morphology, with spherical clusters at 5%$pO_2$ and elongated shapes when cultured at 20% $pO_2$ (Fig. 3). It should be noted that in hypoxia and under basal conditions cells do not form clusters as round as cells in chondrogenic conditions. Samples under normal conditions of oxygen appeared with more fibers deeper in the scaffold, whereas cells were maintained more in the periphery. As opposed, samples cultured in hypoxic conditions showed clusters of cells and fibers throughout the whole scaffold. Toluidine blue staining of cells in scaffolds was weak, probably because, as observed with the hematoxylin/eosin staining, there was little production of matrix within the scaffolds. For this reason, it was difficult for the dye to link to the negative groups of proteoglycans. Due to the existence of small clusters of cells and less matrix at 5%$pO_2$, it was difficult to detect pink staining in both samples, but all samples in hypoxia showed a lighter blue color and a slightly stronger pink staining when compared with basal at 20%$pO_2$ (Fig. 4).

Collagen *type II* staining was also found to be weak, due to the difficulty in visualizing matrix in scaffolds. In addition, chitosan also stained in a soft brown with DAB chromogen, making it difficult to distinguish from matrix staining. However, the presence of brown color in samples stimulated for chondrogenesis at 5% $pO_2$ is evident (Fig. 5 [B]). When compared with basal samples at 20%$pO_2$ it is possible to visualize the increased staining in chondrogenic samples at 20% $pO_2$ and mainly at 5% $pO_2$. These results suggest the presence of collagen *type II* in chondrogenic samples especially under hypoxia conditions. The increased presence of collagen *type II* under hypoxia indicates that hypoxia may enhance the potential for cartilage differentiation.

To quantify the presence of GAGs and viability of the cells, GAGs and DNA content were measured. In figure 6, the amount of GAGs was measured by alcian blue, and values show a slight increase in chondrogenic samples at 5%$pO_2$. These amounts were normalized by DNA content in order to quantify the quantity of GAGs per cell in each condition and compare different samples. There was a decrease in DNA between 14 and 28 days of incubation (Figure 7), suggesting there might be cell death within scaffolds after long periods of incubation. It is possible that cells inside scaffolds, particularly at 5% $pO_2$, will activate a gene-regulation mechanism leading to self-destruction without injury to other cells. However, resazurin tests indicated that there was an increase in the metabolic activity of cells for up to 28 days, indicating that cells maintain their activity, particularly under hypoxia conditions.

When analyzing the amount of GAGs/DNA (Fig. 8) differences were clear: chondrogenic samples at 5%$pO_2$ showed higher amounts of GAGs/DNA than other samples, especially when compared with samples at 20% $pO_2$. These differences were more evident after 28 days of incubation, with GAGs being mostly produced in samples incubated at 5%$pO_2$ for 28 days, in chondrogenic conditions. The differences between values obtained after 14 days or 28 days of culture, in particular in hypoxia, serve to identify the importance of time in differentiation processes of chondrogenesis. In this period of time there is a decrease in the amount of DNA, probably reflecting a decrease in the number of live cells. However, the remaining live cells maintain or even improve the capacity of differentiation in chondrocytes. This potentiality can be important for a possible implantation upon injury, because it can increase and/or improve the regeneration of cartilage tissues.

In summary, the analysis with hematoxylin/eosin, toluidine blue stainings, detection of collagen *type II* and GAGs/DNA quantification evidence the presence of cartilaginous tissue when MSCs were cultured within 3D chitosan scaffolds, in hypoxia or with chemical stimuli. The detection of GAGs accumulation and their quantification were important to understand hypoxia as a stimulus to increase chondrogenesis of hMSCs. However, other important assays (for example, detection and quantification by real time PCR of genes characteristic of cartilage tissue such as, SOX-9, aggrecan and collagen *type II*) must be done to ensure that hypoxia can indeed promote chondrogenesis.

# 5. Conclusion

In summary, it was possible to conclude that under hypoxic conditions of oxygen (5% $pO_2$), hMSCs seeded in chitosan scaffolds aggregated in round clusters, a shape similar to chondrocytes *in vivo.* Furthermore, under hypoxia there was an increase in detection of collagen *type II* and GAGs even without any chemical stimulus for chondrogenesis (dexamethasone and TGF-$\beta_3$). In this context, lack of oxygen stimulus –hypoxia- is an important regulatory factor for cells differentiation and matrix production in chondrogenesis.

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