
Review Article

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Progress in Annulus Fibrosus Regeneration: Connecting Recent Research with Clinical Operation

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Abstract

As a leading course of global disability, the intervertebral disc disorder has brought great burden to the society. Compared to the great input on nucleus pulposus condition, which is the core part of IVD, less attention has been paid on the outer structure, Annulus Fibrosus (AF). However, the failure of AF plays a key role on degenerative disc disease and has shown great influence of the IVD function. In this paper, the author started from the current clinical treatment approach, with the retrospective summary of the publications from lab research, the main type of therapeutic strategies are reviewed. There are natural sourced therapies, cellular-based therapies, growth factor-based therapies and biomaterial-based therapies. Via the introduction of typical methods and trials, the author has put forward the main challenges faced by AF disorder treatment-proper combination by multiple options and the establishment of reliable evaluation in vitro, in vivo and ex vivo models. It is expected that this paper could provide a brief summary for the current strategies on AF treatment and inspire the innovation for better clinical options.

Keywords: Annulus fibrosus; Cellular therapies; Growth factors; Biomaterial; Regeneration; Tissue engineering.

Introduction

As a leading course of global disability, Intervertebral Disc (IVD) disorder has become the largest threaten to human health and caused a high socioeconomic burden. It is not only within ageing population but also the young working population, showing a mean lifetime prevalence of 1-3% [1]. Main symptom including low back pain, leg numbness and weakness are closely related with IVD herniation and degenerative disc disease. Particularly, recurrent IVD herniation has been recognized as the most important factor contributing to persistent pain and disability of patients after surgical discectomy [2] with the damaged IVD structure and function, including the Nucleus Pulposus (NP) and Annu-

lus Fibrosus (AF). At present stage, more attention has been paid on the regeneration of NP [1,3-6] there is only a few studies on AF repair and treatment related issues. However, it has been pointed out that the neo-innervation of AF is one of the key factors that leads to discogenic back pain [7]. The large annular defects are associated with an increased rate of reherniation, which suggested that repair of ruptured Annular Fibrosus (AF) would be essential for large disc protrusion treatment. In this paper, we will provide a comprehensive review on the current situation of AF regeneration and repair strategies from both clinical diagnosis and treatment as well as lab research progress. The advanced AF tissue

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engineering strategies for its structure and function restoration will be summarized and the challenges and prospective will be addressed.

Annulus fibrosus structure and function

Annulus Fibrosus (AF) is the tough circular exterior of the IVD that surrounds the soft inner core of Nucleus Pulposus (NP). Intervertebral Disc (IVD), a complex joint sandwiched between the vertebral bodies, is a tough tissue structure that supports flexible motion including bend, rotate and twist within the spine. IVD acts as a ligament to assemble the vertebrae and also serves as a shock absorber to prevent excessive motion and maintain mechanical stability. Each IVD is a composite tissue of three main parts: the Nucleus Pulposus (NP), a proteoglycan-rich gelatinous center that bears the pressure; the Annulus Fibrosus (AF), a collagen-rich fibrous structure that confines the NP; and the Vertebral Endplates (VEP), made of hyaline cartilage and serve as interfaces between the IVD surrounding the disc on the top and bottom (Figure 1) [8].

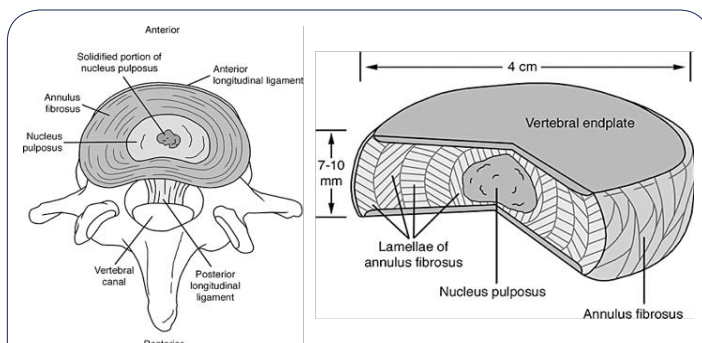


Figure 1: Left: Annulus Fibrosus (AF) is the tough circular exterior of the IVD that surrounds the soft inner core of Nucleus Pulposus (NP); Right: A cut out portion of a normal disc showed the location of the NP, the VEP and the architecture of AF. The human IVD is 4 cm wide and 7-10 mm thick [8].

AF is a largely avascular and aneural tissue composed of highly oriented ligament fibers. It can prevent the NP from herniating or leaking out of the disc by hydraulically sealing the nucleus and evenly distributing any pressure and force imposed on the IVD. The AF mesenchymal cells sparsely distributed and have the characteristics of both fibroblast and chondrocyte and the density of cell declines with growth. These cells synthesize mostly collagen type I in response to deformation while sensory nerve fibers and blood vessels have been evidenced located in the outer AF and transport nutrient to the cells. Collagen type I decreases toward the NP and the collagen type II increases from outer AF to inner AF [9-11].

Current clinical situation on annulus fibrosus

Direct therapies

Besides the traditional bed rest, nonsteroidal anti-inflammatory drugs, physical therapy, the standard surgical treatment of IVD herniation generally reply on the herniotomy or discectomy procedure, which will cause tears, further invasion and possible secondary trauma of AF structure [12]. The restoration of intradiscal pressure through percutaneous intradiscal injection will leave a puncture hole on the AF. For a long time, it was thought that the small defect resulted from the “tiny” needle diameters had little effect on the condition of IVD. However, recent cases

have revealed that even subtle AF impairments could lead to an immediate effect on axial mechanics and disc height owing to the NP pressurization, and may subsequently compromise biologic homeostasis such as metabolism and cell viability [13]. There is high possibility that the remaining wound site would facilitate the reherniation of IVD or leaking of NP matrix, which will reduce the efficiency of coalescence. It has been reported that there are 20-25% of discectomy patients continue to experience unsatisfactory outcome, with recurrent disc herniation as one of the most important factors of discomfort [1].

Suturing and defect blocking of the injured site are the first treatment approach used in AF tissue repair. With specific custom-tailored suturing devices [11], the annular defect site could be fixed with anchors attached to bone, thus keeping the NP from extruding and restoring the tensile strength (Figure 2). However, the small surgical field caused the complexity of procedure and related high cost, which has limited the spread of these suturing devices. As reported previously, the lack of effective AF closure techniques has led to 5-25% rate of complications involving reherniation or recurrent pain at the same level.

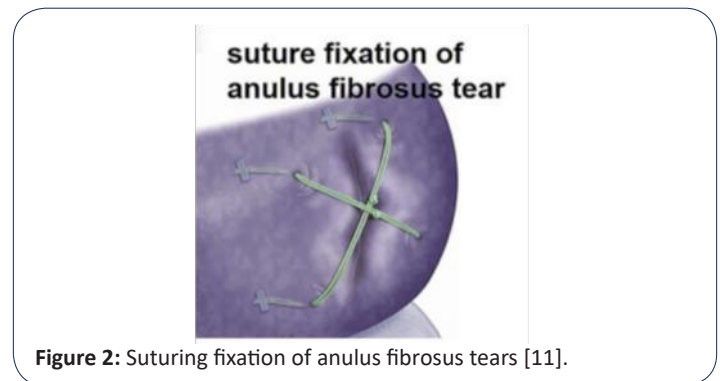


Figure 2: Suturing fixation of annulus fibrosus tears [11].

Indirect precautions and protection

While the major pathological features of IVD degeneration are characterized by a decrease in active cell numbers, a breakdown of extracellular matrix, an altered phenotype of normal disk cells, and the presence of pro-inflammatory mediators [14], clinical reports have reported that lifestyle is closely related to the AF health status. Apart from ageing, the time killer to the health of IVD [15], living conditions and habits play a key role on the status of both NP and AF tissues as described in several clinical reports. Elmasry S and his colleagues have proved that nicotine via tobacco smoking could down-regulate the proliferation rate and glycosaminoglycan (GAG) biosynthesis of both NP and AF cells [16]. The vascular network that distributed in outer AF could be constricted, thus affect the nutrients transport to disc. Inversely, the good habit of physical exercise, which is also recommended worldwide clinically, could significantly improve the IVD condition. Cell densities of AF and NP were increased with higher numbers of 5-bromo-2-deoxyuridine (BrdU)-positive cells and more rapid proliferation [17]. In vitro and animal studies have revealed that successful repair requires that the disc cells remain viable and active; they therefore need an adequate supply of nutrients. Current biologic approaches might place additional demands on an already precarious nutrient supply [18].

Along with the clinical situation of AF therapy, recently researchers in the lab globally are going in two ways to deal with the

repair of AF tissue. On the one hand, they are carrying out studies to deeply explore the underlying mechanism and physiological process during AF failure and regeneration, which may help to provide a reliable and better baseline for the development of possible strategies [19]. On the other hand, they are trying through the cellular, drug-based or suitable biomaterials-based approaches in tissue engineering to restore the structure and function of AF. Tremendous efforts have been contributed and we will do a brief summary in the following.

Tissue engineering strategies for annulus fibrosus regeneration

Natural sourced therapies

Strategies for AF regeneration can be divided into two main types, one is the natural sourced therapy using autogenous tissue with certain modification. One typical option is Demineralized Bone Particles (DBP) [20]. Derived from natural bone tissue, the DBP is composed of substrate, Bone Morphogenetic Proteins (BMP) including proteoglycan and collagen. It was previously proved to be host tissue reaction safe and could even reduce the inflammation reaction and stimulate cell differentiation. In 2015, Song and his colleagues prepared DBP gels with acetic acid solution and mixed with AF cells. After injection under the skins of nude mice after 1, 2 and 3 weeks, 10% DBP gels group showed better cell proliferation, distribution and ECM production in vivo, which indicates its great potential for AF tissue-engineering strategies. Choi, et al compared the DBP sponge and Collagen sponge on proliferation and phenotype maintenance of AF cells. The results suggested that DBP sponges could increase the AF cell viability and the Glycosaminoglycan (GAG) production which is essential for AF structure and function [21]. Jo and his team had successfully fabricated PLGA/DBP hybrid films to improve the AF cell adhesion and proliferation [22,23].

Another option of natural sources is the decellularized AF matrix [24]. In 2014, Xu used three different protocols to remove cells and preserve the matrix components, microstructure and mechanical function. The decellularized AF tissue showed no cytotoxicity and could support AF cell growth. In 2017, Wu et al. decellularized porcine AF tissue under different freeze-thaw temperatures, chemical detergents and incubation times to determine the optimal method for cell removal [25]. They pointed out the importance to ensure greatest retention of GAG and collagen during the decellularization process. With the optimized parameters, they successfully obtained a decellularized AF scaffold with suitable immune-compatibility evidenced by in vivo remodeling and reduction of the alpha-Gal epitope while preserving the biological composition and mechanical properties. Both decellularized AF and demineralized bone particles showed great potential for clinical applications in AF treatment. In addition, there are more and more studies based on these with the introduction of other synthetic materials, cells and growth factors [26].

Cell-based therapy

Previous studies have revealed that the internal disc disruption is accompanied by a decrease in the number of NP cells and AF cells, suggesting that promotion of the related cells could be a promising treatment for the disc regeneration [27]. Cell therapy primarily aims to supplement or replenish the local cell popula-

tion already decreased by ageing, etc [28].

One of the promising cell types is Mesenchymal Stem Cells (MSCs), which could differentiate into nucleus pulposus-like or annulus fibrosus-like cells and synthesizing IVD-mimic Extracellular Matrix (ECM). Pilot fundamental and clinical studies have proved the concept that bone marrow derived MSCs facilitated IVD repair by reducing the extent of fibrosis [29]. Via injection, MSCs could be directly added into the disc as undifferentiated or pre-differentiated cells. Moreover, MSCs could be genetically modified with genes of interest and injected into the IVD as an ex vivo gene therapy strategy [30].

The other main cell type is the inner AF cells. As shown in previous studies, human AF cells have been proved to differentiate into adipocytes, chondrocytes, neurons, osteoblasts and endothelial cells under appropriate stimulation [31]. Jin et al. demonstrated that rabbit AF cells with chondrogenic media significantly increased expression of collagen and aggrecan and may be a target for biological treatment of disc degeneration.

Recent advances in cell therapy have raised the possibility of regenerating the damaged disc via these cells alone or combined. Wang and his colleagues used a mixture of BMSCs and AFCs to transplant into a rabbit IDD model. They proved that the structure of the inner AF was significantly preserved and the boundary between the NP and AF could be clearly seen. The expression of type II collagen and glycosaminoglycan was significantly improved compared to the single-cell group [27,32].

Growth factor-based therapy

Growth factor therapy is another therapeutic strategy showing great potential in the early stage of AF regeneration [33]. Using the advanced RNA sequencing technique, a recent study by Riester, et al. has revealed that three diffusible growth factors, fibroblast growth factor 9 (FGF9), platelet-derived growth factor beta polypeptide (PDGFB), and vascular endothelial growth factor C (VEGFC) play a regulatory role in maintenance of the AF phenotype [34].

Bone morphogenetic proteins (BMPs), a family of growth factors that could promote the proliferation and differentiation of multiple cell lines, may react with IVD cells and stimulate the synthesis of proteoglycan, upregulate the mRNA expression and type II collagen and serve as the mitotic agent [33]. Similarly, stromal cell-derived factor-1 α (SDF-1 α , also known as C-X-C motif chemokine 12, CXCL12), a member of the C-X-C chemokine family of proinflammatory mediators, could induce cellular mobilization as a soluble factor, it is also an anchoring molecule for stem cells in the bone marrow and a homing beacon attached to the ECM, thus directing cells to migrate toward the repair site.

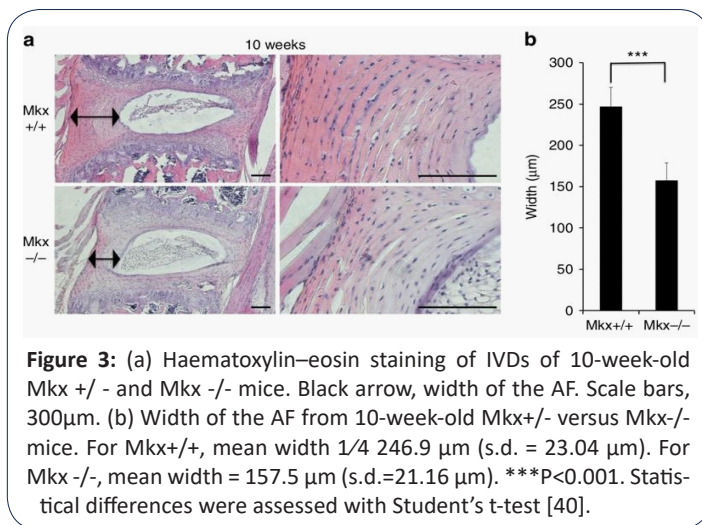
Zhang [29] and his team prepared albumin/heparin nanoparticles as injectable carriers for SDF-1 α and induce the regeneration of annulus fibrosus of Wistar rats tail discs while upregulating the expression of SOX9, Aggrecan and Collagen type II of the migrated stem cells.

As mentioned above, pro-inflammatory mediators are essential for the IVD degeneration. Collectively, IL-1 β expression may be a causative factor in IVD degeneration via a significantly promotive role in all stages of disk degeneration. Thus, the usage of IL-1 β

inhibitors showed the potential as a viable approach for the treatment of IVD degeneration. Candidates include IL-Receptor antibodies (Ra), anakinra, platelet-rich plasma (PRP), epigallocatechin 3-gallate and ligustrazine. They have been identified as potent option to block IL-1 action. However, further studies are necessary for better understanding of these cytokines or growth factors.

Platelet-rich plasma (PRP) is extracted from whole blood and is enriched in mixed growth factors. These platelets are able to release multiple growth factors including PDGF, TGF- β , VEGF, epidermal growth factor (EGF) and insulin-like growth factors (IGF). It has been used clinically to accelerate the healing process on a cellular level. With the native growth factors presenting in a normal biological ratio, PRP may contribute to synergistic effects including the mechanical properties [35]. The simple preparation method, relatively high cost-effectiveness, safety and permanent availability will promote the use in AF as well as IVD regeneration [36-39]. Cho et al. and his team has found in their study that, with the addition of PRP, the tumor necrosis factor- α treated AF cells isolated from porcine IVD tissue showed an increased production of the major matrix components including type II collagen and aggrecan as well as decreased inhibitory collagenase MMP-1 (matrix metalloproteinases). The results address a therapeutic approach for intervening early in AF degenerative process [39].

A recent study reported by Nakamichi proved the homeobox protein Mohawk (Mkx) is specifically expressed in tendon-related and ligament-related tissues [40]. This is a member of the three-amino-acid loop (TALE) superclass of atypical homeobox genes and its expression is maintained even in matured ligament cells. Give the ligament-like properties of AF tissue, the Mkx expression has been proved a key transcription factor that regulate AF development and maintenance. It is mainly expressed in the outer AF of human and mice. As indicated in Figure 3, the deficiency of Mkx will cause a smaller outer AF collagen fibril diameter and more rapid progression of IVD degeneration. However, more insight for the application of AF regeneration therapy via Mkx expression should be provided in future.



Biomaterials-based therapy

Till now, the wide majority of materials used in the AF tissue regeneration are biodegradable polymers. However, the selection of the most suitable biodegradable polymers should be targeted to the envisioned therapeutic approach and to the specific features of the damaged AF tissue. Those materials must possess high adhesion strength (~ 0.2 MPa), approximately 1MPa of compressive strength, 0.3MPa of shear strength and 30 MPa of tensile moduli, respectively. According to the existing publication, the most popular materials such as gelatin, polyurethane (PU), gellan gum, Polycaprolactone(PCL). They are prepared as injectable gel, 2D membrane or 3D scaffolds, etc. for use in different conditions.

For the first process suturing in AF repair, a proper fixation method such as defect filling to match the mechanical property within the native AF defect under dynamic load is needed [46]. It should not only provide temporary mechanical support on site but also improve the biological microenvironment for cell growth. Yang and his team developed a novel injectable hydrogel from gelatin/r-PGA (GP) crosslinked with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (E) to fill the needle puncture defect on AF. As shown in Figure 4, the leakage and saturate pressures could be tuned with different GP/E ratio and that of 10:1 possessed similar property as intact group.

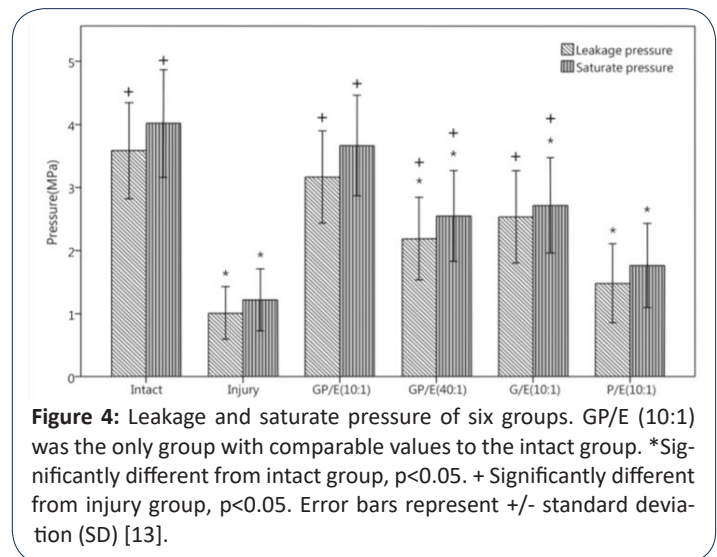


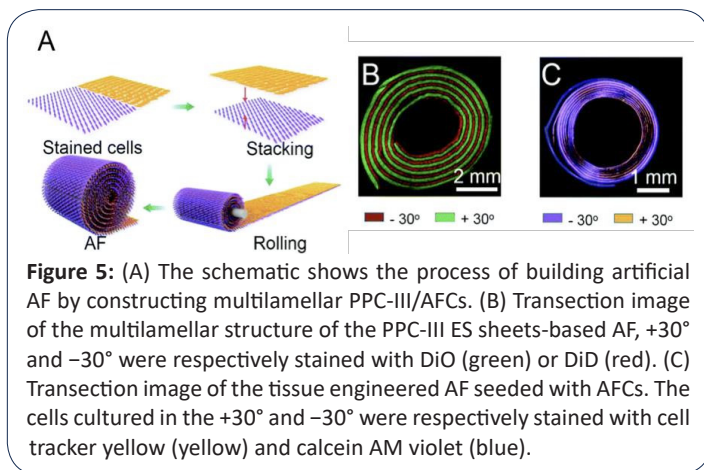
Figure 4: Leakage and saturate pressure of six groups. GP/E (10:1) was the only group with comparable values to the intact group. *Significantly different from intact group, $p < 0.05$. + Significantly different from injury group, $p < 0.05$. Error bars represent +/- standard deviation (SD) [13].

PU, an elastic membrane has been shown to support AF cell growth and function. Pirvu [1] et al. used the PU membrane as a patch sutured on the adjacent AF to maintain their PTMC (poly(trimethylene carbonate)) scaffold seeded with human bone marrow derived mesenchymal stromal cells (MSCs) within the AF defect. The disc height was successfully restored and the typical gene expression of type V collagen, a potential AF marker was significantly upregulated. It could also prevent herniation of NP tissue into AF defect in the annulotomized discs. Pereira, et al. proposed a gellan gum-based construct reinforced with cellulose nanocrystals as a biological self-gelling AF substitute [7]. It showed similar compressive modulus values to human AF tissue and the encapsulated bovine AF cells indicated promoted viability and a physiologically relevant cell morphology in vitro.

An ideal construct will repair the AF by providing physical and biological support and facilitating regeneration. The AF, more

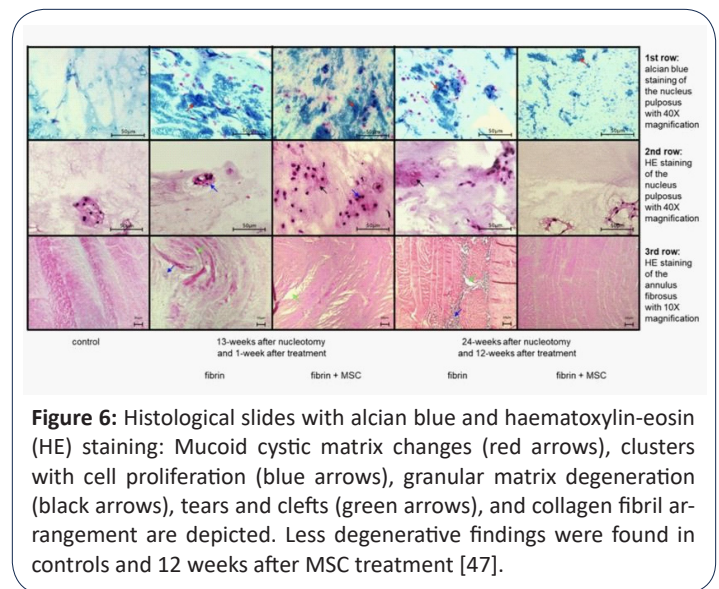
tendon-like and composed by a very organized structure of highly circumferentially orientated collagen fibers type I. Nanofibrous scaffolds are promising approach for AF tissue engineering, since their fibers microarchitecture can be reproduced, and their high length-to-width ratio mimics that of extracellular matrix components, which in turn guides tissue formation, promotes cellular adhesion and improves mechanical properties [41].

Electrospinning, which has been widely used for the fabrication of bioconstructs for tissue engineering [11], could produce tissues with impressive reconstruction of the multilamella structure, fiber alignment, and anisotropic mechanical properties. Xu et al. developed a wet spinning method based on the traditional electrospinning [42] to prepare a circumferentially oriented poly (ε-caprolactone) (PCL) microfiber scaffold. The AF cells could attach on the scaffold, proliferate and infiltrated inside the scaffold. Cells then spread along the microfiber direction and secrete and A related extracellular matrix that also oriented along the microfiber direction. Liu et al. fabricated aligned fibrous polyurethane scaffolds using an electrospinning technique and used them for culturing AF-derived stem/progenitor cells (AFSCs) [43]. The cells were more elongated and better aligned with higher gene expression and matrix production of collagen type I and aggrecan. With the same material PCL [30], Uden and his team developed a rapid prototyping techniques to prepare custom-tailored AF scaffolds via obtaining the IVD reverse-engineered architecture with micro-computed tomography acquisition and Computer-Aided Design (CAD). The scaffold showed higher mechanical values than those of human IVD and had no deleterious cytotoxic effect over the AF cells. To improve the unsatisfied hydrophilic properties, Yang and his colleagues combined PCL with certain percentage of PLGA and collagen to prepare randomly electrospun nanofibers and aligned the sheets into strips to construct the AF implant with a thickness of 40-50 μm (Figure 5). [44] The long-term in vivo implantation assays demonstrate the excellent structural, including shape maintenance, hydration, and integration with surrounding tissues and functional performances including mechanical supporting and flexibility for the IVD regeneration.



Fibrin is clinically employed as a versatile, safe, and clinically applicable sealant and cell carrier. It has been able to support disc cell survival, favor extracellular matrix production, and enhance the efficiency of cell transfer for NP and AF tissues in the intervertebral disc [45]. As reported by Likhitpanichkul et al., an injectable fibrin-genipin adhesive hydrogel (Fib-Gen) was evaluated for its performance repairing large AF defects in a bovine caudal IVD

model using ex vivo organ culture and biomechanical testing of motion segments, and for its in vivo longevity and biocompatibility in a rat model by subcutaneous implantation. Fib-Gen sealed AF defects, prevented IVD height loss, and remained well integrated with native AF tissue following approximately 14,000 cycles of compression in 6-day organ culture experiments. Biomechanically, Fib-Gen fully restored compressive stiffness to intact levels validating organ culture findings [46]. It also has been pointed out that the addition of cells, in particular if terminally differentiated, to the injected fibrin seemed to promote a more physiological matrix in comparison with fibrin alone. A recent work by Omor and his colleagues has proved the effective treatment for AF with fibrin matrix-assisted autologous mesenchymal stromal cells (MSC) in a porcine in vivo model [47]. As indicated in Figure 6, AF showed dense fibrous tissue with tears and clefts and disruption of collagen fibril arrangement, while the 24-week fibrin and MSC group had less fibrosis and better fibril arrangement.



Limitation and prospective

Regarding the IVD disorders, although much have been done on the NP regeneration, there are few on the AF regeneration. An ideal method for AF repair should not only reproduce the mechanical properties, strength, and oriented microstructure of the AF tissue, but also integrate with bone or adjacent AF tissue. Thus, a system that can reproduce such design elements falls into the category of composite tissue engineered approaches and is the subject of ongoing research. As listed above, the cell, growth factors or biomaterials therapies have a common disadvantage as they are usually targeting on one aspect of AF, leaving the challenge of a synergetic effect of tissue repair. Also in practice, treatment such as growth factors are costly and will result in side effects if leaking from the environs of the local site [48]. The proper combination via both or three of these therapies should be taken into consideration with a modified design paradigm. Given the precision during the AF clinical operation, there should be careful choice on the materials, which should be flexible to handle and maintain a relevant stable biological function.

Also, as mentioned above, a reliable in vitro test system or in vivo/ex vivo animal model should be established to better mimic the situation of human IVD. Compared with current pre-clinical

studies, the lack of a strong therapeutic effect suggests the need for an additional study to optimize the number of cells deployed, the use of a carrier biomaterials, and the need for a proper annulus sealant to assist in retention of cells in the discs. In addition, the commonly used caudal spines model for in vivo and ex vivo testing of AF/IVD performance, although sharing most of the properties with the lumbar spine, could not completely imitate the real physiological conditions of the lumbar spine such as mechanics. More accurate and precious animal model should be established in future.

Declarations

Disclosure of interest: The authors declare that they have no competing interest.

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