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Combination of mesenchymal stem cells and bioactive molecules in hydrogels for osteoarthritis treatment

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ABSTRACT

Osteoarthritis (OA) is a chronic and inflammatory disease with no effective regenerative treatments to date. The therapeutic potential of mesenchymal stem cells (MSCs) remains to be fully explored. Intra-articular injection of these cells promotes cartilage protection and regeneration by paracrine signaling and differentiation into chondrocytes. However, joints display a harsh avascular environment for these cells upon injection. This phenomenon prompted researchers to develop suitable injectable materials or systems for MSCs to enhance their function and survival. Among them, hydrogels can absorb a large amount of water and maintain their 3D structure but also allow incorporation of bioactive agents or small molecules in their matrix that maximize the action of MSCs. These materials possess advantageous cartilage-like features such as collagen or hyaluronic acid moieties that interact with MSC receptors, thereby promoting cell adhesion. This review provides an up-to-date overview of the progress and opportunities of MSCs entrapped into hydrogels, combined with bioactive/small molecules to improve the therapeutic effects in OA treatment.

1. Introduction

Osteoarthritis (OA) is a debilitating, chronic disease that affects joints through progressive inflammation and degradation of the articular cartilage, inducing pain, swelling and immobility. This condition is associated with age, obesity and diabetes, and other risk factors that are widespread in our society [1]. Thus, the World Health Organization estimates that the incidence of OA is 9.6% and 18.0% for men and women over sixty, respectively [2]. The knee and hip joints are the most affected areas, but others, such as those in the hands, can also be impaired with OA. This review mainly focuses on the cartilage of the knee, but the principles covered here are also applicable to other joints. The complexity of the joint tissues together with other factors implicated in OA leads to a common strategy that involves pain management, the reduction of risk factors (obesity, mechanical stress, etc.), walking aids or splint use, and ultimately joint surgery [3]. Unfortunately, these treatments in most cases mitigate the suffering of the patients and are not effective when the disease progresses and becomes chronic.

Given this scenario, novel strategies for effecting cures in OA have been recently investigated. The so-called "disease-modifying OA drugs" (DMOADs) show promise in modulating and slowing disease progression, even though only a few of them have reached the market. Small DMOAD molecules such as kartogenin (KGN) [4]. Alternative strategies for cartilage regeneration based on stem cell therapy involve several cartilage degeneration pathways and might be more successful, compared to small molecules that only targets one OA pathway [5–7]. Here, injected Mesenchymal stem cells (MSCs) can differentiate into chondrocytes, which are deficient in the osteoarthritic joint [8,9]. In addition, MSCs secrete both growth factors and extracellular matrix (ECM) molecules that contribute to cartilage regeneration and chondrocyte proliferation [10,11]. A major challenge here is to preserve the integrity of MSCs in the inflammatory environment of the osteoarthritic joint. This issue has been addressed by adding bioactive/small molecules to hydrogel systems to enhance the survival of the injected MSCs

Abbreviations: ADSC, adipose-derived stem cell; BMP-2, bone morphogenetic protein 2; BMSC, bone marrow-derived stem cell; DMOAD, disease-modifying osteoarthritis drug; ECM, extracellular matrix; GAG, glycosaminoglycan; HA, hyaluronic acid; IGF-1, insulin-like growth factor 1; KGN, kartogenin; MMP-1, matrix metallopeptidase 1; MMP-13, matrix metallopeptidase 13; MSC, mesenchymal stem cell; OA, osteoarthritis; PEG, polyethylene glycol; pNIPAM, poly(N-isopropylacrylamide); TGF-β, transforming growth factor β.

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[12]. The construction of these hydrogels often entails the use of polymers that mimic the chemical and mechanical properties of the endogenous ECM [13], generating a local environment able to sustain the viability of MSCs. In addition, incorporated bioactive/small molecules can include growth factors, small molecular weight therapeutic compounds, and ECM molecules [14,15]. These molecules have an adjuvant role since they can control the inflammatory response and/or enhance signaling activities, provide a cell-conducive architecture and even serve as nutrients for MSCs [16–18].

This review highlights the progress made mainly in the past five years to provide new cell-based hydrogel therapeutic formulations for knee OA treatment. The challenges and opportunities for the successful design and development of these formulations will be discussed, taking into account the complex environment of OA.

2. Cartilage degeneration in OA

A joint is a complex system composed of three fundamental structures: i) the bone, ii) the cartilage tissue composed mainly of ECM, chondrocytes and a few MSCs and iii) the synovial cavity and its synovial fluid surrounded by the synovium, with synoviocytes and inactivated macrophages (Fig. 1A) [3,19]. MSCs migrate from the bone toward the superficial cartilage zone adjacent to the synovial cavity, and their chondrocyte progeny appear mainly in the middle and deep cartilage zones. The ECM of cartilage plays a crucial role in cartilage homeostasis and chondrocyte function. ECM contains structural collagen type II fibers and highly hydrophilic proteoglycans, including glycosaminoglycans (GAGs), which perform biomechanical tasks such as structural support and resistance to deformation. Notably, blood nutrients are not directly supplied to this tissue. The lack of vascularization requires local secretion from chondrocytes so that nutrient diffusion through cartilage ECM is a critical survival factor [20].

In OA, the cartilage is progressively damaged by mechanical stress, ECM catabolism, vascular infiltration and activation of macrophages, among others [21,22] (Fig. 1B). This process has two important consequences: cartilage factor loss by leakage into the synovial cavity and chronic inflammation mainly mediated by interleukins [23]. Enzymes such as matrix metallopeptidase 1 (MMP-1) and matrix metallopeptidase 13 (MMP-13) are secreted and lyse collagen from the cartilage matrix [24]. This effect also affects the phenotype and functionality of chondrocytes and MSCs, ultimately triggering hypertrophy and apoptosis of chondrocytes (Fig. 1B). Consequently, layer-by-layer cartilage breakdown occurs, together with endochondral ossification [25–28] that progressively leads to functional collapse of the joint, including the meniscus and adjacent ligaments. Notably, in a non-OA situation, chondrocytes and MSCs actively contribute to spontaneous regeneration of the damaged cartilage. These cells act on ECM remodeling and dissociate crosslinked collagen type I. Chondrocytes secrete new ECM factors, and MSCs and synoviocytes discharge proinflammatory mediators in a controlled manner [29]. Therefore, any spontaneous regeneration is too slow or even insignificant if these cells are compromised through chronic joint misalignment, weight overload, or senescence, and OA develops in joints [8,30]. Despite the efforts made to understand disease pathogenesis, the search for effective treatments is still a major challenge [31–33]. This issue has motivated the implementation of MSCs for OA treatment with the aim of not only reducing inflammation but also regenerating damaged tissue.

3. Therapeutic potential of MSCs in OA

MSCs were introduced in clinical trials more than two decades ago starting with bone marrow stem cell injection during breast cancer chemotherapy [34]. Since then there has been a growing interest in their use in OA (reviewed in [35–36]). The origin and methodology employed to differentiate them into the appropriate cell phenotype are two key issues to consider. The primary origin of MSCs available for treatment modalities is adipose-derived stem cells (AMSCs) and bone marrow-derived stem cells (BMSCs) because of their homogeneity and the ability to supply a considerable therapeutic amount of cells [37]. In 2006 the International Society for Cell and Gene Therapy (ISCT) suggested the

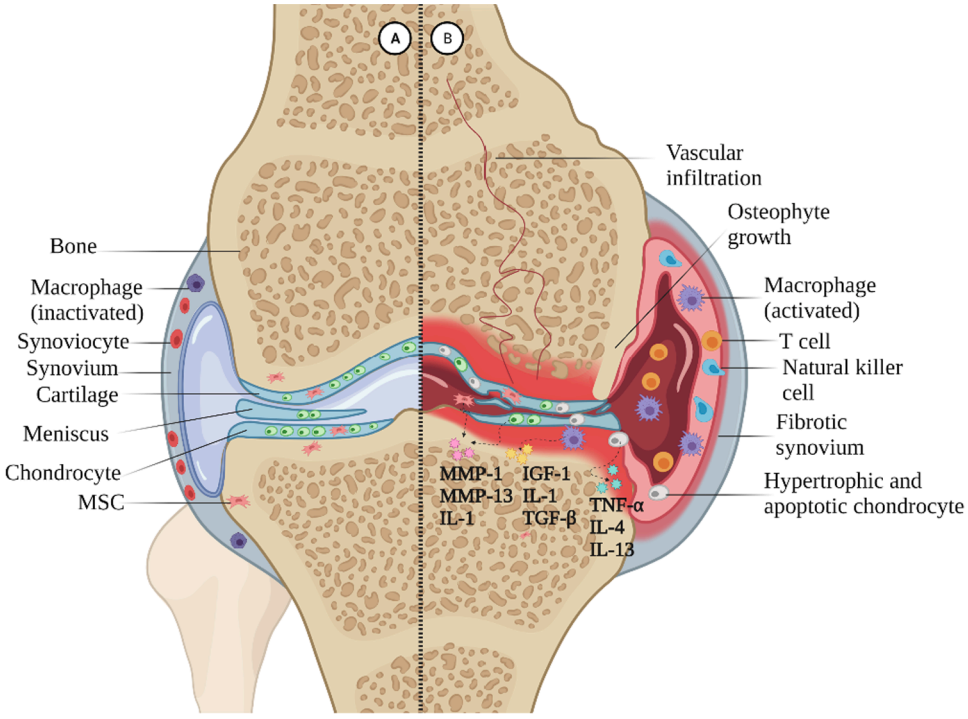

Fig. 1. Healthy (A) and osteoarthritic (B) knee joint. In an osteoarthritic environment, macrophages are activated, secreting several inflammatory factors and collagen degradation enzymes (*e.g.*, IL-1, IL-4, IL-13, TNFα, IGF-1, TGF-β, MMP-1 and MMP-13)*. During OA, the structured zones break down, vascular infiltration occurs and osteophytes grow. The main consequences are cartilage erosion, chondrocyte hypertrophy and apoptosis. *IL-1 = Interleukin-1, IL-4 = Interleukin-4, IL-13 = Interleukin-13, MMP-1 = Matrix metallopeptidase 1, MMP-13 = Matrix metallopeptidase 13, IGF-1 = Insulin-like growth factor-1, TGF-β = Transforming growth factor β, TNF-α = Tumor necrosis factor α.

introduction of four criteria to identify MSCs in heterogeneous populations: i) plastic adherence ii) self-renewal iii) expression of a defined set of surface markers and iv) in the case of OA potential to differentiate into chondrocytes/osteoblasts [38]. A defined set of surface markers diagnostically reveals the capacity of stem cells to differentiate into chondrocytes. Conversely the absence of specific surface markers such as the human leukocyte antigen is essential to avoid recognition by the immune system [39].

In OA, the primary goal of injected MSCs is to induce paracrine signaling that modulates i) the immune response and inflammation, ii) cell migration and proliferation of endogenous MSCs/chondrocytes, and iii) regulation of the ECM environment. With this in mind, what role do MSCs play during cartilage regeneration? MSCs reduce the immune response by secreting soluble factors such as transforming growth factor β (TGF-β), interleukin 6, and prostaglandin E2 [40], among others. These factors inhibit T cell proliferation/function, induce senescent-like natural killer cells and reprogram macrophages from their proinflammatory M1 state to the conducive M2 phenotype (Fig. 2) [41–45]. These anti-inflammatory effects were observed by direct injection of MSC-derived exosomes. Exosome uptake by chondrocytes and synoviocytes led to a significant decrease in inflammatory factors, chemokines, and cytokine secretion [46,47]. During regeneration, growth factors secreted by MSCs, such as stromal cell-derived factor 1 and platelet-derived growth factor, promote the migration and recruitment of endogenous MSCs and chondrocytes (Fig. 2) [48,49]. Here, the hyaluronan receptor CD44 is also essential for MSC migration since the cell homing potential of MSCs in an inflammatory environment is increased when this receptor is overexpressed [50]. Furthermore, MSC-secreted factors activate the anabolic activity of chondrocytes on cartilage and regulate the composition and architecture of the ECM [51,52]. Cosenza *et al.* showed that the levels of type II collagen and aggrecan, two key markers of cartilage regeneration, were increased upon exposure to MSCs. The production of ECM was consequently increased, and joint integrity was preserved in a arthritis mouse model induced by collagenase [53].

The therapeutic use of MSCs in OA is gaining relevance, as evidenced by 6 ongoing phase III clinical trials [54]. In these studies, three different sources of stem cells were used: human umbilical cord blood-derived MSCs (NCT01626677), autologous bone marrow aspirate (NCT04990128, NCT00891501), and autologous microfragmented adipose tissue-derived stem cell aspirate (NCT04427930, NCT04230902). Human umbilical cord blood-derived MSCs were embedded in 4% hyaluronic acid (HA) hydrogels for delivery into the OA joints. In the case of the other clinical trials, it is hypothesized that the aspirate of human tissues "preserves" the function and viability of autologous stem cells. These aspirates contain a heterogeneous mixture of interleukins, platelets, lipids, among others; however, it is unclear which of these factors specifically support MSC survival. In short, approaches that reached the phase III stage have involved hydrogels and/or bioactive molecules. Optimizing the best hydrogel composition, selecting a suitable bioactive molecule and their combination might therefore be a highly promising strategy in OA regeneration therapy.

4. Hydrogel formulation characteristics for MSCs in cartilage regeneration

The ECM is a three-dimensional network that provides mechanical support and facilitates cell signaling [55]. In cartilage, the ECM is dense and has a sparse distribution of MSCs. The presence of polysaccharides (including HA), collagen, glycoproteins, and other proteins [55] contributes to retaining water, which is essential for the unique mechanical properties of cartilage. In addition, chondrocytes and MSCs can sense the mechanical rigidity of the ECM through integrin–mediated interactions between the cells and the matrix. External forces applied on the joint thus influence the factors secreted by cells [56,57]. In a similar way, hydrogels possess cartilage-like features compared to other

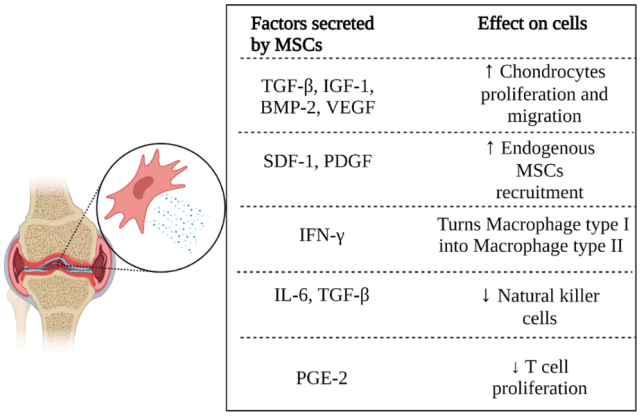

Fig. 2. Therapeutic effects of MSCs in an OA joint. Injected MSCs recruit endogenous MSCs and chondrocytes and increase chondrocyte proliferation. MSCs inhibit T cell proliferation and function. MSCs induce senescent-like natural killer cells and reprogram type I macrophages into type II macrophages. IL-6 = Interleukin 6, TGF-β = Transforming growth factor β, PGE-2 = Prostaglandin E2, IFN-γ = Interferon γ, SDF-1 = Stromal cell-derived factor 1, PDGF = Platelet-derived growth factor, IGF-1 = Insulin-like growth factor 1, BMP-2 = Bone morphogenetic protein 2, VEGF = Vascular endothelial growth factor.

polymers because they can absorb a large amount of water and ensure the diffusional transport of nutrients and signaling molecules (Fig. 3). Their unique characteristics make them suitable injectable scaffolds to support MSCs and act as drug delivery systems. Several aspects have to be considered to design optimal systems for intra-articular injection.

Elastic properties and MSC viability: injection of free MSCs without excipients can be harmful to cells because of the shear/stretching forces and pressure drop experienced by cells during parenteral

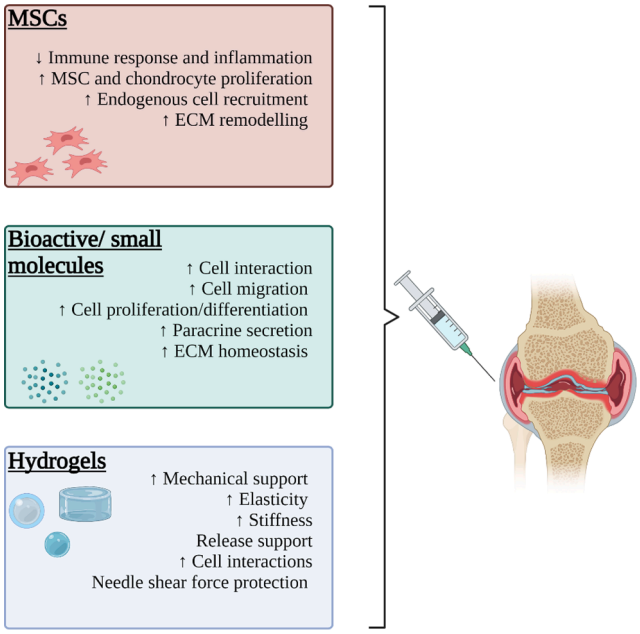

Fig. 3. Characteristics of a suitable hydrogel system design for MSC-induced/promoted cartilage regeneration. Hydrogels provide mechanical support, elasticity, and stiffness and facilitate cell interactions in osteoarthritic cartilage. Hydrogel scaffolds also protect MSCs from shear forces from needles during injection. During OA, MSCs decrease the immune response and inflammation and induce cell proliferation, differentiation and recruitment. MSCs are essential for ECM remodeling. Bioactive/small molecules in hydrogels maintain ECM homeostasis and enhance cell interaction, migration, proliferation, differentiation, and paracrine secretion.

administration. Aguado *et al.* analyzed the membrane damage and viability of MSCs upon injection with and without hydrogels [58]. A clear correlation between the storage modulus (G') of the hydrogel and cell viability was observed. Cell viability increased from 59% for cells injected with PBS to 89% for alginate hydrogels. To better mimic the properties of the ECM, researchers have often added collagen type V and HA to MSC injections [59–61]. In these cases, collagen provided additional mechanical support and biological properties to the MSCs. This approach has influenced the research of polymeric hydrogels and other scaffolds that entrap MSCs while improving the mechanical integrity of the joint [62].

It is essential to consider that native cartilage is anisotropic, and therefore, it is risky to use only one biomechanical parameter to define the similarities between a delivery system and the cartilage. In native anisotropic cartilage, the equilibrium shear modulus, dynamic shear modulus, and compressive modulus are different parameters to consider. Indeed, even if a hydrogel has a high shear modulus close to that of cartilage, its compressive modulus will often be much lower than that of human cartilage [63]. With movement, the equilibrium shear modulus of healthy human cartilage varies between 30 kPa and 230 kPa depending on the measured cartilage zone [64,65]. Therefore, it is crucial to adapt the mechanical forces applied by the matrix to MSCs to support and foster MSC proliferation and differentiation. A recent study showed that MSC chondrogenesis and proliferation were improved under particular conditions *in vitro* [66]. Upon tuning the stiffness of a polyethylene glycol (PEG)-gelatin-methacryloyl gel from 1.6 kPa to 25 kPa, the highest stiffness demonstrated the best chondrogenesis and proliferation results [66]. In this study, the authors only analyzed the equilibrium shear modulus, but the other moduli were not reported, therefore making it difficult to postulate that they mimic human cartilage properties.

In situ forming hydrogels: it is difficult to provide an appropriate highly viscous material while maintaining an appropriate injectability. Some of the polymers designed recently do address this issue. These materials have low viscosity during injection but show viscosities close to that of the cartilage after injection (reviewed in [67]). In these "*in situ* forming hydrogels", the polymer is crosslinked after injection, building its mechanical strength. Following injection, stimuli–responsive polymers may sense specific stimuli that trigger a crosslinking reaction. These stimuli mainly include pH, temperature, light, mechanical forces, or the presence of a small molecule [68]. An example of an *in situ* forming gel is based on methacrylated gelatin, loaded with bone morphogenetic protein 2 (BMP-2) growth factor and AMSC, where chemical crosslinking is triggered by local UV light exposure [69]: the fluid solution of methacrylated gelatin can be easily injected, and subsequent UV photopolymerization leads to the desired viscosity of the hydrogel. With this method, the stiffness, which is then similar to that of human cartilage, was obtained by increasing the crosslinking density to reach a shear modulus of 20 kPa. In addition, gelatin/BMP-2 electrostatic interactions allowed to retain approximately 30% of BMP-2 proteins in the hydrogel for at least 7 weeks. *In vitro* results showed a significant osteogenic differentiation increase from AMSCs when incubated with the hydrogel containing BMP-2. However, the polymerization process may be toxic to cells/tissues, and some difficulties for precise control of UV exposure inside the joint can be anticipated [70]. A less harmful approach for better cell support might be to use a thermoresponsive branched copolymer of HA and poly(N-isopropylacrylamide) (pNIPAM) [71]. At room temperature, this formulation is an aqueous solution. Upon injection in the joint, the body temperature of 37 °C induces a conformational change of the HA/pNIPAM polymer, causing a phase shift to nanoassemblies that increase the viscoelasticity of the scaffold.

Bioactive molecules - incorporation and delivery: hydrogels properties, including the release of loaded bioactive and small molecules, can be tuneable, which makes them suitable candidates as drug delivery systems (Fig. 3). These molecules can be chemically linked to the hydrogel or encapsulated [63]. Indeed, such delivery systems should not only preserve the injected MSCs but also protect the bioactive molecules

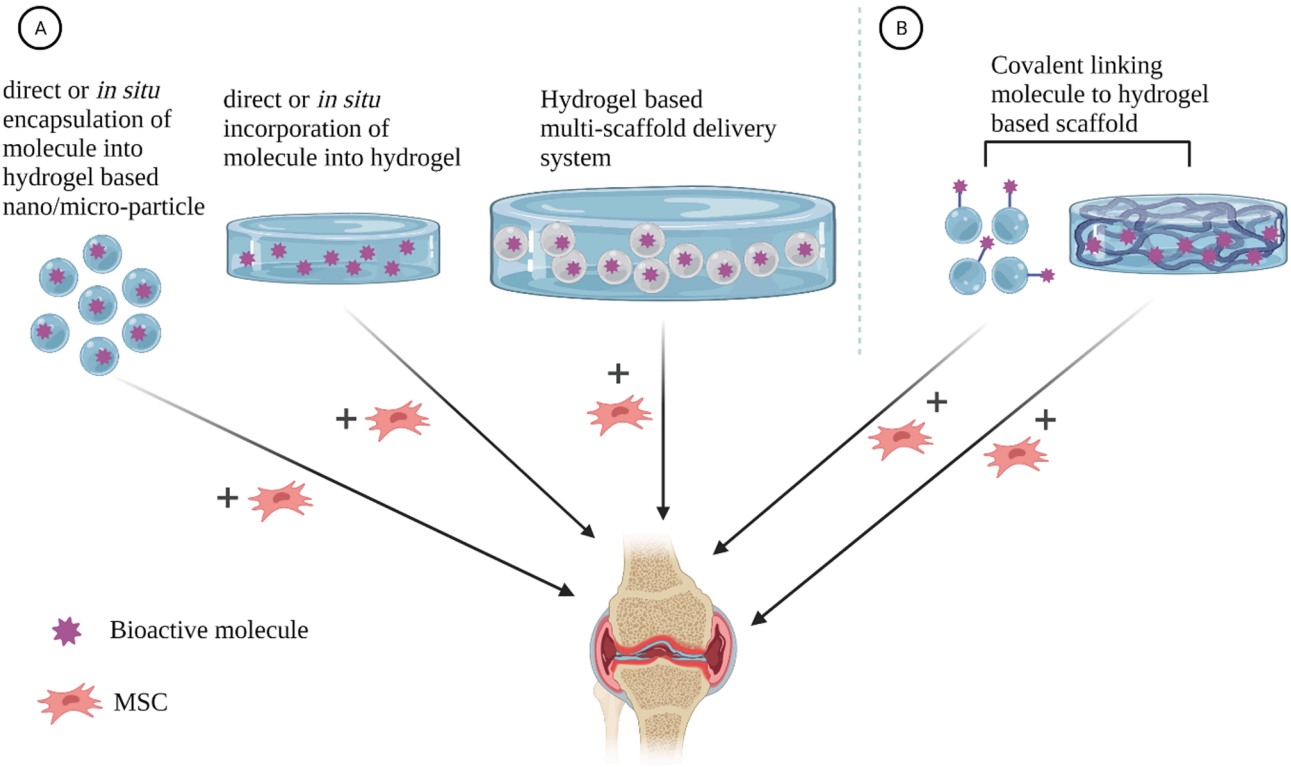

Fig. 4. Methods of incorporation of bioactive/small molecules in a hydrogel system combined with MSCs. Bioactive/small molecules can be loaded into hydrogels by direct encapsulation/incorporation in one or more polymers (A). The molecules can also be covalently bound to different hydrogel moieties (B).

therein from degradation to ensure their controlled release. Controlled timing of the release is achieved by entrapping the molecules in a matrix network [68]. Thus, it is essential to consider the specific method of incorporation of cells and bioactive/small molecules into the hydrogel. As shown in Fig. 4, there are two ways in which hydrogels, MSCs and bioactive/small molecules have been combined: i) by embedding the compound in the gel or nano/microparticles (encapsulation) and iii) by covalently conjugating the compound to the hydrogel [72].

Sterilization. Intraarticular administered formulations for clinical use need a proper sterilization. Moreover, sterile processing is mandatory when handling therapeutic cellular material. However, during high-pressure steam sterilization, such as autoclaving, hydrogel viscosity, structural integrity, and function can be affected when transient exposure to high temperatures (over 100 °C) occurs. For that reason, less aggressive sterilization strategies have been proposed (*e.g.*, ethylene oxide gas, electron-beam technology, or gamma radiation) [73].

Cathal D O'Connell *et al.* performed a screening of sterilisation techniques on 4 hydrogels: gelatin, gelatin methacryloyl, hyaluronic acid, and hyaluronic acid methacrylate [74]. Only methacrylated gelatine passed through the 0.22 μm syringe filters without clogging. Autoclaving and ethylene oxide treatment were therefore compared. Ethylene oxide is a very reactive gas lethal to mycobacteria, bacterial spores, vegetative bacteria (gram-negative and gram-positive), enveloped and naked viruses, fungal spores, and prions. Rheology results showed less impact on the native physical and chemical properties of the gel in comparison with autoclaving. In addition, this group demonstrated that cell viability and differentiation of MSCs were not altered with the ethylene oxide treated hydrogels [74]. We may note that in the case of already commercialized hydrogels such as Ostenil®, a hyaluronan-based product used to restore synovial balance in joints, terminal sterilization is performed in glass pre-filled syringes by autoclave.

Regarding cell therapy, terminal sterilization is not possible for live therapeutics [75]. To ensure the stability and safety of the product and due to the emerging field of cellular therapeutics, the industries adopt two accepted methods for large batch sizes from the United States Pharmacopeia chapters 71 and 63 for sterility testing and mycoplasma testing, respectively. However, the two compendial methods are not well suited to the safety and release testing of low-volume, single-batch products that need to be completed in a short time. Therefore, alternative rapid microbial test methods are developed, such as blood culture systems. The most studied blood culture systems are Bactec [76] and BacT/Alert [77], but Food and Drug Administration (FDA) do not formally approve these methods. Following the European Pharmacopeia (chapter 2.6.27), aerobic and anaerobic enriched media incubated for at least a week at 35–37 °C are recognized as alternatives to microbiological tests.

As explained above, the combination of hydrogels, MSCs and bioactive molecules needs to be designed for the optimal mechanical properties to facilitate injection, MSC survival after injection and effective incorporation and release dynamics of the bioactive molecules. Based on these factors, a plethora of hydrogel systems have been developed in recent years to address all these requirements with different levels of success. Some of these studies are discussed below matching the design and therapeutic effects.

5. Bioactive hydrogel systems for MSCs

Biomaterials of either natural origin or tuneable synthetic origin can be selected to build hydrogel-based MSC delivery systems. Natural hydrogels are biocompatible, most often biodegradable, and often have relatively low immunogenicity [52]. Collagen, gelatin, HA, chondroitin sulfate, and alginate are the most commonly used natural materials. Collagen and HA are, for instance, reported to bind receptors that interact with MSCs, such as integrins, discoidin domain receptors and CD44, mediating cell adhesion [78,79]. Some drawbacks include their mechanical weakness and rapid degradability [56]. Synthetic hydrogels containing PEG, poly N,N-dimethylacrylamide and polyvinyl alcohol, however, have reproducible and more consistent biomechanical properties [80]. Another advantage is that they rarely elicit an immunogenic response [81]. Furthermore, synthetic polymer properties are often easily tuneable for adequate degradability and low toxicity [82]. Hereafter, some of these hydrogel formulation designs containing bioactive/small molecules suitable for MSC therapy for OA are discussed.

5.1. Hydrogels with growth factors and MSCs

Cartilage is a source of a large variety of growth factors, cytokines, and chemokines, such as TGF-β, BMP, fibroblast growth factor, insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor, among others [83]. Thus, the development of advanced hydrogels can be achieved by incorporation of these bioactive molecules in their matrix together with MSCs [84]. The aim of this approach is to generate a positive feedback loop with the native factors released by the injected MSCs, thereby enhancing the regenerative process [11]. A controlled release profile over a defined period of growth factors is required for signaling to be effective and promote regeneration. For example, *in vitro*, cells must be repeatedly supplied with TGF-β due to its short half-life (less than 0.5 h). Consequently, a repeated supply of TGF-β every 1–3 days results in an effective concentration window (usually 10 ng/mL) to induce and maintain chondrogenesis [85,86]. Tables 1 and 2 summarize naturally derived hydrogel and synthetic hydrogel systems incorporating growth factors reported since 2016.

In OA, the ECM is damaged so that the homeostasis of growth factors is altered. When comparing the intra-articular injection of MSCs *vs.* MSCs + TGF-β in a rat partial-thickness cartilage defect model, Choi *et al.* reported a significant increase in cartilage regeneration [87]. However, high doses of TGF-β might cause osteophyte development and other pathological damage [88,89]. All these observations uncovering undesired effects caused by deleterious growth factors at an incorrect dosage reveal that regenerative growth factor signaling requires a careful, finely controlled supply of biomolecules in appropriate delivery systems [90].

The most common strategies to incorporate growth factors consist of embedding the bioactive molecule directly in the gel or encapsulating it into nano/microparticles (Fig. 4). Due to its simplicity, the direct incorporation of factors within scaffolds is an attractive method to protect molecules from premature degradation [72]. Encapsulation into nano- and microparticles may further prolong its long-term release. However, burst release is often a common limitation. It has been reported that such bursts are influenced by particle size, porosity, and polymer molecular weight [91]. To reduce this process, Cho *et al.* encapsulated IGF-1 in poly(ethylene adipate)/heparin coacervates that were afterward embedded into a natural gelatin hydrogel [92]. The release profile of IGF-1 from the coacervate hydrogel was slower than that from the hydrogel alone [92]. Tissue regeneration was assessed in a rabbit knee osteochondral defect model. The authors evaluated the intra-articular injection of the hydrogel ± ADSCs together with a bolus of free IGF-1 or its coacervate counterpart. Goebel's macroscopic scoring system and the International Cartilage Repair Society (ICRS) score showed significant osteochondral tissue regeneration for the group with MSCs and IGF-1 coacervates compared to the other groups. Furthermore, subchondral and chondral histological scores revealed significant tissue regeneration.

Covalent linking of bioactive factors was consistently reported to provide a prolonged release compared to the abovementioned entrapment methods. Likewise, TGF-β was recently conjugated to a PEG derivative subsequently crosslinked to HA *in situ* via pH-triggered Michael-type addition [16]. The activation of target genes of the corresponding signaling pathway mediating chondrogenesis with GAG and collagen type II *in vitro* expression was investigated. MSCs in this hydrogel with covalently bound TGF-β showed higher expression of GAGs (26 μg/μg)

Table 1
Naturally derived hydrogels with growth factors combined with MSCs for cartilage regeneration in OA.

Growth factor loaded	Function	Naturally derived hydrogels	Release profile	Effect on MSCs	*In vivo* model	Refs
TGF-β	Stimulates chondrocyte differentiation in early stages and inhibits terminal hypertrophic differentiation	Collagen type I-tyramine and HA-tyramine	NA	↑ GAGs	Full thickness cartilage defect rat model	[95]
		i) fibrin/ECM hydrogel ii) fibrin/gelatin hydrogel	1.5% released over 21 days 2.5% released over 21 days	↑ GAGs and collagen type II	Nude mouse model	[96]
		Calcium alginate	No release, BMSCs were pretreated with TGF-β before injection	↑ collagen type II, proteoglycan ↓ collagen type I, RUNX2, chondrocyte hypertrophy	Full thickness cartilage defect rat model	[97]

Table 2
Synthetic hydrogels with growth factors combined with MSCs for cartilage regeneration in OA.

Growth factor loaded	Function	Synthetic hydrogels	Release profile	Effect on MSCs	*In vivo* model	Refs
TGF-β	Stimulates chondrocyte differentiation in early stages and inhibits terminal hypertrophic differentiation	Methacrylated gelatin with acrylated β-cyclodextrins	Continuously released over 28 days	↑ expression of aggrecan, collagen type II, GAGs and SOX9, ↓ Runx1, collagen type X	Osteochondral defect mouse model	[98]
		poly-lactic-coglycolic acid microcarriers in collagen matrix	Sustained release over 21 days (4.6 ng/mL at day one, 3.3 ng/mL/day)	↑ collagen type II, proinflammatory cytokines (TNF- α, IL-1b), anti-inflammatory cytokines (IL-10, TGF-β) ↓ collagen type I and collagen type III	*In vitro*	[99]
		poly(glycidol) acrylate (modified PEG) and HA	8% released over 21 days	↑ GAGs and collagen type II, aggrecan, collagen type I	*In vitro*	[16]
IGF-1	Important for cartilage homeostasis. Regulates proteoglycan synthesis and breakdown	Gelatin and poly(ethylene glycol) diacrylate in a CoA complex (poly (ethylene arginylaspartate diglyceride) and heparin)	48% release for 4 weeks, no burst release	↑ collagen type II, aggrecan, SOX9 ↓ collagen type I	Osteochondral defect rabbit model	[92]
PDGF	Powerful chemotactic and mitogenic factor for MSCs and chondrocytes	Hydroxyethylmethacrylate- pNIPAM	70% released in 2 weeks	↑ ADSC adhesion, chondrogenesis, proliferation, aggrecan, collagen type II, SOX9,	*In vitro*	[100]

SOX9 = SRY-Box Transcription Factor 9, PDGF = Platelet-derived growth factor, TNF-α = Tumor necrosis factor α, IL-10 = Interleukin-10, IL-1b = Interleukin-1b.

and collagen type II (7 μg/μg) than those with noncovalently bound TGF-β (GAG: 13 μg/μg and type II collagen: 3 μg/μg) *in vitro*. TGF-β bioactivity was enhanced when conjugated to a network, possibly because in this way, the TGF-β protein–complex cannot be easily eliminated by endocytosis and lysosomal degradation [16,93]. However, this covalent linking implies a chemical modification, which may diminish bioactivity and/or worsen interactions with the environment [72,94].

Whether covalently bound or not, the release of the growth factor is always affected by the biodegradability of the hydrogel. Biodegradability should ideally proceed as new tissue forms. Complete resorption ensures long-term safety under physiological conditions [68]. Recently, the biodegradability of a slowly degradable chitosan hydrogel was accelerated by photocrosslinking of silk fibroin [82]. After 7 days in culture, the amounts of TGF-β released from the chitosan gel and the chitosan gel crosslinked to silk fibroin were 7% and 31%, respectively. Cell attachment was also enhanced after combination with the TGF-β-loaded crosslinked hydrogel, suggesting that this formulation holds promise for cartilage regeneration. In short, for optimal growth factor release from a hydrogel, different parameters, such as the growth factor dose, the release rate, the bioactivity of the released growth factor, and the biodegradability of the hydrogel, must be taken into account. TGF-β has been the most investigated due to its ability to increase the production of ECM and the differentiation capacity of MSCs. However, contribution of other factors released by the MSCs deserves further investigations, specifically with respect to anti-inflammatory properties.

5.2. Hydrogels with small molecules and MSCs

An alternative strategy to growth factors is to deliver small molecules. Small exogenous molecules enable more precise and controllable dosing and delivery than larger bioactive molecules. At least 20 of them have been reported to promote MSC chondrogenesis and ECM regeneration [14]. Three of these compounds are already on the market for cartilage treatment: glucosamine, estrogen, and icariin. These well-known molecules are thought to increase collagen type II levels and reduce cartilage oligomeric matrix proteins (COMPs), which cause chondrocyte hypertrophy and bone loss [101–104]. Their biological effect is fast, reversible, dose-dependent, and thus beneficial at a low cost.

Small compounds have an anti-inflammatory effect in cartilage [14]. Such is the case of KGN [105]. This molecule recently drew the attention of many researchers who combined KGN with MSCs in a matrix scaffold [17,106]. KGN combined with MSCs triggers chondrogenic effects similar to those of growth factors, representing a potential alternative to TGF-β. MSCs exposed to KGN differentiate into chondrocytes, promoting cartilage regeneration. KGN binds the actin-binding protein filamin A [105], which, in turn, binds the core-binding factor subunit β, promoting chondrogenesis through the RUNX1 pathway [107]. The similarities

between TGF-β and KGN have been demonstrated in a recent study assessing cartilage regeneration in a rat osteochondral defect model. KGN or TGF-β was encapsulated in MSC-laden gelatin hydrogels [98]. There was no significant difference between the two compounds in cartilage regeneration as quantified by GAG and collagen type II content and macroscopic appearance after 6 weeks, demonstrating their similarities.

For sustained release of small molecules from their matrix, molecules can be immobilized via hydrophobic interactions within the hydrogel. For example, hydrophobic KGN can be combined with a chitosan-based system, which results in a slower release of KGN through the matrix. Dehghan-Baniani *et al.* took advantage of KGN's hydrophobicity to incorporate KGN into their chitosan hydrogel and added it to an AMSC culture. While this formulation has not yet been used in injection assays, naturally derived chitosan confers low viscoelasticity to the gel matrix compared to cartilage. When adding β-glycerophosphate to the formulation, thermosensitive crosslinking of the polymer can be achieved, providing a shear modulus of 78 kPa (at 37 °C), which is in the range of the dynamic shear modulus of human cartilage (30–230 kPa) [108]. This addition improves the mechanical properties and provides a dense network that can slow KGN diffusion and release. Additionally, KGN has a negatively charged carboxyl acid group that interacts with the positively charged amine groups in chitosan, making chitosan an excellent candidate to delay the diffusion of KGN in the matrix. Finally, KGN has three hydrogen bond acceptor groups and two hydrogen bond donor groups that can provide H–bonding with chitosan, allowing KGN to be slowly released. *In vitro* results showed chondrogenic differentiation of ADSCs [108].

Another recent study aimed to control the fate of MSCs by encapsulating the cells in gelatin and HA with chondrogenic and osteogenic inducers (KGN and melatonin) [109]. The small molecules were entrapped *via* a modified β-cyclodextrin. First, a photocrosslinked hydrogel containing gelatin, melatonin, and MSCs intended for bone regeneration was injected into the subchondral area of an osteochondral interface defect rabbit model. In a second step, cartilage regenerating combinative hydrogel (HA, KGN, MSCs) was administered. Melatonin protects cartilage from OA-induced degradation by targeting a specific microRNA (microRNA-140-5p) involved in this degradation process [110]. Rabbit knees analyzed at weeks 4, 8, and 12 revealed a significant increase in the bone volume/tissue volume ratio and a considerable decrease in the Osteoarthritis Research Society International (OARSI) score.

Glucosamine sulfate and chondroitin sulfate are drugs given daily by oral administration (1500 mg/day) to patients with OA for more than 20 years. These two molecules are components of cartilage and synovial fluid and have an anti-inflammatory effect. They reduce interleukin-1 expression, which results in a subsequent reduction in cyclooxygenase-2 (COX-2), nuclear factor-kappa B and prostaglandin E2 expression [111]. Surprisingly, their oral administration can be controversial since bioactivity has not consistently been demonstrated [112]. In addition, this treatment might increase eye pressure and the risk of glaucoma [113,114]. Nevertheless, a long-term follow-up showed a significant reduction in cartilage volume loss in OA patients treated with glucosamine and chondroitin sulfate for 6 years (n = 1593) [115]. To overcome oral administration drawbacks, several studies analyzed the effect of intra-articular administration of glucosamine. MSCs were affected when treated with glucosamine: aggrecan expression was upregulated by over 30%, and significant inhibition of degradation enzymes from cartilage (MMPs) was observed compared to that of untreated MSCs [116]. Combining MSCs with glucosamine in the same hydrogel might represent an effective formulation for intra-articular OA treatment. In one such study, glucosamine was grafted to a PEG hydrogel containing BMSCs to induce chondrogenesis. The authors implanted subcutaneously this formulation into NOD/SCID (nonobese diabetic/immunodeficient) mice and observed that BMSCs increased collagen type II and GAG expression after 8 weeks [117]. Furthermore, the glucosamine-PEG hydrogel promoted BMSC proliferation. Increased resistance to mutations and small cell clusters were also observed when treating the cells with the hydrogel. Consistent with this finding, another experiment using a PEG hydrogel with glucosamine and embryonic stem cells showed similar results in terms of ECM production of embryonic stem cells upon glucosamine exposure [118]. In addition to KGN, the most studied molecule, and glucosamine, other well-known molecules, such as resveratrol and melatonin, have also been combined with stem cells and synthetic or naturally derived hydrogels to enhance MSC healing properties (Tables 3 and 4).

The latest molecule introduced in clinical trials for OA is lorecivivint [119]. This compound inhibits the Wnt pathway, which is overactivated in OA. Wnt proteins are involved in endogenous MSC differentiation and the induction of protease production that degrades the ECM [120,121]. Thus, MSCs previously treated with lorecevivint *in vitro* induced mature chondrocyte differentiation that expressed both collagen type II and aggrecan after 21 days. Lorecevivint also reduced the expression of catabolic enzymes in cytokine induced MSCs and chondrocytes [122]. However, no study has yet reported an adequate delivery system combined with MSCs for this promising drug, although clinical trials are already taking place (NCT03928184).

5.3. Hydrogels with ECM-cellular recognition for MSCs

ECM factors combined with hydrogels can play key roles in signaling mediated by proteoglycans, among others, and provide cell interaction and adhesion properties similar to the endogenous ECM. Recently, a photopolymerizable hydrogel was developed and bound to 3 different ECM compounds: MMP–2 peptide (involved in ECM breakdown), chondroitin sulfate (GAG), and RGD (Arg-Gly-Asp, a cell-adhesive peptide) [18]. MSCs embedded in this hydrogel were then injected into the critical-size chondral defect of rabbit joints. Injecting MSCs together with the biomimetic hydrogel resulted in low collagen type II and proteoglycan expression but efficient attachment to the subchondral bone, indicating efficient ECM-cell binding interaction without chondrogenesis. In a similar study, an ECM-mimetic peptide containing the "HAVDI" sequence derived from the first extracellular domain of N-cadherin was photocrosslinked to form a self-assembling peptide hydrogel [131]. In this case, the crosslinked part was the extracellular domain of N-cadherin, a transmembrane glycoprotein that mediates cell–cell adhesion. MSCs embedded in this hydrogel promoted chondrogenesis, correlating with the increase in GAG and proteoglycans. Interestingly, the Wnt/β-catenin pathway, which activates anti-chondrogenic factors under OA conditions, was decreased by N-cadherin inactivation.

Li *et al.* used rat tail collagen type I as an ECM bioactive molecule for their hydrogel system [132]. The objective was to induce MSC differentiation into chondrocytes by simulating the early stage of differentiation (*e.g.,* condensation) via cell-to-cell contacts. This group postulated that microencapsulated MSCs within the collagen matrix mimic the best condensation state of the cells that favor chondrogenesis. Different MSC concentrations were microencapsulated into collagen (0.5 to 1 $\times$ 10^6 cells/mL). *In vitro* results revealed that condensation and chondrogenic markers (peanut agglutinin and SOX9) were increased. However, their data also showed hypertrophy of the cells, probably attributed to excessive inductive signals (chondrogenic differentiation medium). This study further demonstrated the importance of gathering a high cell density to induce MSC condensation, the first step toward cell chondrogenic differentiation. For that task, collagen is undoubtedly a suitable candidate to allow cell-to-cell contacts due to its high affinity for MSCs. In comparison, MSCs do not interact with other polymer hydrogels composed of polyethylene glycol or alginate because they do not exhibit ligands for cell binding. Nevertheless, collagen hydrogels affect MSC immunological properties and undergo rapid degradation [133,134].

Table 3
Naturally derived hydrogels incorporating small molecules combined with MSCs for cartilage regeneration in OA.

Small molecule	Function	Combined with	Naturally derived hydrogels	Release profile	Effect on MSCs	*In vivo* model	Refs
Kartogenin	Induces MSC chondrocytic differentiation through the RUNX1 pathway	Chondrocyte	Transglutaminase crosslinked gelatin	NA	↑ proliferation, collagen type II, GAG, lubricin	Cartilage defect rat model	[123]
			Silk-fibroin-chitosan	NA	↑ SOX9, collagen type IX, collagen type II ↓ collagen type I	*In vitro*	[124]
Resveratrol	↑ MSC viability and self-renewal capacity and inhibit oxidative damage		Gelatin	No release, MSCs pretreated with resveratrol before injection.	↑ SOX9, aggrecan, SIRT1 ↓ collagen type X, alkaline phosphatase, collagen type I	Osteochondral defect rabbit model	[125]
Ginsenoside-Rb1	↓ inflammation, induces MSC proliferation	TGF-β	Silk fibroin-coated gelatin scaffold	Rb1: 46% released after 7 days TGF-β: 85% released over 28 days	↑ SOX9, aggrecan, collagen type II ↓ collagen type I, MMP13, collagen type X, IL-1β	OA-induced rat model	[126]

SIRT1 = Sirtuin 1, SOX9 = SRY-Box Transcription Factor 9, IL-1β = Interleukin-1β.

Table 4
Synthetic hydrogels incorporating small molecules combined with MSCs for cartilage regeneration in OA.

Small molecule	Function	Combined with	Synthetic hydrogel	Release profile	Effect on MSCs	*In vivo* model	Ref
Kartogenin	Induces MSC chondrocytic differentiation through the RUNX1 pathway		Methacrylated gelatin with acrylated β-cyclodextrins	Continuously released over 28 days	↑ aggrecan, collagen type II, GAGs and SOX9, Runx1, collagen type X	Rat [90] and rabbit [104] osteochondral defect model	[98,109]
			N-(β-maleimidopropyloxy) succinimide ester - modified chitosan	20% burst release and sustained release over 40 days	↑ collagen type II, SOX9, aggrecan. no change of collagen type I and collagen type X	*In vitro*	[108]
		Curcumin	PLGA microparticles in methacrylated gelatin	30% burst release and sustained release (80%) over 32 days	↑ (KGN effect): collagen type II, aggrecan, GAGs ↓ (curcumin effect): collagen type I, collagen type X, MMP13, MMP1	Osteochondral defect rabbit model	[127]
		RGD	2-layerhydroxides in a PEG poly(l-alanine)-poly(l-aspartate) thermogel	90% released over 21 days	↑ collagen type II, SOX9, GAGs	*In vitro*	[128]
		Histidine-alanine-valine and RGD	PLGA microspheres in methacrylated HA	Sustained release (80%) over 50 days	↑ GAGs, collagen type II, collagen type I	Nude mouse model	[129]
Melatonin	Induces chondrocyte and osteocyte differentiation via the Wnt pathway and maintains the self-renewal capacity	KGN	Methacrylated HA	Sustained release over 21 days	Melatonin: osteogenesis related expression KGN: Chondrogenesis related expression	Osteochondral defect rabbit model	[109]
		Methylprednisolone	Oxidized alginate, carboxymethyl chitosan and PEG microparticles	Melatonin released over 90 days, metylprednisolone released in 24 h	↑ GAGs, collagen type II, aggrecan ↓ collagen type I from Day 0 to Day 14 and then ↑	Cartilage defect rabbit model	[130]
Glucosamine	Inhibits MMP-13 expression, ↑ MSCs and chondrocyte proliferation/ migration		Diacrylated PEG	NA	↑ GAGs, SOX9, collagen type II, aggrecan ↓collagen type I, collagen type X and MMP13	NOD/SCID mouse model	[117]

PLGA = Poly (lactic-co-glycolic acid), SOX9 = SRY-Box Transcription Factor 9, IL-1β = Interleukin-1β.

5.4. Hydrogels for MSC nutrient supply

MSCs reside in a nonvascularized environment deprived of nutrients. A little explored strategy to enhance the activity of MSCs is to add compounds to the hydrogels to nurture them. The frequently reported limited *in vivo* survival following injection is partly due to the scarcity of nutrients. The primary source of cellular energy is glucose, and it has been shown *in vitro* that the lack of glucose is a limiting factor for MSC survival since they are glycolytic [135]. Thus, several studies examined the effect of different glucose concentrations on the biology of stem cells [136–138]. Concerning cell proliferation, differentiation, and the secretion of growth factors, the role of glucose is still unclear. In that

sense, encapsulation of glucose in microspheres by means of emulsification/internal gelation of alginate was hypothesized to improve the viability and aggregation of MSCs [139]. Glucose was entrapped into microspheres, which could retain 20% of the glucose in solution. Glucose was released from the microspheres steadily over three days. The affinity of glucose for alginate microsphere is still unclear, considering that glucose is a small hydrophilic molecule that should be released quickly. *In vitro* results showed increased cell survival upon incubation with glucose microspheres, although it was no longer significant after 36 h. This result is thought to be related to the rapid release of glucose, which warrants further studies to enhance the release profile of glucose toward enhanced cell viability. Therefore, finding a suitable energy supply delivering system able to release glucose in a controlled manner to effectively induce MSCs anti-inflammatory factors and ECM compound secretion over time would be a promising strategy for OA treatment.

6. Concluding remarks and future perspectives

Innovative and effective techniques for cartilage repair in OA that involve the injection of MSCs still lack suitable cell-supporting strategies. So far, the latest phase III clinical trials reports mentioned the implementation of hydrogels or human tissue aspirates to extend the lifespan of MSCs once injected. Indeed, hydrogels can be excellent hosts for MSCs when designed to provide an appropriate environment able to mimic the ECM and the mechanical intra-articular characteristics. Several studies aimed for hydrogels with shear modulus values close to the ones in the cartilage to maintain the stem cells alive and preserve their therapeutic effect. *In situ* forming hydrogels enabled enhanced survival of MSCs thanks to the low shear during injection, and then provided a high shear modulus once in the joint (*e.g.,* the knee). In this context, *in situ* crosslinking and thermoresponsive copolymer (*e.g.,* pNIPAM) hydrogels hold promise for ameliorating patients' compliance and quality of life. On the other hand, it is important to note that the success of these approaches also relies on the ability of these gels to modulate the fate of encapsulated bioactive molecules, which are crucial for the survival and modulation of MSCs function. In addition, the large number of studies combining the encapsulation of DMOADs and growth factors such as KGN and TGF-β, respectively, shows the growing interest for approaches targeting multiple OA pathways towards a more efficient therapy.

The *in vivo* preclinical experience gathered is very valuable in view of future clinical steps. Nevertheless, large efforts are still needed to elucidate the best hydrogel rheological characteristics and the associated release kinetics of the bioactive molecules. Only by adjusting these key knobs and exploiting the available techniques will lead to efficient combinations of bioactive hydrogels and MSCs for OA regenerative therapy. For the moment, the use of anti-inflammatory drugs is still strongly recommended by the last OA guidelines. However, even the technological forefront extended-release dexamethasone microparticles formulations such as Zilretta® are only showing modest long-term benefits. MSCs are progressively emerging as realistic alternatives for OA due to their disease-modifying ability and chondrogenic potential (i.e., to not only reduce pain but also to detain/reverse the disease). The clinical trials ongoing up to this date (6 in phase III but also around by 51 in phase II) hold for promise towards the introduction of stem cell therapies into the current guidelines in the next future. Over 10,000 patients have been treated with MSCs in almost 1000 clinical trials, but the outcome is still variable, and FDA approval has not yet been granted. Encountering a suitable and standardized environment for MSCs might be a critical step before receiving an eventual FDA approval. Combining hydrogels and active molecules to MSCs could help to improve stem cells' therapeutic activity and also homogenize manufacturation protocols.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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