In-situ hydrogel polymerization for articular cartilage regeneration

Francisco Rodríguez-Fontán,[*#] Cecilia Pascual-Garrido[**]
[*]*University of Colorado Anschutz Medical Campus, Denver (Colorado, USA)*
[#]*School of Medicine, University of Buenos Aires (Buenos Aires, Argentina)*
[**]*Washington University Orthopedics, St. Louis (Missouri, USA)*

ABSTRACT

A significant number of young active adults are affected by focal chondral lesions. These lesions, if left untreated, will progress to osteoarthritis (OA). OA is one of the main debilitating musculoskeletal diseases and leads to a high economic and social burden. Despite surgical cartilage repair for focal chondral lesions, which improve patient-reported outcomes at short and mid-term, there is a risk of early OA progression. Biological treatments (i.e., stem-cell therapy, bioengineering) have made great progress in the last years. Tissue engineering is an evolving field for articular cartilage repair which could potentially be used for the treatment of focal chondral lesions, promoting regeneration and preventing joint surface degeneration. Stem cells and hydrogels may provide a functional, dynamic and biologically equivalent tissue that promotes tissue regeneration while being gradually degraded and replaced. The standard approach to tissue engineering consists in delivering cells within a hydrogel or a three-dimensional printed biomaterial scaffold into the chondral lesion to induce regeneration. This review focuses on the current and future use of hydrogels and tissue scaffold bioprinting for the treatment of focal chondral lesions, and provides preliminary data from two pilot animal studies.

Keywords: Focal chondral lesions; osteoarthritis; hydrogels; bioprinting; regeneration; stem cells.
Level of evidence: V

Hidrogeles de polimerización *in situ* para la regeneración de cartílago articular

RESUMEN

Una significativa cantidad de adultos jóvenes activos sufre lesiones condrales focales. Estas lesiones, si no se tratan, pueden progresar hacia la artrosis, que es una de las principales enfermedades musculoesqueléticas debilitantes y de gran carga económica que afectan a toda sociedad. Pese a los tratamientos quirúrgicos disponibles para la reparación de defectos condrales focales sintomáticos que mejoran la calidad de vida a mediano plazo, hay un mayor riesgo de progresión hacia la artrosis prematura. Los tratamientos biológicos (células madre, bioingeniería tisular) han avanzado a grandes pasos en los últimos años. La bioingeniería es un área que ha progresado en la regeneración de cartílago articular y que potencialmente podría progresar en el terreno de tratamientos articulares, promoviendo la regeneración y evitando la degeneración. Las células madre y los hidrogeles pueden proveer un tejido símil biológico de comportamiento dinámico-funcional equivalente que induce la regeneración tisular al ser degradado y reemplazado gradualmente. El abordaje consiste en colocar un hidrogel precursor o un biomaterial tridimensional impreso dentro del defecto condral por ocupar para inducir la regeneración. Esta revisión se focaliza en el uso actual y futuro de hidrogeles y bioimpresión tridimensional para la regeneración de cartílago articular en el tratamiento de lesiones condrales focales y proporciona datos preliminares de dos estudios piloto en animales.

Palabras clave: Lesiones condrales focales; artrosis; hidrogeles; bioimpresión; regeneración; células madre.
Nivel de Evidencia: V

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Articular cartilage supports weight and friction. It is mainly composed of an extracellular matrix (ECM) and, to a lesser extent, of chondrocytes (~5%), and its main function is to maintain the ECM through the synthesis and degradation of its components, which balance is directed by the compressive modulus of this tissue.[1] The ECM is mainly composed of collagen II and proteoglycans, such as aggrecan and chondroitin sulfate (CS).[2] Unfortunately, being an avascular tissue (its only vascular supply is of subchondral nature) and due to its low cellularity, its intrinsic regenerative ability is low. Chondral lesions do not usually regenerate as well as osteochondral one—which have a subchondral vascular supply that allows it. However, the latter tend to produce fibrocartilage, comprised mainly of collagen I, with lower resistance to compression and friction.[3]

Articular cartilage lesions are more common in athletes and active people than in the general population.[4,5] Most of these lesions progress to osteoarthritis if not treated promptly.[6,7] Certain risk factors have been associated with progression to osteoarthritis, such as a high body mass index, female sex, trauma, genetic predisposition and joint misalignment, among others.[8] In addition, certain sports seem to make people more prone to osteoarthritis, such as football, fighting, long distance running, contact sports and weight-lifting.[9] Surgical treatments for cartilage lesions have evolved from microfracture to cell transplantation and viable osteochondral implants. However, none of these therapies allows the regeneration of normal and native hyaline cartilage, and even less in large lesions.

Microfracture allows the recruitment of stem cells from the subchondral bone, but is usually used for defects measuring <2 cm^2 and has limited potential, since it heals by forming fibrous tissue. The transfer of autografts or osteochondral allografts can restore the subchondral bone and the articular cartilage more efficiently in a surgical procedure, but its major disadvantages are the availability of autografts or allografts, its morbidity rate (not applicable when an allograft is used) and the consistency of irregular surfaces in large defects. In the case of allografts, it should also be added that there is a risk of disease transmission, graft rejection and partial or incomplete incorporation into the joint.[6,10]

Autologous chondrocyte implantation is a surface procedure performed in two stages, and it has limited capacity to restore the subchondral bone, to keep the chondrocytes differentiation in a culture medium and to be used in defects measuring >6-8 mm deep.[11] It should be mentioned that this procedure evolved and gave rise to a new technique: matrix-induced autologous chondrocytes implantation on collagen type I/III, which allowed to prevent the dedifferentiation of chondrocytes that happens with the autologous chondrocyte implantation technique.[12] Brittberg *et al.* obtained good clinical results at 5 years in the treatment of osteochondral knee defects measuring ≥3 cm^2.[12] This technique was approved in December 2016 by the United States Food and Drug Administration to treat chondral knee defects in patients <55 years old.

Some of these therapies produce fibrocartilage, which is unfit for performing the original function of the joint. Hydrogels can be used to treat these defects by circumventing these limitations, and also provide a chondrogenic substrate for cell therapy (e.g., stem cells, chondrocytes). Recent clinical studies using different compositions of polymers and cell sources have efficiently treated osteochondral defects >2 cm^2, but results are usually better in minor single injuries.[13]

Tissue engineering through the use of polymers combined with cell sources could provide a therapeutic tool that would advance the field, allowing a regeneration of the native articular cartilage or native simile.

This review is focused on the use of hydrogels for the engineering of articular cartilage and their potential use combined with stem cells to treat symptomatic focal chondral lesions and early osteoarthritis. Here, we present some preliminary results in animals. The reader can also reference more comprehensive reviews.[14,15]

TISSUE BIOENGINEERING

Cell sources

The use of cells in hydrogels can result in faster and stronger hyaline cartilage regeneration.[14] A large number of cells have been evaluated for cartilage repair and can be categorized by their level of differentiation: fully differentiated chondrocytes or undifferentiated (pluripotent or multipotent) stem cells (Table).

Table. Cell types available. Sources and factors for and against their use

Cell type	Subtype	Tissue source	Implant	Positive	Negative
Chondrocytes	NA	Autologous: from another joint or surrounding the lesion.	ACI,[16-18] MIACI[19-21]	- More than 20 years of experience. - Good clinical results with the MIACI technique in knee injuries.[12,22,23] - MIACI prevents de-differentiation. - MIACI was recently approved by the FDA.	- 2-stage procedure. - ACI: de-differentiation of cells in culture media. - Patients >50 years, lower chondrogenic capacity.[6,24]
Stem cells[25]	Mesenchymal	Autologous: bone marrow, synovial, adipose, peripheral blood, etc.	*In-situ* polymers or 3D polymers.	Most studied cell line in tissue engineering.[26-31]	- Differentiation and replication capacity decreases with age and aging.[32,33] - Chondrogenic potential depends on the cell source (synovial: the one with the highest chondrogenic potential).[25]
	Induced pluripotent	Autologous (e.g., dermal fibroblasts). [34,35]		- ESC [*sic*] simile. - There is genetic integration-free cell reprogramming.[36,37] - Good non-clinical results for chondrogenesis.[38,39]	- Method of genetic integration: risk of teratomas.[34,40]
	Embryonic	Allogeneic (early embryo).[41]		- Pluripotent; unlimited replication without loss of differentiation capacity. - Good non-clinical results for chondrogenesis.[42-45]	- Their use is morally wrong.[46]

NA = not applicable, ACI = autologous chondrocyte implantation, MIACI = matrix-induced autologous chondrocyte implantation, FDA = US Food and Drug Administration.

POLYMERS AND HYDROGEL BEHAVIOUR

Bioengineering is the combination of cells with polymers in the form of hydrogels or three-dimensional (3D) structures for promoting tissue regeneration. Recent advances in bioprinting have guaranteed the ability to assemble hydrogels in anatomically functional tissue forms or in organ parts. In addition, hydrogels can be used as drug delivery systems, through controlled and sustained intra-articular release, for weeks and even months,[47] in order to treat inflammatory joint diseases, such as osteoarthritis or rheumatoid arthritis.

Hydrogels are insoluble crosslinked polymers, which are hydrated in aqueous media. They can be broadly divided into natural or synthetic, and biodegradable or not.[48] Polymers generate a microenvironment that resembles specific tissues and stimulates native regeneration by promoting cell-matrix and intercellular interactions which regulate targeted cell differentiation and tissue growth.[43,49,50] Synthetic polymers usually have mechanical and shear properties similar to those of articular cartilage.[51,52] Certain studies have shown the ability to easily embed cells and growth factors into synthetic hydrogels.[53-59] Even more promising is the combination of synthetic and natural polymers as a better approach to create biomimetic or cartilage mimetic hydrogels.

These can be designed to mimic fundamental aspects of the native environment to provide appropriate signals to the cells that are sown in it, while precisely adjusting the mechanical, chemical and degrading properties of the hydrogel.[60-64] For example, the percentage of hydration can also be regulated to resemble that of the native cartilage (~80%) and favour the exchange of substrates and peri- and extracellular products among cells.[14]

The advantages of natural polymers are their biocompatibility, their biochemical similarity with native cartilage and their ease of degradation (e.g., DNA, RNA, hyaluronic acid, collagen, fibrin, elastin, actin and myosin). As for non-biodegradable examples, there is soy, alginate, silk, agarose and cellulose.[65] Due to their high ductility, synthetic polymers are preferred, since they allow greater control over macroscopic, micro-environmental and degradation properties. Some examples of synthetic polymers that have been used for cartilage regeneration are polyethylene glycol (PEG) and polyvinyl alcohol (PVA).

The challenge of designing a hydrogel that supports joint weight-bearing and facilitates tissue growth simultaneously until its gradual degradation persists. An ideal hydrogel should: 1) fill the defect, 2) withstand weight-bearing on the joint (compressive modulus simile cartilage: from 240 to 1000 kPa),[66] 3) integrate into the surrounding tissue, 4) gradually degrade and 5) transfer the weight-bearing stimulus to the new forming tissue in a balanced dynamic way. It is possible to match degradation with new tissue formation if the properties and the initial formulation of the hydrogel are carefully adjusted. Different factors can be regulated and affect the behaviour of the hydrogel and, therefore, of the new tissue. Crosslinking and the use of degradable bonds affect the rate of degradation of the hydrogel (e.g., the higher the crosslinking, the slower the degradation). However, a high density of crosslinking is required to withstand weight-bearing on the joint, but it will slow down degradation and negatively affect macromolecular diffusion, including growth factors and newly synthesized ECM molecules, especially aggrecan and collagen, which are too large to be transported through the hydrogel crosslinking and, as a result, degradation must occur before a new tissue forms.[54,67,68] This has been achieved using hydrolysis-susceptible hydrogels[69,70] and enzymes (e.g., metalloproteinases and aggrecanase).[71,72]

Optimal integration with the surrounding tissue is another critical factor. The integration phenomenon works as a "nexus" or "bridge" between the biomimetic tissue and the defect surface, allowing cells to migrate out of the platform or hydrogel, or inwards, from the surrounding tissue.[73] This is allowed by *in-situ* cross-linked molecular or chemical polymerization between the adjacent cartilage and the polymer, or by the addition of adhesive groups (e.g., aldehyde).[74] Without efficient integration, the hydrogel will not be able to properly transfer the weight-bearing capacity to the forming tissue and, consequently, a solution of discontinuity appears between the surrounding tissue and the biomimetic tissue that could lead to implant failure.[65]

One way of applying hydrogels *in vivo* that offers a great advantage due to its easy application and its ability to fill the defect, is to use a liquid pre-polymerized solution of the hydrogel, which is then polymerized *in situ* (Figure 1). This *in-situ* polymerization (thermosensitive or photosensitive) allows greater adhesion between the hydrogel and the surrounding tissue.

Bryant *et al.* developed and worked with a promising hydrogel cartilage simile for *in-situ* application and photopolymerization (photosensitive).[75] This natural/synthetic polymer is formed by PEG and ECM analogues (CS and arginylglycylaspartic acid [RGD])[75], and it allows the encapsulation of cells (e.g., stem cells, chondrocytes) during their formation. The addition of norbornene to the PEG allows it to be light-cured with 405 nm visible blue light (Figure 1). CS is the main glycosaminoglycan in cartilage, and creates a hyperosmotic microenvironment and promotes tissue synthesis under dynamic compression.[76-78] RGD is a chondrogenic peptide that acts on the $\alpha 5\beta 1$ cellular integrin and, as a mechanosensor, cells survey rigidity of the substrate and dynamic compression.[79-81] The advantages include temporal and spatial control during the formation of the hydrogel, the ability to polymerize it at a physiological pH and temperature, and in a quickly manner, in seconds to minutes.[82]

In addition, this hydrogel can be formed with multiple layers that mimic the different properties of native tissue layers. In order to be used for the treatment of osteochondral lesions, which involves replicating different mechanical properties, Steinmetz *et al.*, using PEG-based hydrogels and changing the type and concentration of the ECM analogues, as well as local rigidity within each layer, have found that, under compression, the variation in the rigidity of the hydrogel within each layer produced high tension in the soft layer (cartilage simile), low tension in the rigid layer (bone simile) and moderate tension in the interface. This allows the possibility of directing differentiation of stem cells embedded in the hydrogel.[83] Therefore, in order to treat complex lesions that involve the combination of "layers" with different structural properties, it is possible to combine different polymers with different sources of stem cells and guide them towards the desired differentiation.

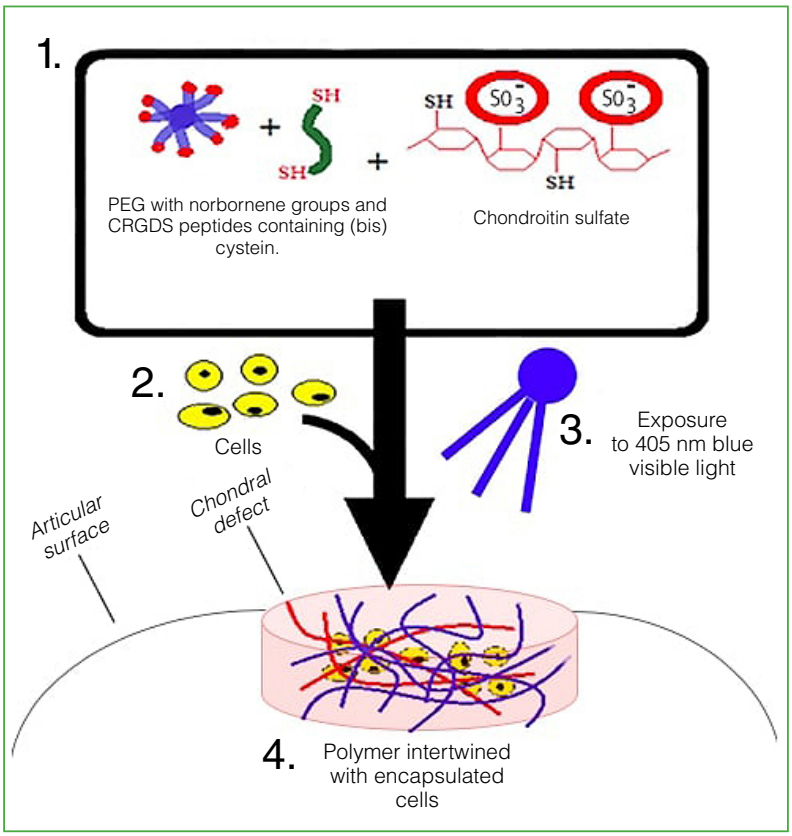

Figure 1. Biomimetic hydrogel architecture during its *in-situ* formation in a chondral defect. In this case, the hydrogel is composed of polyethylene glycol (PEG), chondroitin sulfate (CS) and a biodegradable peptide for cell adhesion (CRGDS). First, the hydrogel precursor is inoculated in its liquid form and then, under visible light, polymerization is initiated and the hydrogel undergoes *in-situ* gelation. Cells or growth factors can be added during pre-inoculation and polymerization preparation.

PRELIMINARY RESULTS

Following the guidelines of the International Cartilage Repair Society (ICRS) proposed by Hoemann *et al.* for the histological evaluation of cartilage repair,[84] we have studied a photopolymerizable hydrogel composed of PEG-CS-RGD, in experimental models of chondral lesions in rabbits and horses. In two recent pilot studies carried out by Pascual-Garrido *et al.*, promising results have been obtained with the same hydrogel to repair chondral knee injuries.[85]

The first study evaluated critical bilateral osteochondral knee lesions, measuring 3 mm wide x 2 mm deep, in 10 adult male white New Zealand rabbits (n = 20). Three groups were treated randomly: group 1, hydrogel (n = 5); group 2, hydrogel + MSC (n = 5); group 3, controls, untreated (n = 10). The group that was treated with the hydrogel presented greater chondrogenesis and partial integration to the adjacent cartilage after six months of surgery (Figure 2). The hydrogel can be administered and photopolymerized in a sterile manner. It filled the defect without showing inflammatory signs and with good chondrogenesis in three cases of group 1. Surprisingly, the addition of MSC to the hydrogel did not potentiate chondrogenesis of the hydrogel and, in some cases, the result was less than that of the control group. This suggests that MSCs take different stimuli from the surrounding environment, truncating their chondrogenic differentiation. Although it was not statistically significant, group 1 was the one with the best chondrogenic performance as measured by the modified O'Driscoll scale (MODS)[86] (group 1: 17.4 ± 4.7; group 2: 13 ± 3; group 3: 16.7 ± 2.9; p = 0.11), and it should be noted that it was the one with the highest glycosoaminoglycans staining (percentage safranin-O red staining, group 1: 49.4% ± 20; group 2: 25.8% ± 16.4; group 3: 36.9% ± 25.2; p = 0.27).[85]

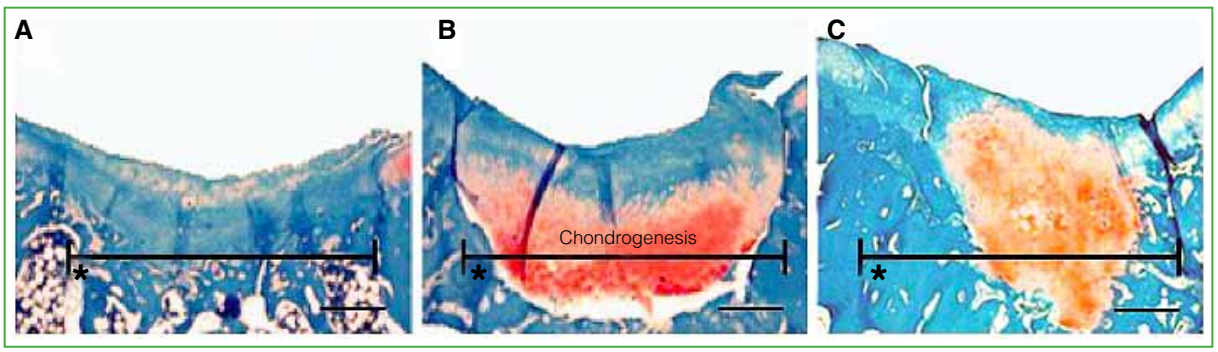

Figure 2. Safranine-O staining Original magnification x4; the scale (black) equals 500 µm. **A.** Control (not treated with hydrogel): limited chondrogenesis. **B.** Hydrogel: moderate chondrogenesis due to its higher glycosaminoglycan content (red). **C.** Hydrogel + MSC: certain minor chondrogenesis compared to group 1.
Black bar: defect area.

The second study (not yet published) was carried out in critical bilateral osteochondral knee injuries (x2, one proximal and one distal) of the medial femoral condyle measuring 15 mm wide x 5 mm deep, in 3 adult mares of 2.5 years (n = 12). They were divided into 5 groups: group 1, hydrogel (n = 3), group 2, hydrogel + MSC (n = 3); group 3, microfracture (n = 1); group 4, microfracture + hydrogel (n = 3); and group 5, microfracture + hydrogel + MSC (n = 2). Six months after the intervention, the mares were humanely slaughtered. Again, the hydrogel could be administered and photopolymerized in a sterile manner. In this case, despite not being statistically significant, group 5 had better MODS results (group 1: 13 ± 3.6; group 2: 13.3 ± 5.8; group 3: 10 ± 0; group 4: 10 ± 2.6; group 5, 14 ± 2.8; p = 0.61). The microfracture procedure showed no inflammatory signs and produced fewer subchondral abnormalities (fibrosis, cysts, neovascularization). On the other hand, the group treated with hydrogels had the greatest vertical and horizontal integration to the surrounding tissue, but presented moderate inflammation. This did not happen in the study conducted in rabbits. Defects treated with microfracture + hydrogel showed more glycosoaminoglycans, less inflammation (vs. hydrogel alone) and less subchondral abnormalities (vs. microfracture alone) (Figure 3).

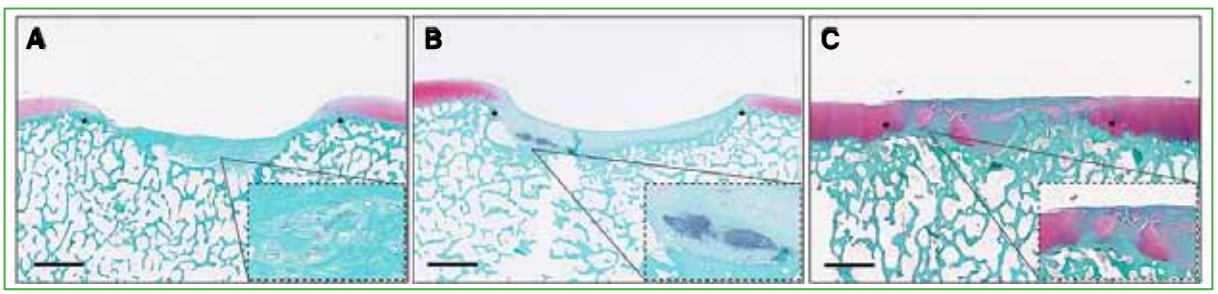

Figure 3. Safranine-O staining. Nanomicroscopic images. Original magnification: x4; the scale (black bar) equals 3000 µm.
A. Microfracture: shortage of glycosaminoglycans; subchondral abnormalities and vascularization in magnified area.
B. Hydrogel: inflammation and good vertical integration. The magnified area shows inflammatory infiltrates. **C.** Microfracture + hydrogel: more glycosaminoglycans (red staining), less inflammation and less subchondral abnormalities in magnified area.
Between *: defect area.

BIOPRINTING

It is a key instrument for future biological therapeutical approaches, since it allows the incorporation of polymers into cells that remain functional and create 3D structures to be applied in tissue lesions, fill the solution of continuity and promote tissue regeneration. Natural polymers, such as collagen, alginate, gelatine and hyaluronic acid, or synthetic ones, such as PEG, can be used and combined widely. Bioprinting allows recreating interfaces either "layer by layer" or continuously, depending on the printer used.[87] Therefore, the creation of structures with different rigidity gradients that best reflect the native cartilage or bone allows the recreation of

the tissue microenvironment. The ability to reproduce precisely the wide range of chondral forms, and then limit cartilage resection in repair surgeries, can potentially yield greater surgical results with minimal removal of healthy cartilage. In this regard, focal chondral defects have been efficiently treated by this approach.[88-91]

Osteoarthritis, one of the main debilitating conditions and with greater personal and social-financial burden (knee and hip being the most affected), is more difficult to treat.[92-94] The possibility of remodelling joint surfaces completely, mimicking the anatomical form, with similar biomechanical and biological potentiation properties, is a challenging task that is being carried out in experimental studies.[95] Moutos *et al.* described the formation of functional cartilage based on a crosslinked 3D polymerized structure that can be used to reshape the whole articular surface, with the biological ability to protect affected joints from inflammation through the expression of anti-inflammatory molecules. This approach could dramatically change the current treatment of polyarticular joint disorders.[95]

The combination of chondrogenic hydrogels with 3D bioprinted structures prior to inoculation of the hydrogel within the defect could be a promising therapy. 3D structures provide a stronger platform until the defect regenerates. In addition, 3D printed structures could promote integration of the hydrogel into subchondral bone and surrounding tissue.[95,96] The proposed approach is to inject the hydrogel precursor between the 3D printed structures (Figure 4).

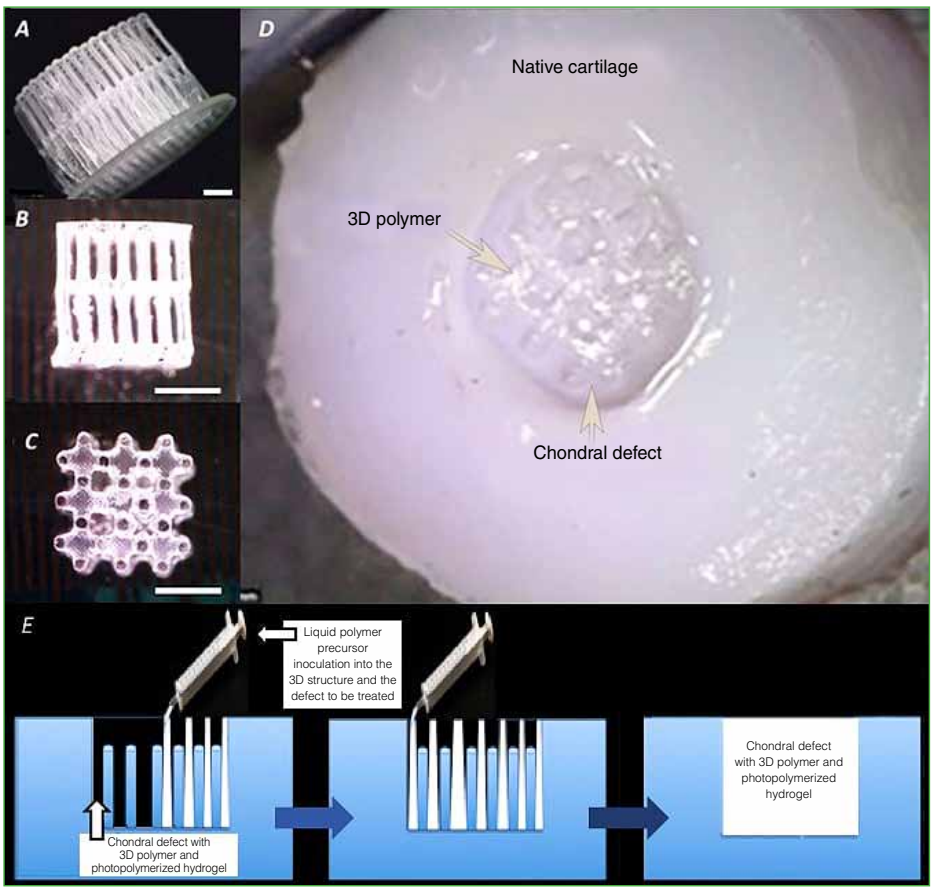

Figure 4. A-C. Note the different 3D printed structures before *in vitro* inoculation. Scales (blank bars): **A**, 2 mm; **B and C**, 5 mm. These structures are usually formed by pillars that connect to each other. The space between the pillars can be filled by another polymer. This combination provides different mechanical properties and tries to mimic the interfaces of the native tissue. **D.** 10-mm osteochondral defect created in the knee of a pig. The 3D structure (**C**) was placed in the defect, and a pre-polymerized hydrogel precursor was injected over it, covering the 3D structure, and then polymerized under 405 nm visible blue light. **E.** Representation of a procedure based on the application of a pre-polymer together with a 3D polymer and its *in-situ* polymerization for cartilage regeneration. The pre-polymer is injected between the 3D printed structures (blue pillars). Then, it polymerizes *in-situ*; both polymers, the hydrogel and the 3D structure, will gradually degrade, stimulating and generating the new tissue.

USE OF HYDROGELS IN ORTHOPEDICS AND TRAUMATOLOGY

Hydrogels could eventually be used to repair different structures, for example, meniscus tears,[97] growth plate fractures[98] and bone fractures,[99] among others. However, this report is focused on the use of hydrogels for articular cartilage regeneration. It is important to emphasize that, to expand the scope of the use of hydrogels in different musculoskeletal tissues, their main components would have to adapt to the biological characteristics of the tissue to be regenerated.

The most immediate application of hydrogels in Orthopedics is for the treatment of symptomatic focal chondral lesions. There are clinical reports on the use of chondrogenic hydrogels in combination with microfracture for the treatment of articular cartilage lesions.[74] The proposed approach is to use these hydrogels as a sealing agent at the site of the microfracture. After this, hydrogels add a chondrogenic stimulus and allow the clot and endogenous stem cells to remain in place.[74,100]

Few clinical studies have been carried out with these new chondrogenic hydrogels for the treatment of focal chondral lesions. Encouraging results have been achieved in a pilot study using microfracture and hydrogels to treat a local chondral lesion after six months.[74] In addition, in another randomized study that compared the use of microfracture versus microfracture and BST-CarGel® (PiramalLife Sciences, Bio-Orthopedics Division; Smith & Nephew plc, London, United Kingdom) for the treatment of chondral knee injuries, better symptomatic and regenerative results evaluated by MRI were achieved in the second group.[101] It is key to understand that there is a huge need for further studies in this area of bioengineering. Numerous considerations must be addressed according to the composition of the hydrogel: biodegradation time, type of polymer that is ideal for the regeneration of different tissues, and how to reproduce the gel to fill the defect in a morphological fashion. In addition, it is necessary to optimize the biological environment with the most favourable cell lines, growth factors and anti-inflammatory agents.

CONCLUSIONS

Biodegradable and biomimetic hydrogels have multiple advantages for the treatment of chondral lesions and early osteoarthritis, and they are also promising for treating other orthopedic conditions. Advantages include their ability to be injected, controlled *in-situ* polymerization and controlled degradation times that mimic the formation times of new tissue. A promising area worth exploring is their ability to send chondrogenic signals that would impact on the differentiation of endogenous or exogenous stem cells. Given the promising results, researchers should continue to study the potential of stem cells within hydrogels, such as their multiple formulations based on cell density, the combination of natural and synthetic polymers, and the addition of growth factors. Finally, bioprinting offers the opportunity to print structures that most closely resemble native anatomy and that will eventually allow the formation of the surface of an entire joint. Hydrogels will continue to evolve, and there is hope that their use will impact articular cartilage regeneration and the treatment of other orthopedic conditions.

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C. Pascual-Garrido ORCID ID: http://orcid.org/0000-0001-7487-4753

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