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Mesenchymal Stem Cell-Derived Exosomes in 3D-Bioprinted Hydrogel Scaffolds for Cartilage Regeneration: Advances, Mechanisms, and Translational Challenges

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Abstract

Despite the current advances in the field, articular cartilage defects and osteoarthritis remain a challenge in the clinic due to the fact that mature cartilage lacks blood supply, is poorly cellular and has a weak intrinsic repair response. Although conventional treatments focusing on the symptoms can alleviate pain and surgical methods can restore some function in a few lesions, regeneration of hyaline cartilage with native zonal architecture is rare. Exosomes, or small extracellular vesicle-enriched products, derived from mesenchymal stem/stromal cells have been the focus of much interest as cell-free regenerative signals due to their ability to contain regulatory microRNAs, proteins, lipids and other biomolecules that can affect chondrocytes survival, extracellular matrix production, macrophage phenotype and inflammatory signalling. Freely injected EVs, however, are diluted, their location is unpredictable, there is no consistency in dosage and very little control over the release of EVs at the injection site. Three dimensional bioprinted hydrogel scaffolds provide a complementary biotechnology approach, where the defect-matched architecture, matrix-like support, mechanical protection and controlled spatial distribution of bioactive cargo are provided. This is a very important research paper that assesses the progress, mechanisms and translational hurdles of using MSC-derived exosomes for the development of 3D-bioprinted hydrogel scaffolds for cartilage regeneration. The analysis suggests that the most scientifically justifiable approach is an integrated exosome-bioink platform in which the design of vesicles, their identity and potency, the mechanics of the hydrogel, the release kinetics of the vesicles, the printability of the bioink, the sterility and regulatory grade manufacturing are all designed together. Preclinical evidence is in favor of chondroprotective, anti-inflammatory and pro-regenerative activity, but human evidence is preliminary and is not sufficient for the routine clinical use. However, before translation, standardised reporting of extracellular vesicles, reproducible potency assays, clinically relevant cartilage models, long-term studies in animals and GMP-compatible manufacturing and meticulously controlled clinical trials will be required.

Keywords: Mesenchymal Stem Cells; Exosomes; Extracellular Vesicles; 3D Bioprinting; Hydrogel Scaffold; Cartilage Regeneration; Osteoarthritis; Tissue Engineering and Regenerative Medicine

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Abbreviations

ADAMTS are enzymes that degrade hyaluronidans; BM-MSC: Bone Marrow Mesenchymal Stem/Stromal Cell; COL2A1: Collagen type II Alpha 1 chain; ECM: Extra-Cellular Matrix; EV: Extracellular Vesicle; FDA: The Food and Drug Administration of the United States (FDA); GelMA: Gelatin Meth Acrylol; GMP: Good Manufacturing Practice; HA: Hyaluronic Acid; MISEV: Minimal Information for Studies of Extracellular Vesicles: MSC: Mesenchymal Stem/Stromal Cell; OA: Osteoarthritis; sEV: Small Extracellular Vesicle

Introduction

Articular cartilage is a specialised connective tissue which allows movement of the joints with low frictional forces and protects the subchondral bone from the load. In its extracellular matrix, it is rich in Type II Collagen and Aggrecan and Chondrocytes only take up a small proportion of the tissue volume. This composition provides cartilage with very good compressive and lubricating properties, but also hinders repair as there is no direct vascular, lymphatic or neural supply, and

injury to the cartilage results in a limited migratory and proliferative capacity of the resident cells [1]. As a result, many times the focal cartilage injuries and osteoarthritic degeneration will heal with fibrocartilage, which is a mechanically inferior tissue that is not as organised as native hyaline cartilage.

Cartilage regeneration is a major focus of biotechnology and regenerative medicine, due to the disease burden. Today, osteoarthrosis is known as a disorder of the whole joint, which includes breakdown of the cartilage matrix, synovial inflammation, subchondral bone remodeling, osteophyte formation and pain-related disability [2, 3]. It is estimated that approximately 595 million people were suffering from OA in 2020, and that by 2050, this number will continue to grow, as a consequence of population ageing, obesity and joint-injuries [4]. These numbers highlight the need for interventions which don't only provide temporary relief of symptoms, but biological meaningful tissue repair.

Existing clinical tools are important and useful, but limited. Symptoms may be improved with exercise and weight control; some focal defects may be improved with microfracture, osteochondral grafting and autologous chondrocyte implantation and analgesia/ intra-articular injections. However, these techniques are limited by the development of fibrocartilage, donor site morbidity, inconsistent graft incorporation and complexity of procedure and poor restoration of native zonal cartilage. Joint replacement is an effective treatment for end-stage disease, but not a biological regeneration treatment and is less appealing for younger patients or early-stage defects.

In order to shift the paradigm of regenerative medicine to approaches that incorporate biological signalling and engineered biomaterials, a platform is needed that enables both. To enable the integration of these approaches, a platform is needed that allows the integration of both biomaterials and biological signalling. Although MSCs were originally studied for their reparative properties due to their secretion of trophic and immunomodulatory factors, direct cell transplantation has issues with survival, control of their differentiation, storage, immunogenicity and batch to batch consistency of manufacturing. Many MSC effects have been shown to be mediated by EVs, such as preparations of small EVs enriched for exosomes (Ther y et al., 2018) [5]. Simultaneously, 3D bioprinting has been used to control the production of hydrogel scaffolds that are capable of mimicking cartilage relevant matrix cues and defect geometry [6, 7]. As such, the combination of the MSC-derived exosomes and 3D-bioprinted hydrogels is a promising avenue for scientific research and a relevant and timely translation.

Research Aim, Objectives and Questions

This paper aims to discuss critically the potential of MSC-derived exosomes as a tool to enhance cartilage regeneration, focusing on biological mechanisms, scaffold design, the quality of available evidence and the barriers to translation.

Objectives

To provide biological basis for the application of cell-free regenerative signals (exosomes) derived from MS cells in cartilage repair.

1) To assess the potential for enhancement of local retention, mechanical support, spatial organisation and controlled release of 3D-bioprinted hydrogel scaffolds.

2) To integrate existing data in the areas of mechanisms, preclinical results, early clinical translation and biotechnology platform design.

3) To pinpoint the key manufacturing, safety, regulatory and clinical issues that need to be overcome prior to the routine use.

Research Questions

1) What are the effects of exosomes derived from MSC on cartilage regeneration and osteoarthritis associated inflammation?

2) With regard to the use of EXOSOMEs in a cartilage scaffold in the context of hydrogel and bioprinting, what are the most desirable/ essential properties of the EXOSOMEs?

3) How can the activity of the exosome with scaffolds be explained in the context of cartilage repair?

4) What are some of the translational challenges that need to be addressed for this biotechnology strategy to become a clinically robust one?

Methodology

This paper takes a critical narrative review design approach and is not a laboratory experiment or a comprehensive systematic review. The field of extracellular vesicles, cartilage pathophysiology, hydrogels, 3D bioprinting, preclinical and regulatory translation is an area in which a narrative synthesis is suitable. The review thus focuses on conceptual integration and critical evaluation instead of pooling of the heterodox studies in a meta-analysis.

A literature base was created from the peer-reviewed literature that studied properties of MSC-derived EVs, exosome biology, cartilage repair, osteoarthritis, hydrogel scaffolds, 3D bioprinting, EVs loaded into hydrogels, and regulation in clinical practice. Recent publications from 2017 onwards were given priority and older publications were only included if they were important for the understanding of cartilage and osteoarthritis. Hence, articles published by the DOI, PubMed indexed articles and official clinical trial and regulatory documents were preferred to ensure that the base of the references is credible and traceable.

Sources were analysed as if they were in the context of biotechnology. Product identity, mechanism of action, scaffold design, manufacturing quality and clinical feasibility and regulatory classification have all been considered equal. This is a way of overcoming the typical limitations in terms of a single injection or 3D bioprinting as a mechanical scaffold technology. Instead, it poses the topic as an integrated platform, which needs to meet both the biological and engineering needs.

Biological basis of cartilage degeneration and repair

The healthy articular cartilage is composed of a superficial, middle, deep and calcified zone. Each of these zones plays a role in lubrication and shear resistance, load distribution and deep zone attachment to subchondral bone. The restoration of the quantity of the matrix, tissue organisation, integration and mechanical function is therefore essential for effective regeneration. An improvement in the histology does not necessarily mean that it will be mechanically improved if it is done without restoring the architecture, which is the production of collagen or proteoglycan.

Osteoarthritis causes a disturbance of the cartilage homeostasis by inflammatory, mechanical and metabolic mechanisms. Catabolic enzymes - matrix metalloproteinases and aggrecanases - are stimulated by pro-inflammatory mediators, such as interleukin-1 beta

and tumor necrosis factor-alpha. Oxidative stress, cellular senescence and synovial inflammation are other factors that further impair repair responses in addition to these enzymes [2, 3]. A viable regenerative therapy should thus tackle the three aspects of inflammation, catabolism, and chondrocyte survival and matrix reconstruction at the same time.

MSCs derived exosomes are desirable due to their ability to carry multiple biological signals as opposed to one. Based on preclinical experiments, MSC-derived vesicles are involved in the promotion of chondrocytes proliferation, the decrease of chondrocytes apoptosis, the stimulation of the synthesis of chondrocytes matrix as well as the regulation of immune activity [8, 9]. It is not that they are destined to become cartilage cells or that they actually replace them that is their theoretical value, but that they are able to provide a coordinated paracrine message that can change an osteoarthritic microenvironment to one of repair.

MSC-Derived exosomes as cell-free regenerative signals

Extracellular vesicles are extracellular particles which are lipid bilayers and are released by cells and involved in intercellular communication. Small EVs obtained from endosomal compartments are commonly referred to as exosomes, but if researchers don't have evidence of vesicle biogenesis, they should avoid using the term exosome, as advised by the MISEV guidelines [5]. To that end, this paper employs the term "MSC-derived exosomes" instead of the often-used term "MSC-derived small EV-enriched products" in keeping with the common research topic, but keeping in mind the nature of the products.

The advantages of the exosomes derived from MSCs are that they can be endowed with many paracrine properties of MSCs without introducing living cells. Vesicles could be more convenient to store, mix with biomaterials, process control and dose to measurable particle/cargo parameters as compared to whole cells. They also decrease worries associated with the uncontrolled differentiation. But they are not so easy chemical drugs. They are dependent on the source of the donors, culture conditions, passage number, oxygen tension, inflammatory priming and isolation method of their cargo. This is one of the reasons why they have therapeutic potential and is a major hurdle to clinical translation [10].

A number of studies are indicative of cartilage-protective activity. Cosenza *et al.,* [8] found that bone marrow MSC-derived exosomes and microparticles had chondroprotective and anti-inflammatory effects in the models of OA. The miR-140-5p-overexpressing synovial MSCs proved to have exosomes that could enhance cartilage regeneration and inhibit the progression of OA in rats [11]. Through various mechanisms, such as promoting proliferation, inhibiting apoptosis and regulating immune reactivity, Zhang *et al.,* [9] concluded that MSC exosomes can promote cartilage repair. Mao *et al.,* [12] also showed that the exosomal miR-92a-3p promoted the chondrogenesis and blocked the degeneration of cartilage through the targeting of WNT5A.

The evidence still is largely preclinical, however. There are a number of studies with different methods and sources of vesicles, doses, outcome measures and follow up durations, and animal models can only partially mimic the process of chronic osteoarthritis in humans. Although recent reviews and meta-analyses indicate potential for therapy, the necessity for improving clinical validation and risk of bias control [13] highlights the need for further research. The focus of the field has thus shifted from the potential effect of MSC-derived EVs on cartilage cells to the reproducible production, characterisation and delivery of EVs.

3D-Bioprinted hydrogel scaffolds for cartilage engineering

A high-water content (hydrogels), polymer networks can be engineered to possess some of the properties of the native cartilage ECM. Natural polymers such as hyaluronic acid, collagen, gelatin, alginate and chitosan are biocompatible and cell-interactive, and can be combined with synthetic polymers with tunable chemistry and mechanical control like polyethylene glycol. Gelatin meth acryloyl is a popular choice of material due to its printability and shape fidelity, which are provided by the photo cross linkable groups, while the biological motifs derived from gelatin are also present [6, 14].

Three dimensional bioprinting brings architectural control which is not easily achieved by conventional injection/moulding or casting. It allows for the defined geometry of pores, multiple layers, stiffness gradients and spatial arrangement of cells, vesicles and/or bioactive factors. These features are of importance as articular cartilage is not a homogenous structure. A construct can be bioprinted that mimics superficial, middle and deep zones and is similar to the geometry of the defect. In the review of cartilage-mimicking bioprinted constructs, the key role of biomimetic architecture for improved function and integration is highlighted [7, 15].

The challenge in the main engineering aspect is to balance the printability, biological compatibility and mechanical performance. It is important to have an appropriate bioink as too soft a bioink may collapse after printing, and too crosslinked bioink may inhibit diffusion, cell migration and release of vesicles. Native cartilage has high compressive stiffness, low friction, is anisotropic and is lacking in mechanical strength unless reinforced. To tackle this challenge, a number of hybrid hydrogels and composite systems, such as GelMA-hyaluronic acid, GelMA-alginate, collagen-based matrices, de-cellularised cartilage matrix and double-network hydrogels, have been studied [6, 16].

Results

Rationale for integrating MSC exosomes with 3D-Bioprinted hydrogels

The delivery of free intra-articular exosome injection is simple and yet, it is not biologically efficient. Vesicles may get lost in synovial fluid, be removed from the joint, be absorbed by nontarget cells or be degraded prior to any permanent repair. The drawback of these systems is overcome by injectable hydrogel and scaffold systems that keep vesicles close to the defect site, and provide a local depot for vesicles to be released gradually [17]. With the incorporation of 3D bioprinting, the scaffold can also be shaped and zoned, to match a particular cartilage or osteochondral defect.

The combination can be considered as doing double duty as a regenerative platform. The hydrogel scaffold, which can be used to fill the defect, protects the mechanical integrity, offers an extracellular matrix-like supporting environment and spatial organisation. The bioactive signals delivered by MSC-derived exosomes could help to inhibit inflammation and induce matrix synthesis and modulate immune responses. The broader field of hydrogel-exosome research lends credibility to the idea that there are benefits to making vesicles retain in the area longer and to increase the activity of the vesicles in the area [18, 19]. The exosome systems that can be bioprinted could be more complex than being just an injectable depot. Vesicles can be

Table 1: Hydrogel bioink options for exosome-loaded cartilage scaffolds.

Bioink option	Advantages	Key limitation
GelMA	Good printability, photo crosslinking and cell-interactive motifs.	Photo-initiators and crosslinking conditions may affect EV integrity.
Hyaluronic acid	Cartilage-relevant matrix component useful for lubrication and cell signalling.	Often needs chemical modification or blending for stable printing.
Alginate	Rapid ionic gelation and good shape fidelity.	Limited cell adhesion unless modified or blended.
Collagen/gelatin	Biologically familiar ECM cues and good cell compatibility.	Mechanical weakness and thermal sensitivity can limit fidelity, which can lead to inaccurate measurements.
Chitosan	Biocompatible and possibly anti-inflammatory.	Can be difficult to achieve solubility and printability.
PEG-based hydrogels	Highly tunable synthetic chemistry and controlled degradation.	Requires biofunctionalisation to improve cell interaction.
dECM hydrogels	Closest biochemical similarity to cartilage matrix.	Batch variability and processing complexity are major concerns.

directly incorporated in bioinks, affinity immobilized, incorporated in microgels, postprinted or printed in gradients. The emergence of exosome-based bioinks and exosome bioprinting is due to vesicles being non-viable and thus do not have the viability restrictions of cell-laden printing [20]. Fabrication, however, needs to be done to ensure that vesicles are not subject to shear stress, temperature variations, photo-initiators and crosslinking chemistry.

Evidence synthesis and current advances

These pieces of evidence can be categorized into three intertwined streams: MSC-exosome biology, EV-loaded hydrogel delivery and 3D-bioprinted cartilage engineering. Mechanistic support for chondroprotection and immunomodulation are provided by the MSC-exosome studies. Retention and sustained release studies are done in the case of hydrogels. The studies of Bio-printing give architectural control and biomimetic design of scaffolds. The best future research will be integrated research between all three streams that will not view them as separate technologies.

Initial clinical data show promise, but are not conclusive. Bolandnazar *et al.,* [21] conducted a triple-blind, randomized study on the use of placental mesenchymal stromal cell-derived extracellular vesicles for knee osteoarthritis, which found them safe, but failed to show superiority over saline placebo at the evaluated time points. An open-label phase 1 pilot study to assess the safety of allogeneic MSC-derived small EVs for treating mild to moderate knee osteoarthritis is also registered on http://ClinicalTrials.gov [22]. These new findings indicate the clinical translation is starting, but also reveal that the field is not prepared for the therapeutic claims to be made routinely. There is no single hydrogel which is suitable for all needs. The most practical approach is a hybrid bioink consisting of two different polymers, one with good print fidelity and the other with cartilage-relevance, and the vesicles with controlled-release behaviour. The choice of the type of hydrogel should thus be determined by the type of defect, the type of loading, the printing conditions, the desired degradation behaviour and release profile (Table 1).

Mechanisms of scaffold-based exosome cartilage repair

Firstly, chondrocytes are taken up and stimulated to anabolism. MSC-derived vesicles can be used to transfer regulatory cargo which can enhance expression of cartilage matrix markers, like SOX9, COL2A1 and aggrecan, and decrease the expression of catabolic mediators, like MMP-13 and ADAMTS5. This is of importance since cartilage regeneration involves both the deposition of new matrix and reduction of matrix breakdown [8, 9].

The second is the anti-inflammatory signalling. Osteoarthritic (OA) joints have catabolic pathways which are driven by cytokines and suppress repair. In preclinical models, MSC-derived exosomes have been shown to have anti-inflammatory effects and regulate NF-kB, MAPK, and Wnt signaling pathways [23]. One way to improve this mechanism is to maintain the vesicles in the microenvironment for a sufficient period of time to be effective; this can be done with a hydrogel scaffold, which keeps the vesicles in place for a long time before they are rapidly cleared.

Immune-modulation is the third mechanism. Macrophage phenotype is of particular interest, as M1 like macrophages have the potential to drive inflammation, and M2 like is to repair. Sang *et al.,* [24] found that the chondrocytes-derived exosome-laden thermosensitive hydrogel can facilitate the repair of cartilage, in part through the regulation of the polarisation of macrophages. Although the study was not carried out with MSC-derived exosomes, but with chondrocyte-derived exosomes, this study suggests that EVs locally derived from the hydrogel can influence the immune behaviour of the osteoarthritic tissue, supporting the overall concept.

The fourth type of regulation is related to cargo. The highly cartilage homeostasis-related miR-140-5p was found to be closely related to the cartilage repair, and Tao *et al.,* [11] reported that exosomes derived from synovial MSCs with miR-140-5p overexpression promoted cartilage repair in a rat model. Using cartilage development and degeneration model mice, Mao *et al.,* [12] demonstrated that exosomal miR-92a-3p targets WNT5A to control cartilage development and degeneration. Based on the data presented herein, the MSC exosomes could be optimised in the future to become cartilage specific.

The fifth mechanism is "spatial guidance. A 3D-bioprinted scaffold enables the creation of regions of vesicle accumulation, stiffness and degradation. This is important as cartilage repair is not biochemical, it's also biomechanical. The scaffold should have the ability to retain its shape, be resistant to compression, and be able to incorporate into the host tissue and release vesicles in the same rate as the repair biology. So, the most protected one is not a single pathway, but the concerted action of the vesicle cargo, scaffold mechanics and the joint microenvironment.

Proposed biotechnology research platform

The first step to a high-quality research platform is to have a defined MSC source. There are several reasons why synovial MSCs may be cartilage-specific, umbilical cord MSCs are readily available and can be expanded, adipose MSCs are easily accessible and bone marrow MSCs have a vast literature in orthopaedics. The chosen source should have a high yield of exosomes, be ethically acceptable, have a consistent source of donors, and have the potential to generate

Table 2: Translational barriers and recommended solutions.

Barrier	Recommended solution
EV heterogeneity	Use defined MSC banks, controlled culture conditions and MISEV-aligned characterisation.
Dose uncertainty	Report particle number, protein content, cargo markers and mechanism-linked potency.
Burst release	Use affinity binding, microgel encapsulation or layered printing to control release kinetics.
Weak hydrogel mechanics	Use reinforced hybrid or double-network hydrogels while preserving biological activity.
Bioprinting stress	Optimise nozzle size, extrusion pressure, viscosity, temperature and crosslinking chemistry.
Regulatory complexity	Design GMP-compatible processes and define product identity, safety and potency early.

good chondrogenic potency, while also being feasible for the production of clinical-grade exosomes.

The isolation and characterisation of EVs should be done following the principles of MISEV2023. A good workflow should involve the use of defined culture conditions, EV-depleted or serum-free media, removal of cells and debris, concentration, purification (either by size-exclusion chromatography or density-based methods), and assessment using nanoparticle tracking analysis, electron microscopy, protein markers and negative controls. Since the method cannot substantiate the claim, it is not recommended to use the word "pure" in the report. Based on the cartilage requirements, hydrogel platform needs to be chosen. A hybrid system of GelMA and HA (or GelMA and alginate) appears to be a good choice as it offers the benefits of printability, crosslinking control and cartilage relevance. Mechanical reinforcement can be added via the addition of chondroitin sulphate, nanocellulose, silk fibroin, collagen fibres or double network architecture. Additionally, the design should prevent the vesicles from severe crosslinking, and enable a prolonged release time within a clinically relevant time window.

Evaluation of performance should be multi-dimensional. Print fidelity, swelling, degradation, compressive modulus, vesicle release kinetics, vesicle integrity after printing, chondrocyte viability, inflammatory challenge assays, matrix gene expression and glycosaminoglycan production and histological staining are all required tests. The progression from chondrocyte/ cartilage explant to orthotopic small-animal models to large-animal cartilage defect models should be done in preclinical testing. This staged approach is important because, if not taken, strong in vitro data can fall off the table when it comes to translation.

Translational challenges, safety and regulation

The first big hurdle is on the heterogeneity. The characteristics of the exosomes derived from MSCs can be changed by the age of the donors, the source of the tissues, the conditions of the culture, the number of passages, the oxygen tension, inflammatory priming and isolation method. Products with the same labelling can have different populations of vesicles and/or biological cargo. This is because it is hard to reproduce the work and it is thus important to share the results in a standardised manner [5].

The second challenge is the dosage and strength of the product. Dose is reported in various different ways, including particles, protein mass, number of parent cells or volume injected, making it difficult to make direct comparisons. An evaluation based on particle number alone will not guarantee biological activity and protein mass can be contaminated with non-vesicular proteins. A clinically relevant potency assay should be related to mechanism including inhibition of IL-1 beta induced MMP-13, up-regulation of COL2A1 and aggrecan, macrophage polarization markers and/or cartilage matrix deposition in cartilage explants.

The third challenge is getting to manufacturing scale up. There are several requirements for clinical grade vesicles: GMP-compliant cell banking, validated culture, serum free media, closed system processing, sterility testing, endotoxin testing, identity testing as well as potency testing and stability data. The research scale ultracentrifugation is not suitable for the mass production. While bioreactors, TFF and SEC might help in scaling up the processes, every process needs to be validated for yield, purity and function [10].

The fourth challenge is one that has to do with scaffold processing. Alteration of vesicle function is possible through sterilisation, freeze-thaw cycles, UV light, photo-initiators, extrusion pressure and storage. Just because vesicles are printed onto a scaffold, does not make it translational; investigators must demonstrate that the printed vesicles are active and functional throughout the printing, storage and delivery processes. This is crucial when manufacturing bioprinted constructs as steps can harm the nanoscale vesicles.

Care needs to be taken to ensure safety as well. Although exosomes are more complex than cells, they are sometimes referred to as safer than cells. Potential side effects include contamination, off target immune effects, pro-fibrotic signaling, unwanted angiogenesis, donor variability and biodistribution. In 2020, the U.S. Food and Drug Administration (FDA) alerted people that exosome treatments for disease should be properly regulated and that no exosome product is FDA approved for any usage.

EV heterogeneity Use defined MSC banks, controlled culture conditions and MISEV-aligned characterisation. Dose uncertainty Report particle number, protein content, cargo markers and mechanism-linked potency. Burst release Use affinity binding, microgel encapsulation or layered printing to control release kinetics. Weak hydrogel mechanics Use reinforced hybrid or double-network hydrogels while preserving biological activity. Bioprinting stress Optimise nozzle size, extrusion pressure, viscosity, temperature and crosslinking chemistry. Regulatory complexity Design GMP-compatible processes and define product identity, safety and potency early. The translational hurdles are not distinct from the science, but rather are the factors that will be the deciding factor in whether or not a promising concept can become a clinically credible therapy. Therefore, the central scientific issues and not the final stage administrative concerns must be a part of a rigorous paper, which will evaluate manufacturing, dose, sterility and storage and regulatory classification (Table 2).

Limitations

The literature available is good but there are inconsistencies. Small animal models, short follow-up period and varying OA induction methods are used in many studies and it is difficult to compare results

between studies. There are some experiments that show histological improvement but not enough biomechanical testing; there are others that have been done on the molecular markers without any long-term tissue integration shown. These are important limitations as they need to be able to bear repeated loading over time in the joint.

Another constraint is there is not a consistent description of EV products. The term exosome can be applied to studies regardless of the type of isolation techniques used that yield a mixture of small EVs. Since there are no detailed reports of characterisation and purity of the vesicles, it is hard to assess if the effects observed are due to vesicles, soluble proteins, protein aggregates or other co-isolated materials. This will be the reason why MISEV2023 reporting standards will be crucial for future work. Clinical evidence is, too, young. Useful, but there is no clear superiority of the product over the placebo in at least one randomised trial, which means that the dose, product potency, patient selection and endpoints of the trials should be improved [21]. It should not be inferred from this that the field has failed, rather that there is a need for better product definition, and study design to translate into the clinic.

Future Directions

The next step in the research will likely be to engineered extracellular vesicles. To further improve the releasable cargo of cartilage relevance, parent MSCs can be preconditioned with hypoxia, inflammatory cytokines, mechanical stimulation or specific miRNA engineering. Engineered vesicles could also be themselves engineered to incorporate ligands and/or binding peptides that target cartilage in order to enhance retention. But, the more engineered a product is, the more necessary it is to have safety testing, dose control as well as regulatory clarity [25].

Another direction that is promising is the design of smart hydrogels. Future scaffolds could be *pH* responsive, and/or inflammatory enzymes or mechanical loading responsive, releasing vesicles when there is a need for it in the local joint environment. By using zonal bioprinting, it may be possible to achieve various release profiles in superficial, middle and deep cartilage. They can also be packaged with anti-inflammatory molecules, lubricating molecules or even molecules that regulate the genes, but combination products will need to be carefully tested to prevent uncontrolled interactions.

Disease models also are lacking in the field. While simple 2-D chondrocyte cultures are good for screening, they are not able to replicate the mechanical and inflammatory complexity of the joint. However, there is a need to fill the gap between cell culture and animal studies and this could be achieved using cartilage-on-chip systems, osteochondral organoids, synovium-cartilage co-cultures and bioprinted disease models. These platforms can help to minimize unnecessary animal use, and provide more realistic evaluations of release kinetics and inflammation and matrix formation.

The next key focus is on translation of disciplines. The manufacturability, sterility, storage, cost and regulation should be considered in the design of the promising constructs from the beginning. A scaffold that works well in the lab, but is not eligible for mass production will not be a clinical therapy. Together, however, the future of MSC-exosome loaded 3D-bioprinted hydrogels is promising, with the potential for biotechnology scientists, materials engineers, orthopaedic clinicians, regulatory experts and biomanufacturing specialists to work together.

Conclusion

Exosomes derived from MSCs (Mesenchymal Stem Cells) and integrated with 3D-bioprinted hydrogel scaffolds are among the most promising integrated biotechnology strategies that could be used for cartilage regeneration. The approach is scientifically strong as multiple barriers are tackled at once, including cell-free bioactive signalling, retention and controlled release (hydrogels) and biomimetic architecture and spatial organisation (3D bioprinting). These components combine to make the platform more complex than just free injection of exosomes or acellular implantation of a hydrogel.

But it is still a field of translation and not a clinical field. Although preclinical data point towards chondroprotective, anti-inflammatory and pro-regenerative mechanisms, there are still limited human data and the therapeutic use is not yet established. Biological barriers are not the only ones, as EV nomenclature, product heterogeneity, dose definition, potency assays, GMP manufacturing, scaffold processing, storage, sterilisation, regulation and long-term safety are all important factors to consider. Challenges should be viewed as the key research questions and not just the technical details.

Finally, there are no overall clear trends in the direction of the research, except for the standardised exosome-bioink platform with the aim of its clinical translation and cartilage-specific potency from the start. With careful EV characterization and well-designed clinical trials in the future, MSC-exosome loaded 3D-bioprinted hydrogels may prove to be an important future treatment strategy for cartilage defects and early onset osteoarthritis. The technology needs to be marketed as a potential Regenerative Platform until then, not as a clinical cure.

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