SOCIEDADE BRASILEIRA DE ORTOPEDIA E TRAUMATOLOGIA

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Update Article

Regenerative potential of the cartilaginous tissue in mesenchymal stem cells: update, limitations, and challenges☆

Ivana Beatrice Mânica da Cruz[a,b], *Antônio Lourenço Severo*[c], *Verônica Farina Azzolin*[b], *Luiz Filipe Machado Garcia*[b], *André Kuhn*[c], *Osvandré Lech*[c,*]

[a] *Universidade Federal de Santa Maria (UFSM), Centro de Ciências da Saúde, Santa Maria, RS, Brazil*
[b] *Universidade Federal de Santa Maria (UFSM), Laboratório de Biogenômica, Santa Maria, RS, Brazil*
[c] *Universidade Federal da Fronteira Sul (UFFS), Hospital São Vicente de Paulo, Instituto de Ortopedia e Traumatologia, Passo Fundo, RS, Brazil*

ARTICLE INFO

Article history:
Received 12 February 2016
Accepted 15 February 2016
Available online 6 December 2016

Keywords:
Stem cells
Cartilaginous tissue
Regenerative potential

ABSTRACT

Advances in the studies with adult mesenchymal stem cells (MSCs) have turned tissue regenerative therapy into a promising tool in many areas of medicine. In orthopedics, one of the main challenges has been the regeneration of cartilage tissue, mainly in diarthroses. In the induction of the MSCs, in addition to cytodifferentiation, the microenvironmental context of the tissue to be regenerated and an appropriate spatial arrangement are extremely important factors. Furthermore, it is known that MSC differentiation is fundamentally determined by mechanisms such as cell proliferation (mitosis), biochemical-molecular interactions, movement, cell adhesion, and apoptosis. Although the use of MSCs for cartilage regeneration remains at a research level, there are important questions to be resolved in order to make this therapy efficient and safe. It is known, for instance, that the expansion of chondrocytes in cultivation, needed to increase the number of cells, could end up producing fibrocartilage instead of hyaline cartilage. However, the latest results are promising. In 2014, the first stage I/II clinical trial to evaluate the efficacy and safety of the intra-articular injection of MSCs in femorotibial cartilage regeneration was published, indicating a decrease in injured areas. One issue to be explored is how many modifications in the articulate inflammatory environment could induce differentiation of MSCs already allocated in that region. Such issue arose from studies that suggested that the suppression of the inflammation may increase the efficiency of tissue regeneration. Considering the complexity of the events related to the chondrogenesis and cartilage repair, it can be concluded that the road ahead is still long, and that further studies are needed.

☆ Study conducted at the Institute of Orthopedics and Traumatology of Passo Fundo, Passo Fundo, and at the Health Sciences Center, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil.

* *Corresponding author.*
E-mails: lech@lech.med.br, ensino@iotrs.com.br (O. Lech).

http://dx.doi.org/10.1016/j.rboe.2016.11.005

Potencial regenerativo do tecido cartilaginoso por células-tronco mesenquimais: atualização, limitações e desafios

Palavras-chave:
Células-tronco
Tecido cartilaginoso
Potencial regenerativo

RESUMO

Os avanços nos estudos com células-tronco mesenquimais (CTMs) adultas tornou a terapia regenerativa tecidual uma ferramenta promissora em diversas áreas da medicina. Na ortopedia, um dos principais desafios tem sido a regeneração do tecido cartilaginoso, sobretudo em diartroses. Na indução de CTMs, além da citodiferenciação, o contexto microambiental do tecido a ser regenerado, bem como uma disposição espacial adequada, são fatores de extrema importância. Além disso, sabe-se que a diferenciação das CTMs é basicamente determinada por mecanismos como proliferação celular (mitose), interações bioquímico-moleculares, movimento, adesão celular e apoptose. Apesar de o uso de CTMs para a regeneração da cartilagem estar ainda em âmbito de pesquisa, existem questões importantes a serem resolvidas para tornar essa terapêutica eficaz e segura. Sabe-se, por exemplo, que a expansão de condrócitos em cultura, necessária para aumentar o número de células, pode produzir fibrocartilagem, e não cartilagem hialina. No entanto, os últimos resultados são promissores. Em 2014, foi publicado o primeiro ensaio clínico fase I/II para avaliar a eficácia e a segurança da injeção intra-articular de CTMs na regeneração de cartilagem femorotibial e houve uma diminuição das áreas lesadas. Uma questão a ser explorada é o quanto modificações no próprio ambiente inflamatório articular poderiam induzir a diferenciação de CTMs já alocadas naquela região. Tal incógnita parte do princípio de estudos que sugerem que a supressão da inflamação articular aumentaria, potencialmente, a eficiência da regeneração tecidual. Considerando a complexidade dos eventos relacionados à condrogênese e ao reparo da cartilagem, conclui-se que o caminho ainda é longo, são necessárias pesquisas complementares.

Introduction

The human body fundamentally originates from embryonic stem cells: ectoderm, mesoderm, and endoderm. It is from these three leaflets that the 230 cell types found in the body are differentiated. In a differentiated organism, many tissues retain adult stem cell lines that work in tissue replacement and regeneration; the most abundant ones are of mesodermal origin, the mesenchymal stem cells (MSCs). MSCs are found in various places in the body, such as in the red bone marrow, hair follicles, muscle, umbilical cord, dental pulp, adipose tissue, bone, and cartilage, among others.[1] With the increased knowledge on adult MSCs, their clinical use for tissue regeneration has become quite attractive. However, understanding and effectively and safely handling MSCs is still a considerable challenge, especially in tissues with difficult regeneration, such as cartilage.

In this context, this review aimed to provide an update on the main processes related to morphodifferentiation and its potential role in the regeneration of cartilage tissue. Therefore, the information contained herein is based on scientific journal articles indexed in the databases of PubMed-MEDLINE and SciELO.

Cartilaginous tissue and challenges for regeneration

In structural terms, the articular cartilage is rich in extracellular matrix, in which chondrocytes are distributed, whether isolated or arranged in clonal groups in small cell colonies.[2] Chondrocytes are responsible for secreting cartilage matrix components such as collagen, proteoglycans, and glycoproteins. Cartilage tissue receives nutrition *via* capillaries contained in the perichondrium, a connective tissue that surrounds the cartilage and has adult MSCs termed chondroblasts.

Nonetheless, as the cartilages lining the bones of movable joints do not have perichondrium, they receive nutrition through the synovial fluid present in the joint cavities. Synovial fluid is a plasma ultrafiltrate that passes through the synovial membrane, where it receives mucopolysaccharides that contain hyaluronic acid and a small amount of high molecular weight proteins. Therefore, even with a large amount of collagen protein, the small amount of cellular components in cartilage tissue hinders its regeneration capability leading repetitive joint injuries toward a tendency to become chronic.[3]

In order to understand the role of MSCs in adult tissue regeneration, it is important to remember that the human organism is made of cells that have distinct differentiation and functions.[4] It should be noted that tissue regeneration is not only focused on the induction of undifferentiated MSCs in a differentiated cell. Each type of tissue has an extracellular matrix, with a key role in body homeostasis. Thus, the microenvironmental context (extracellular matrix) of the tissue being regenerated must be taken into consideration.

Five causal mechanisms are crucial for cell differentiation in tissues and organs, as well as the tissue regeneration process itself, namely: cell proliferation (mitosis), biochemical and molecular interactions, movement, cell adhesion, and apoptosis. As these mechanisms are of great importance for handling MSCs with the perspective of developing cartilaginous tissue regeneration techniques, they will be the focus of this review.

Proliferation and cellular senescence

To understand in greater depth the biology of MSCs, it is necessary to understand some of the mechanisms associated with the cell cycle. Eukaryotic cells divide by mitosis, considered the final stage of the cycle, in which the two newly born cells will perform their respective metabolic functions.

Nonetheless, the mitotic division is not an unlimited process. As they divide, most specialized cells lose their proliferative capacity. Some cells that will no longer divide remain constantly in the gap1 phase (G1) of mitosis, which is the case of the vast majority of chondrocytes. New chondrocytes are formed from chondroblasts from the perichondrium; it is through this mechanism that the cartilage tissue is renewed, albeit slowly when compared, for example, with bone tissue. Conversely, some mature specialized cells undergo the entire cell cycle until they lose their ability to proliferate, by a process termed replicative senescence or cellular aging (Fig. 1).

Cellular aging is triggered by changes occurring in the terminal region of the chromosome, known as telomere, which comprises a single-stranded deoxyribonucleic acid (DNA) molecule, in contrast with the double-stranded structure present in the remainder of the genetic material. Telomeric DNA consists of a sequence of six nucleotides – thymine, thymine, adenine, guanine, guanine, guanine (TTAGGG) – which repeats thousands of times. This chromosomal region is synthesized by the reverse transcriptase enzyme telomerase, which synthesizes DNA and uses a ribonucleic acid (RNA) molecule as a scaffold.

In cell division, a small telomere shortening is always observed. In embryonic cells, the telomere is reconstituted by the action of telomerase. In specialized cells, telomerase gene is silenced; therefore, when telomeric shortening occurs, telomere reconstitution is not possible. Over the divisions (approximately 50–80 mitoses), the telomere becomes too short and starts to inhibit mitosis, thus constituting cellular senescence or Hayflick limit.

Unlike specialized cells, in MSCs, the telomerase gene is active; therefore, within the body, such cells do not present a sharp cellular aging. However, the MSC proliferation rate is extremely low and thus the number of these cells in body tissues is quite limited. This is an important challenge to be overcome in regenerative therapy.

Some studies have suggested that the induction of *in vitro* MSC proliferation can be done by exposure to reactive oxygen species (ROS) molecules, such as hydrogen peroxide. The study by Bornes et al.[5] showed that *in vitro* chondrogenesis of MSCs induced in sheep bone marrow increased proliferation and cell differentiation. However, it appears that, despite the increase in cell growth, cells start to present significant DNA damage, indicating chromosomal instability.[6] The study by Machado et al.[6] supports the results of the research conducted by Brand et al.,[7] suggesting that *in vitro* exposure to oxidative stress induces cellular senescence in chondrocytes.

In turn, the study by Machado et al.[6] showed a reversal of replicative senescence markers in human MSCs collected through liposuction by supplementing culture medium with a hydroalcoholic guarana (*Paullinia cupana*) extract. The guarana seed, used for the production of the extract, is rich in caffeine, theophylline, theobromine, and catechins.

Another very surprising result was described by Sadeghiet et al.,[8] who investigated the effect of estrogen supplementation on chondrogenesis induced in MSCs derived from adipose tissue. It was observed that the presence of estrogen had negative effects on the chondrogenesis process by inhibiting the expression of the collagen 2 gene and reducing the expression of the aggrecan protein gene.

Cell differentiation

All body cells and tissues are formed from the zygote, in a highly controlled transcriptional regulation process. In general, the DNA of the eukaryotic gene has an initial sequence of nucleotides known as the promoter region. It is in this region that the signaling molecules bind, allow (or not) the transcription, and determining the amount of RNA to be transcribed. This modulation is known as gene regulation, a mechanism through which each cell type is formed by the production of proteins in different shapes and quantities. Endogenous molecules, such as transcription factors and hormones, can differentially regulate genes. Likewise, molecules derived from the diet, such as resveratrol (present in grapes), induce the production of sirtuins, proteins that increase cell life.

Under *in vitro* conditions, the induction of MSC differentiation in the presence of certain molecules is very well known. Notwithstanding, when MSCs are placed in an injured organ, it is not always possible to know whether the microenvironmental conditions will favor the induction of differentiation (even when the inducing agents are co-inserted with the cells).

Other molecules that regulate the maintenance of the undifferentiated state of MSCs, ensuring their pluripotentiality and self-renewal, have been identified. This is the case of Oct-4, Nanog, and Sox-2, which are found both in humans and in mice.[9] When this protein is no longer expressed, the cell has entered the process of differentiation.[10]

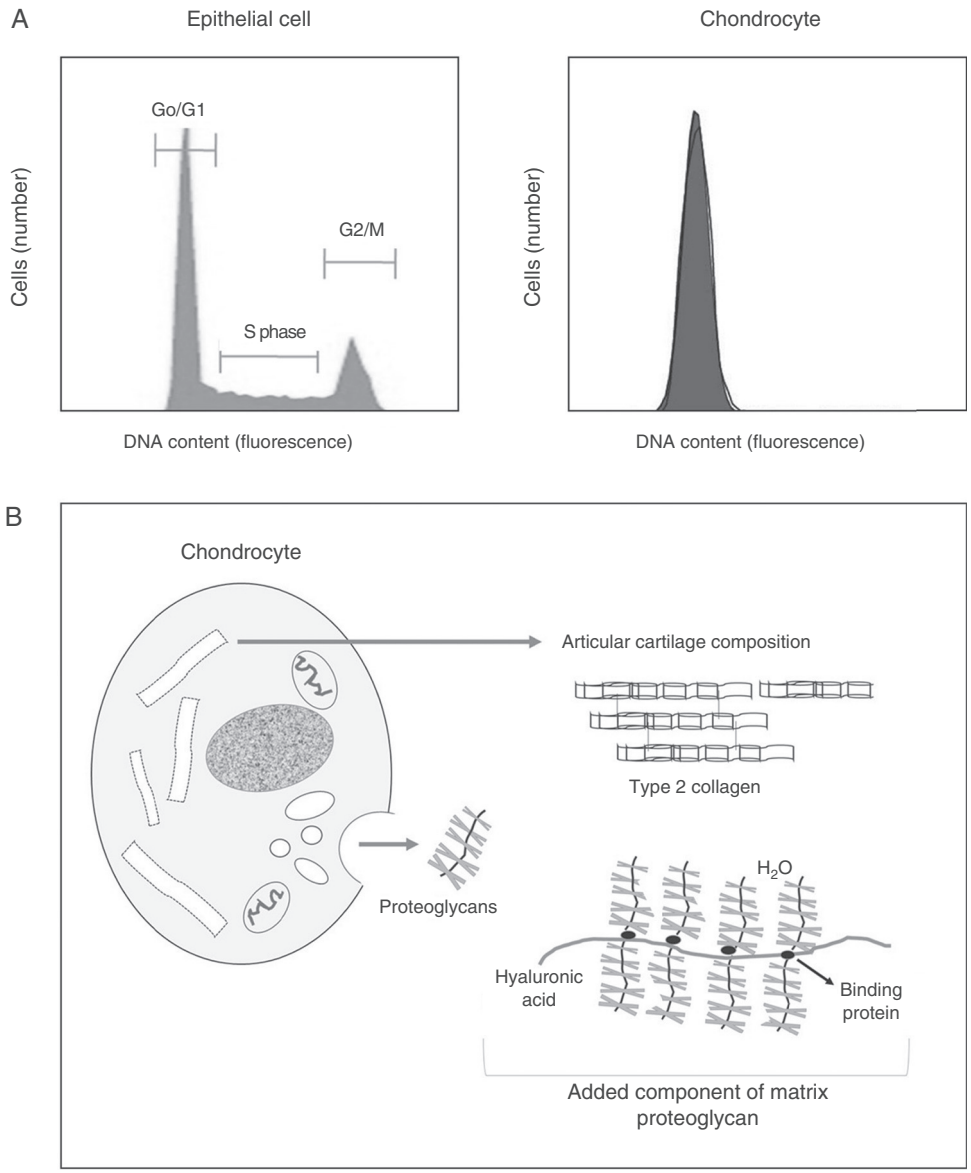

Fig. 1 – Comparison of the cell cycle of an epithelial cell and of a chondrocyte, assessed by flow cytometry. (A) In an epithelial tissue, cells are found in phase G0/G1, S, and G2/M, while in the cartilage tissue most chondrocytes are in the G1 phase. Only chondroblasts from perichondrium will present a complete cell cycle. (B) Chondrocytes, once formed, are usually clustered in about eight cells that continuously secrete an extracellular matrix composed mainly of type 2 collagen, proteoglycans, and hyaluronic acid.

To induce chondrogenic differentiation, MSCs are cultured without the presence of fetal or adult serum (animal or human), which is typically used to nourish the cells, and upon exposure to growth factor b3.[11] Thus, the cells develop a multilayer with a proteoglycan-rich extracellular matrix. In 10–14-day cultures, cells begin to produce type 2 collagen, characteristic of the articular cartilage. Moreover, they present positive surface markers for chondrocytes and typical cell gaps visible at optical microscopy. The chondrocytes remain viable until approximately 90 days after initiation of differentiation.[12]

Chondrogenesis is induced through various inducing molecules, especially through supplementation of culture medium with TFN-β3, IGF-1, BMP-2, and BMP-6. Chondrodifferentiation induction is confirmed by the identification of markers such as type 2 collagen, Sox-9, and aggrecan, through analysis of gene expression using real-time quantitative polymerase chain reaction (PCR).

In addition to differential regulation of gene expression, methylation is an epigenetic modification that usually occurs in the promoter region of the gene to be silenced. This process is mediated by the DNA-methylases (DNMTs) enzymes.

Genes can also be silenced *via* the acetylation process, which prevents histones from becoming relaxed when the DNA is exposed to transcriptional regulation.[13]

Adhesion and cell movement, and production of scaffolds

During embryogenesis, in addition to differentiation process, cells need to migrate or grow toward a specific location and remain there to perform their roles. Cell adhesion and movement, which occur by chemical and spatial signals, are of vital importance for combining individual cells in a three-dimensional format, such as in body tissues and organs.

Cell adhesion mechanisms are highly regulated during tissue morphogenesis. Reversible phosphorylation by protein kinase C (PKC) is a key event in cell adhesion and migration during chondrogenesis.[14]

Adhesion and cell movement are also related to the architectural formation of tissues and organs. *In vitro* studies have shown that MSCs respond to their environmental format and that *in vivo* cells are also induced to differentiate by the topographical characteristics of the tissue in which they are arranged. Such evidence boosted the field of tissue engineering, which combines cellular therapy with use of biomaterial scaffolds. This area involves the use of compatible and biodegradable materials that act as a matrix for cell growth. Scaffolds are simple media in which cells are cultivated to create a tissue *in vitro*.

Apart from providing mechanical support and spatial orientation for cell growth and differentiation, the structure of the scaffold must allow the transport of nutrients, metabolites, growth factors, and other important regulatory molecules to the cells and extracellular matrix. Scaffolds can be produced from natural or synthetic molecules. Among natural biomaterials, collagen, hyaluronic acid, hydroxyapatite, and glycosaminoglycans are noteworthy.[15]

Electrospinning produces scaffolds formed by fibers that can physically mimic a natural extracellular matrix. This condition creates a suitable microenvironment for cell and tissue differentiation. The creation of fibers of different diameters by electrospinning is performed using polymer solutions applied to a magnetic field. The polylactic-co-glycolic acid (PLGA) polymer has been widely used in the production of scaffolds by electrospinning, because it is biodegradable, bioabsorbable, and biocompatible. The use of PLGA-based scaffolds has been approved in humans by the US Food and Drug Administration (FDA). Investigations have shown that this biomaterial can induce growth in different cell types, such as fibroblasts, osteoblasts, and chondrocytes.[16]

Another technology derived from electrospinning is bioelectrospinning, which uses the processing of cellular suspensions that are subjected to a high intensity electric field and induced to pass through a sharp needle, generating fine droplets that contain cells. Thus, the scaffold is built with cells already integrated. This technique allows for a homogeneous distribution of MSCs in the scaffold and therefore, a greater regenerative potential.[15] Considering the limited regenerative capacity of cartilaginous tissue, the mixture of biomaterials and stem cells appears to be the most promising option, although further studies are needed to confirm the efficacy and safety of this method.

Apoptosis and inflammation in cartilage degeneration and regeneration

Cells have the ability to self-regulate not only the rate of proliferation and differentiation, but also their death in many situations, from an event known as apoptosis or programmed cell death. Unlike necrosis and autophagy, apoptosis is a highly coordinated mechanism and does not cause a specific inflammatory process.[17] However, evidence shows that chronic inflammation induces disorganization of the extracellular matrix and apoptosis of chondrocytes, which consequently leads to cartilage destruction. This occurs in many degenerative diseases, such as rheumatoid arthritis and osteoarthritis.[18]

It is known that monocytes/macrophages are essential components of the innate immune system and have a variety of functions. They control the onset and resolution of inflammation by phagocytosis, release of inflammatory cytokines, reactive oxygen species (ROS), and activation of the acquired immune system. Under normal circumstances, monocytes circulate in the bloodstream for a short period before spontaneously entering in apoptosis. The presence of stimulatory factors inhibits the apoptosis of monocytes that differentiate into macrophages, which can live for a long time in tissues.[19,20]

Macrophages produce many substances that are relevant to immune response and coordinate the inflammatory process (inflammatory cytokines L-1β, IL-6, TNFα, and the anti-inflammatory cytokine IL-10). Furthermore, they produce factors that are critical in combating microorganisms (such as oxygen metabolites and nitric oxide) and factors that promote tissue repair (such as fibroblast growth factor), among others.[21] Nowadays, two types of macrophage activation in the inflammatory response are recognized: the “classical activation” and “alternative activation” (Fig. 2).

In this process, when an increase in the activation of M1 macrophages compared to M2 macrophages is observed, there will be poor tissue repair with continued destruction in tissues with low regenerative capacity, such as cartilage.

In osteoarthritis, the occurrence of intra-articular inflammation with synovitis indicates that synovial fluid can be the source of inflammatory cytokines and proteolytic enzymes. In synovitis, there is release of proinflammatory cytokines such as IL-1β and TNFβ; these molecules have an inhibitory effect on the type 2 collagen and aggrecan production by chondrocytes. Furthermore, these cytokines cause the release of metalloproteinases and aggrecanases that degrade the matrix, which results in the destruction of cartilage. Other molecules, such as M IL-1β and TNFβ, may also be involved in apoptosis of chondrocytes by increasing the release of nitric oxide and prostaglandin E2 (PGE2).[18]

Although there are MSCs in joint tissue, synovial membrane, tendons, and cartilages, the induction of these cells

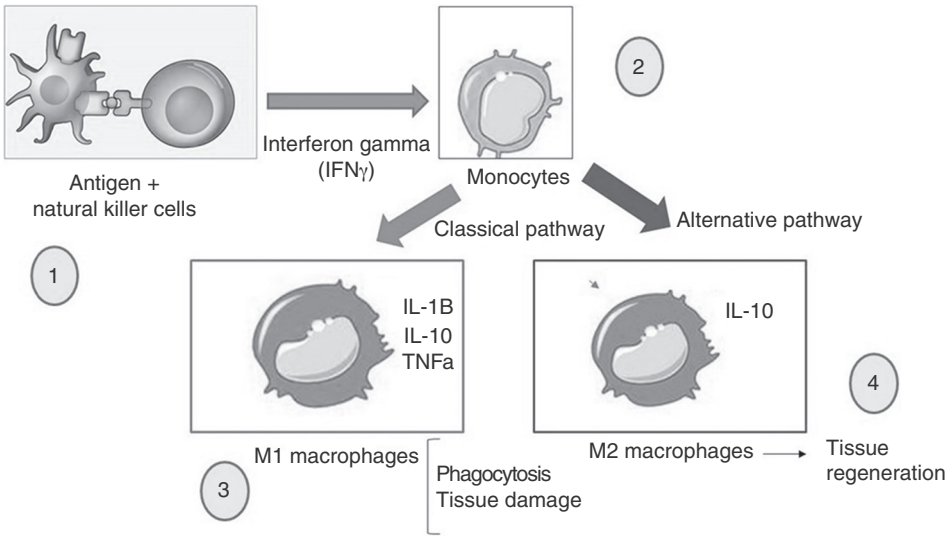

Fig. 2 – Macrophages activated by the classical pathway act as inflammation inducers and are called M1. These macrophages produce high levels of IL-2 and low levels of IL-10. Studies have also shown that the activation of macrophages is dependent on the stimulation of inflammatory cytokines produced by helper lymphocytes or NK cells, in particular gamma interferon (INFγ). The activated macrophages that have microbial and tumoricidal activity are characterized by secreting large amounts of cytokines and proinflammatory mediators. In this inflammatory response, these macrophages release proinflammatory cytokines, such as IL-1, IL-6, TNFα, and also produce reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide, as well as reactive intermediates, such as nitric oxide. Shortly after phagocytosis, the macrophages die by programmed cell death, known as apoptosis. The "alternative activation" involves the stimulation of macrophages by molecules such as the interleukins IL-4 and IL-13, which leads to an increase in IL-10 anti-inflammatory cytokine levels and induces tissue repair (anti-inflammatory response). In the body, the proinflammatory immune response is usually followed by an anti-inflammatory immune response. This is important for tissue repair after a microbial infection or physical injury. The imbalance between the two responses can lead to chronic diseases such as osteoarthritis.

to regenerate cartilage tissue has not been fully elucidated. It is known that the chondrogenesis process is triggered by factors such as bone morphogenetic proteins (BMPS) and growth factors such as TGF-β. These factors act on genes, such as transcription factor SRY-box 9 (Sox9), which is essential for chondrocyte differentiation. Sox9 controls the transcription of genes that synthesize extracellular matrix molecules, such as type 2 collagen and aggrecan, while also suppressing the formation of hypertrophic chondrocytes.

Chronic inflammatory processes appear to negatively influence the differentiation of MSCs into chondrocytes. In this case, the cytokine IL1β and TNF-aβ have a suppressive effect on chondrogenesis. This is because these cytokines inhibit the expression of the Sox9 gene by suppressing the expression of the TFG-β molecule (an important initiation factor in the differentiation of chondrocytes) and increasing the expression of the Smad7 molecule (a chondrogenesis inhibitor). The inflammatory cytokine IL-17, which is a key molecule in chronic inflammation processes, also has the ability to suppress chondrogenesis. This molecule suppresses the phosphorylation of Sox9 protein and prevents its regenerative action.[22]

Therefore, considering all the evidence on the important role of chronic inflammation in the regenerative process of cartilage, it is clear that, in an inflammatory environment with high levels of IL-1β, TNFβ, and IL-17, MSCs may not respond adequately to regenerative therapy. This is because these cells can be induced to apoptosis before they differentiate into chondrocytes.

Clinical applications of stem cells in cartilage tissue regeneration

Many preclinical and clinical studies involving potential cartilage regeneration with MSCs are conducted for various diseases, including osteoarthritis. Although the use of MSCs for cartilage regeneration is still at the research level, there are important issues to be resolved to make this an effective and safe therapy.

The implantation of chondrocytes from one region of the body to the injured region has the disadvantage of requiring two surgical procedures. The need for growing chondrocytes in culture to increase the number of cells to be implanted is also another major problem, as these cells may dedifferentiate and produce fibrocartilage instead of hyaline cartilage.[23–26]

In an attempt to minimize these problems, some authors began to research the effect of intra-articular MSC injection in the treatment of osteoarthritis. This procedure apparently

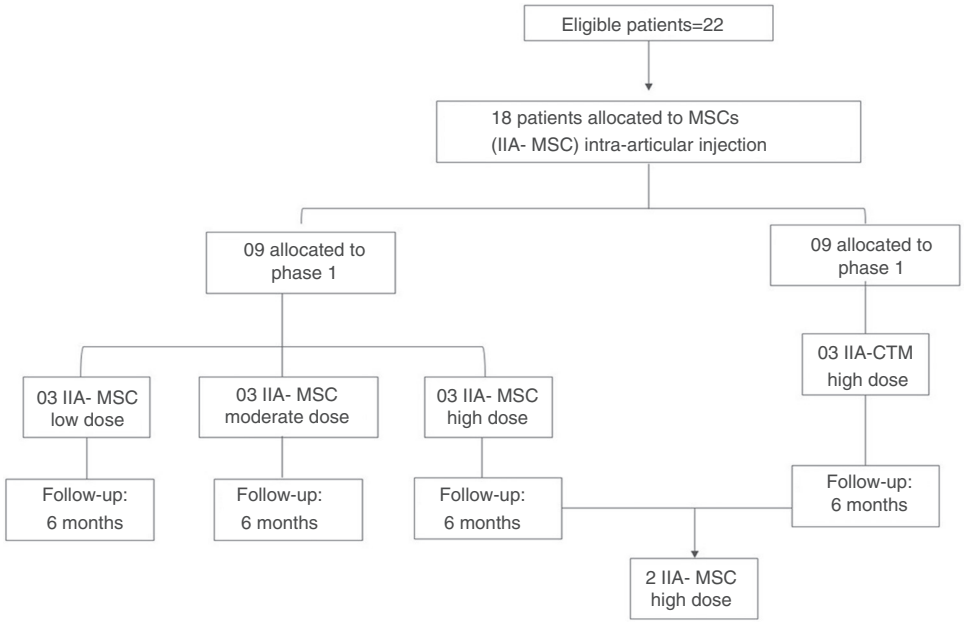

Fig. 3 – Overall experimental design of the study by John et al.[33] (2014), who assessed, in a phase I/II clinical trial the effect of intra-articular injection on the regeneration of the knee cartilage in patients with osteoarthritis. MSCs were obtained from abdominal lipoaspirate, cultured in the laboratory, and injected in the joint after three weeks. Low dose = 1×10^7; moderate dose = 5×10^7; high dose = 1×10^8 cells in saline. Pharmacological therapy was discontinued, with the exception of ketoprofen administration.

has many advantages, since it would avoid surgery in many cases.[27–32] Nonetheless, the first phase I/II clinical trial to evaluate the efficacy and safety of intra-articular injection in knee articular cartilage regeneration through clinical, laboratory, radiological, arthroscopic, and histological analyses was only published in 2014, by Jo et al. (Fig. 3).[33]

The injected MSCs were obtained by liposuction of subcutaneous abdominal fat; the obtained MSCs were tested for their viability, purity (with evaluation of CD31 markers, CD34, CD45), identity (with evaluation of CD73 markers, CD90), sterility, and lack of contamination with endotoxins or mycoplasma.

The procedures for intra-articular injection were performed in the supine position with spinal anesthesia three weeks after liposuction. A standard arthroscopic examination was performed and the knee articular cartilage lesions were measured with a calibrated and graduated arthroscopic probe, in accordance with the International Cartilage Repair Society (ICRS) classification of cartilage lesions. The MSCs diluted in saline were injected, without debridement, synovectomy, or meniscectomy during the procedure. No serious adverse effects were reported and the quality of knee condition was assessed by the Western Ontario and McMaster Universities Arthritis Index (WOMAC), and showed significant improvements in patients receiving high concentration of intra-articular MSCs. The cartilage defect size decreased in the medial femoral and tibial condyles, and also in the groups receiving high doses of MSCs. Thus, the authors concluded that intra-articular injection of 1×10^8 cells improved the function of osteoarthritic knee and pain, without causing adverse effects, through the reduction of cartilage defects by regenerating tissue similar to hyaline cartilage.

Final considerations

Although the results of Joet et al.[33] are encouraging, Kondo et al.,[18] in their review on the subject, pointed out that the results of additional studies involving multiple protocols are needed in order to truly prove the efficacy and safety of this procedure. Furthermore, these latest authors commented that further investigations are being conducted, aiming to improve efficiency of MSC differentiation into chondrocytes by analyzing the supplementation of culture media with various regulatory molecules, such as TGF-β1–3; BMP-2, -4, -6, -7; FGF-2, and IGF-1, among others. In addition to these, some compounds such as dexamethasone and ATP have shown a positive effect on chondrogenesis.

Another important question concerns the inflammatory microenvironmental conditions of the MSC injection site. Evidence shows that MSCs have immunosuppressive and anti-inflammatory effects. Nonetheless, suppression of joint inflammation could potentially increase the efficiency of tissue regeneration.

In this case, what remains unclear, requiring further exploration in future studies, is how changes in the inflammatory

joint environment itself could induce the differentiation of MSCs already allocated in the region. The answer to this question could lead to the development of regenerative techniques associated with the already well-established surgical techniques, without transplantation of MSCs to other parts of the body. However, considering the complexity of the events related to chondrogenesis and cartilage repair, the road ahead is still long and a considerable volume of additional research is needed.

Conflicts of interest

The authors declare no conflicts of interest.

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