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INTERVERTEBRAL DISC DECELLULARISATION: PROGRESS AND CHALLENGES

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Abstract

Intervertebral disc (IVD) degeneration and the consequent low-back pain (LBP) affect over 80 % of people in western societies, constituting a tremendous socio-economic burden worldwide and largely impairing patients' life quality. Extracellular matrix (ECM)-based scaffolds, derived from decellularised tissues, are being increasingly explored in regenerative medicine for tissue repair. Decellularisation plays an essential role for host cells and antigen removal, while maintaining native microenvironmental signals, including ECM structure, composition and mechanical properties, which are essential for driving tissue regeneration.

With the lack of clinical solutions for IVD repair/regeneration, implantation of decellularised IVD tissues has been explored to halt and/or revert the degenerative cascade and the associated LBP symptoms. Over the last few years, several researchers have focused on the optimisation of IVD decellularisation methods, combining physical, chemical and enzymatic treatments, in order to successfully develop a cell-free matrix. Recellularisation of IVD-based scaffolds with different cell types has been attempted and numerous methods have been explored to address proper IVD regeneration.

Herein, the advances in IVD decellularisation methods, sterilisation procedures, repopulation and biocompatibility tests are reviewed. Additionally, the importance of the donor profile for therapeutic success is also addressed. Finally, the perspectives and major hurdles for clinical use of the decellularised ECM-based biomaterials for IVD are discussed. The studies reviewed support the notion that tissue-engineering-based strategies resorting to decellularised IVD may represent a major advancement in the treatment of disc degeneration and consequent LBP.

Keywords: Decellularisation, recellularisation, intervertebral disc, tissue engineering.

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List of Abbreviations

ACAN	aggrecan
ADSCs	adipose-derived stem cells
AF	annulus fibrosus
AMSCs	amniotic stem cells
APR	aprotinin
bFGF	basic fibroblast growth factor
BM-MSCs	bone-marrow-derived mesenchymal stem cells
BSA	bovine serum albumin
Ca12	carbonic anhydrase XII
CCK8	cell counting kit 8
CD	ctuster of differentiation
Col1	collagen type 1
Col2	collagen type 2
COL2A1	collagen type II alpha 1 chain
COL3	collagen type 3
COL5A1	collagen type V alpha 1 chain
DAF-G	decellularised AF-based hydrogel
DAPI	4′,6-diamidino-2-phenylindole
DMMB	1,9-dimethylmethylene blue
DMEM	Dulbecco's modified Eagle medium
dsDNA	double-stranded DNA
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FBLN1	fibulin-1
FBS	foetal bovine serum
FCT	Portuguese Foundation for Science and Technology
FDA	Food and Drug Administration
Foxf1	forkhead box F1
g-DAF-G	genipin-crosslinked DAF-G
GAG	glycosaminoglycan
Gal	galactose-a-1,3-galactose
Gpc3	glypican 3

Gdf10	growth differentiation factor 10
H&E	Hematoxylin & Eosin
HYP	hydroxyproline
Ibsp	integrin-binding sialoprotein
IF	immunofluorescence
IHC	immonohystochemistry
IVD	intervertebral disc
K19	keratin 19
Krt19	keratin 19
LBP	low-back pain
MAC387	macrophage monoclonal antibody clone 387
MHC	major histocompatibility complex
Mmp13	matrix metalloproteinase 13
MSCs	mesenchymal stem/stromal cells
Ncam1	neural cell adhesion molecule 1
NP	nucleus pulposus
NPC	NP cells
Pax1	paired box 1
PEEK	polyetheretherketone
PKH26	Paul Karl Horan 26
RT	room temperature
SB	sulphobetaine
SDC	sodium deoxycholate
SDS	sodium dodecyl sulphate
SOX9	SRY-box transcription factor 9
TGF-β	transforming growth factor beta
TGF-βR	TGF-β receptor
TIMP	TIMP metallopeptidase inhibitor
TNMD	tenomodulin

IVD degeneration and LBP

The human spine contains 24 IVDs localised between the vertebrae (Anderson and Tannoury, 2005) providing flexion, extension and rotation during daily activities (Tomaszewski *et al.*, 2015). The IVD is composed of an external region, called AF, an internal region, named NP, and the endplates that enclose the disc (Fig. 1) (Tomaszewski *et al.*, 2015). The AF, formed by 15-25 concentric lamellae that resist tensile stress (Colombier *et al.*, 2014), is constituted mostly by Col1 (Oegema, 1993). In turn, the hydrogel-like NP is mainly composed of water, proteoglycans (Galbusera *et al.*, 2014; Iatridis *et al.*, 2007), Col2 (Urban and Roberts, 2003; Whatley and Wen, 2012) and elastin (Galbusera *et al.*, 2014; White and Panjabi, 1990). Due to this composition, it supports the high compressive loads generated during daily activity (Mwale *et al.*, 2004; Urban and Roberts, 2003; Whatley and Wen, 2012). Finally, the endplates, consist of hyaline cartilage (Raj, 2008; Tomaszewski *et al.*, 2015) and control the diffusion of solutes and water (Tomaszewski *et al.*, 2015). The disc ECM comprises different molecules that are crucial for tissue function (cellular support, proliferation, survival, morphogenesis, differentiation and signal transduction, among others), as reviewed elsewhere (Molinos *et al.*, 2015; Newell *et al.*, 2017).

Degeneration of the IVD is one of the most frequent causes of LBP (Vos *et al.*, 2012; Waddell, 1996) and occurs with ageing. Disc degeneration is characterised by morphological modifications (*e.g.* collapse of intervertebral space, loss of hydration, sclerosis of endplate and osteophyte formation) that affect disc biomechanics, particularly spine flexibility (Galbusera *et al.*, 2014). As a result of degeneration, the IVD can start to bulge leading to disc herniation, with associated radiculopathy and discogenic pain (Martin *et al.*, 2002). Moreover, cellular and biochemical changes occur as a consequence of cell density decline and altered matrix turnover (Galbusera *et al.*, 2014). In the context of ECM composition, important age-

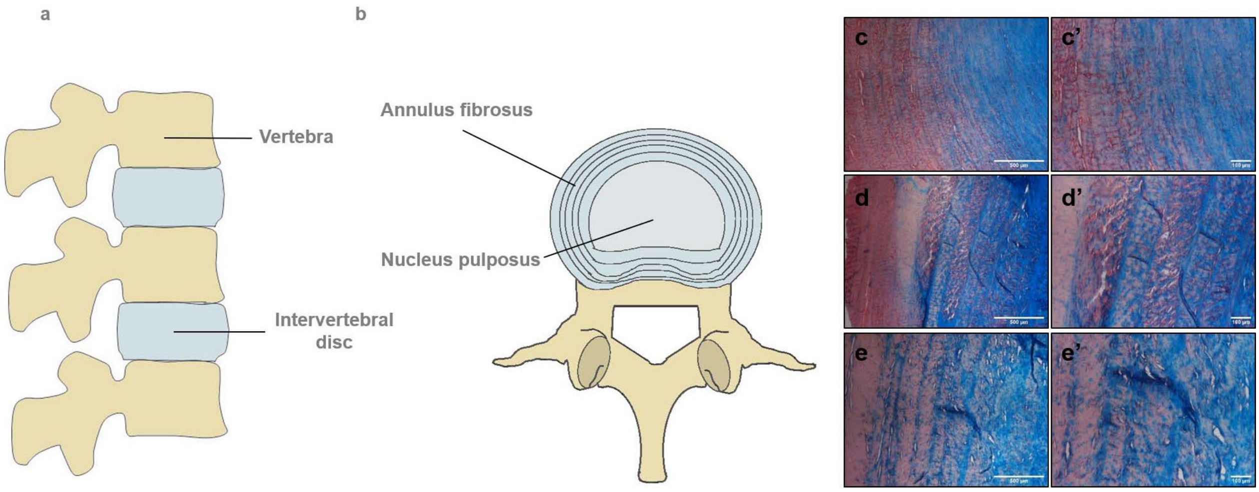

Fig. 1. Gross anatomy of the IVD and histological comparison of different-age bovine IVDs. (**a**) Lateral view of the spine illustrating the IVD laying between adjacent vertebrae. (**b**) Upper view with the different disc regions – the AF formed by concentric rings organised around the central NP. Picrosirius red, alcian blue and H&E staining of (**c**,**c′**) foetus, (**d**,**d′**) young and (**e**,**e′**) old disc samples. GAGs are identified in blue, whereas collagens are coloured in red. For each image, the left region represents the outer AF and on the right is the NP. Note the smaller interlamellar space in foetal discs. In older IVDs, the layers present an irregular distribution with an increase in the interbundle spaces (optically empty spaces). Images were acquired at magnifications of 5× (**c**,**d**,**e**, scale bar 500 μm) and 10× (**c′**,**d′**,**e′** scale bar 100 μm).

associated alterations in the NP matrisome have been recently observed. The amount of fibronectin and prolargin increase with age, whereas collagen type XII and XIV are almost exclusively expressed in bovine foetal NPs (Caldeira *et al.*, 2017). Others have also described a decrease in proteoglycan content, which results in water loss, increased expression of proteases as well as changes in collagen crosslinking and synthesis patterns (Adams and Roughley, 2006; Colombier *et al.*, 2014; Cs-Szabo *et al.*, 2002; Duance *et al.*, 1998; Takaishi *et al.*, 1997). As a result of such a homeostatic imbalance, the NP becomes much more fibrous and cartilaginous, affecting cell phenotype and ECM synthesis, in a degradative cascade that triggers LBP (Adams and Roughley, 2006).

Current therapies for LBP and IVD degeneration are mostly conservative, being addressed to control inflammation and relieve pain (Bydon *et al.*, 2014). However, they do not eliminate the underlying pathology and, hence, cannot be considered long-term clinical solutions. Surgical treatment, namely discectomy, arthroplasty and lumbar fusion, is usually considered when the other options fail (Bydon *et al.*, 2014). Nevertheless, these invasive treatments have limitations (Nasser *et al.*, 2010; Onesti, 2004; Swann *et al.*, 2016). Subsequent disc degeneration and recurrent herniation are major problems following surgery (Swartz and Trost, 2003). Spinal fusion, for instance, has been associated with long-term adverse consequences, such as dehydration, disc space narrowing, osteophyte formation and progressive degeneration of the adjacent segment (Schizas *et al.*, 2010). Although PEEK cages have been introduced as an alternative to metallic implants (Novotna *et al.*, 2015), due to them presenting a more adequate load transfer and increased fusion success rate (Schimmel *et al.*, 2016), their hydrophobic surface does not allow for protein absorption or cell adhesion, thus requiring further modifications to enhance cell attachment and biocompatibility (Novotna *et al.*, 2015). IVD total replacement by a non-biological prosthesis represents an alternative but long-term results are limited due to prosthesis wear, often requiring revision surgery. With the lack of effective long-term solutions, there is an urgent need to develop novel therapeutic strategies that target IVD functional regeneration, improving LBP patients' lives.

IVD regeneration has been attempted using different strategies including protein injection (Masuda *et al.*, 2006; Walsh *et al.*, 2004), gene transfer (Leckie *et al.*, 2012; Yue *et al.*, 2016) and cell implantation (Okuma *et al.*, 2000; Sakai *et al.*, 2005). Still, only few treatment options have been effectively translated into the clinic (Veruva *et al.*, 2014). Cell-based therapies, namely with MSCs, have been used in clinical trials, decreasing pain but without signs of tissue regeneration (Orozco *et al.*, 2011). Obstacles remain to cell transplantation, including cell leakage, which potentially causes undesired extra-discal bone formation (Vadalà *et al.*, 2012) and poor cell survival in the harsh IVD microenvironment (acidic pH, low oxygen and limited access of nutrients). Several biomaterials have also been developed but much work is still needed to obtain clinically successful alternatives. Natural hydrogels (*e.g.* alginate, chitosan, agarose, collagen, chondroitin sulphate) are close to the NP matrix composition but do not meet its mechanical requirements (van Uden *et al.*, 2017).

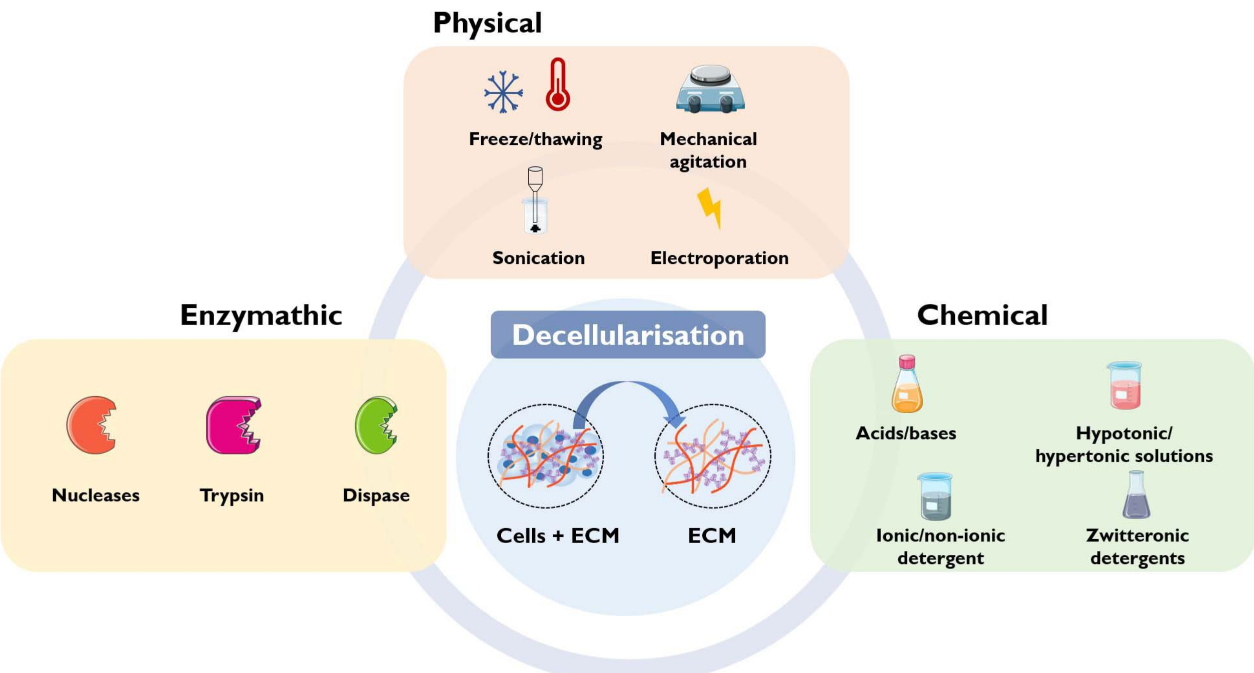

Fig. 2. Overview of decellularisation strategies. Tissue decellularisation can be performed by using physical (freeze-thawing, mechanical agitation, sonication, electroporation), chemical (acids or bases, hypotonic or hypertonic solutions, ionic or non-ionic detergents and zwitteronic detergents) and enzymatic (nuclease, trypsin, dispase) methods. For details, see Table 1.

Table 1. Overview of agents and methods commonly used for decellularisation.

Agent/methods	Mechanism of action	ECM effect	References
Physical methods			
Freeze-thawing	Induces cell lysis by ice crystal formation	Preserves ECM proteins and mechanical properties. ECM can be disrupted with rapid freezing.	Crapo *et al.* (2011) Gilbert *el al.* (2006) Gilpin and Yang (2017)
Mechanical agitation	Promotes diffusion of solutions into the tissue and removal of cellular debris	ECM structure damage with aggressive agitation or sonication	Gilbert (2012) Gilbert *et al.* (2006)
Electroporation	Disrupts cell membranes by electrical pulse.	Disrupts ECM structure	Crapo *et al.* (2011)
Chemical agents			
Acids and bases	Denature proteins, solubilise cytoplasmatic elements and disrupt nucleic acids	Damage ECM components, particularly collagen, GAGs and growth factors	Crapo *et al.* (2011) Fu *et al.* (2014)
Hypotonic and hypertonic solutions	Provoke cell lysis by osmotic shock and disruption of cell membranes	Enable cell debris washout from the tissue	Gilbert *et al.* (2006) Zahmati *et al.* (2017)
Ionic detergents: sodium dodecyl sulphate	Solubilises membrane proteins	Successfully removes cells from dense tissues and organs Perturbs tissue structure resulting in loss of GAGs and collagen integrity	Boccafoschi *et al.* (2017) Crapo *et al.* (2011) Fu *et al.* (2014) Seddon *et al.* (2004)
Ionic detergents: sodium deoxycholate	Solubilises cells and nucleic membranes	Denatures ECM proteins, resulting in GAGs and collagen loss	Cheng *et al.* (2009) Seddon *et al.* (2004) White *et al.* (2017)
Non-ionic detergent: Triton X-100	Disrupts lipid-lipid and lipid-proteins interactions and, to a lesser degree, protein-protein interaction	Less effective in DNA removal Disrupts ECM structure with GAGs loss.	Boccafoschi *et al.* (2017) Crapo *et al.* (2011)
Non-ionic detergent: zwitterionic detergent	Mixed properties of non-ionic and ionic detergents	Removes cells from tissue with minimum disruption More efficient in thin (*e.g.* lung) rather than thick tissues	Crapo *et al.* (2011)
Biological enzymes			
Nucleases	Hydrolyse ribonucleotide and deoxyribonucleotide chains	Remove DNA from tissues Enzyme residuals can provoke an immune response	Crapo *et al.* (2011) Keane *et al.* (2015) Vernengo *et al.* (2020)
Trypsin	Cleaves peptide bonds on the carboxyl side of arginine or lysine	Effective as a decellularisation adjuvant; however, long exposure can disrupt tissue structure and remove ECM proteins	Crapo *et al.* (2011) Keane *et al.* (2015)
Dispase	Cleaves fibronectin and collagen IV	Used for several tissues (*e.g.* porcine skin and corneas), however needs to be combined with additional agents to perform efficient decellularisation It can damage ECM structure and remove fibronectin and collagen IV with long exposure	Crapo *et al.* (2011) Keane *et al.* (2015)

In turn, synthetic materials (*e.g.* polyethylene glycol and polyvinyl alcohol) provide better biomechanical properties but have poor biocompatibility.

Decellularised ECM-based scaffolds, have received significant attention and started to be widely used in different tissues (cardiac valves, vascular grafts, cornea, *etc.*) (Mercuri *et al.*, 2011). Given their pro-regenerative potential, they are currently being commercialised for many different therapeutic applications and could be a promising alternative for IVD regeneration (Gilbert *et al.*, 2006). Recently, the combination of decellularised ECM and bioprinting has started to be explored for IVD and cartilage. Although this strategy is still at an early stage, the use of this novel technique may improve the design of IVD-based scaffolds (Vernengo *et al.*, 2020). The present review summarises recent advances in IVD decellularisation and discusses the need for novel therapeutic solutions for disc regeneration.

IVD decellularisation methods

Decellularisation is the technique used to remove host cells from tissues or organs (Londono and Badylak, 2015). Decellularised scaffolds should provide the same or similar microenvironment for seeded cells as native ECM (Xu *et al.*, 2014). However, most decellularisation methods affect ECM properties at least to some extent. Several decellularisation procedures and agents have been investigated to overcome matrix disruption and preserve its composition. Optimal decellularisation methods vary from different tissues or organs, depending on specific features: tissue size, thickness, shape, cell and matrix density (Vernengo *et al.*, 2020; White *et al.*, 2017). Following decellularisation, the efficiency of the process can be evaluated considering several aspects, including the presence of DNA and cell removal. As such, acellular scaffolds should have less than 50 ng dsDNA/mg ECM dry weight, less than 200 bp DNA fragment and no visible cell nuclei (Gilpin and Yang, 2017). Also, matrix proteins content (collagen, laminin, fibronectin, GAGs, growth factors) as well as mechanical properties should be analysed and maintained as close to native tissue as possible (Gilpin and Yang, 2017). In the end, even if cell residues such as DNA, RNA, cell membrane and debris remain, the decellularised scaffold needs to be biocompatible to avoid immune or inflammatory reactions (Crapo *et al.*, 2011). Cell removal should be maximised while minimising adverse effects on ECM composition, biological activity, integrity and biomechanical properties (Hoshiba *et al.*, 2010). An overview of the most commonly used decellularisation methods (physical, chemical and enzymatic) is shown in Fig. 2 (for further details see supplementary Table 1).

In recent years, decellularisation is being widely investigated as a novel strategy to develop functional substitutes for allogenic transplantation and resolve the major problems encountered in the clinic, such as donor shortage and immunosuppression (Tapias and Ott, 2014). Although several decellularised ECM scaffolds have already been approved by the FDA and are being commercialised for clinical applications (Alloderm®, SurgiSIS®, Restore®, ACell, Synergraft®) (Gilbert *et al.*, 2006), many challenges remain. Several attempts have been made to develop an ideal IVD decellularisation protocol through the combination of numerous enzymatic, physical and chemical methods (Table 2) but a satisfactory method is still to be defined. GAGs loss after decellularisation is one of the major issues to be solved. Large amounts of GAGs could improve IVD biomechanical properties after decellularisation, namely by increasing ECM compressive properties. Recent research is focusing on the development of more efficient and milder protocols that could preserve GAGs in IVD-based scaffolds (Vernengo *et al.*, 2020).

NP tissue decellularisation

Simionescu's group was the first to establish a decellularisation protocol for the IVD (Mercuri *et al.*, 2011). They were able to create a porcine decellularised NP-based scaffold by using a combination of Triton X-100 and deoxycholic acid detergents, ultrasonication and nucleases (DNAse and RNAse). Although the protocol was efficient in removing cells from the NP, it also affected tissue ultrastructure as well as ECM composition, leading to a 49 % GAGs loss. Decellularised scaffolds contained nearly twice as much collagen as fresh tissues (decellularised NPs: 75.24 µg/mg; fresh NPs: 36.20 µg/mg). This apparent increase corresponds to a decrease in other tissue components (Mercuri *et al.*, 2011). After optimising decellularisation conditions, they showed higher Col2 expression in porcine explants seeded with human ADSCs, when cultured in a differentiation medium as compared to normal DMEM at days 7 and 14 (9-fold *vs.* 2-fold increase, respectively). Expression of *PAX-1*, *SOX9*, *COL3* and *TIMP-1* was also upregulated. Additionally, GAG content of seeded scaffolds cultured in differentiation medium was significantly higher than for non-seeded ones, after 7 d (non-seeded NPs: 18.3 µg/mg; repopulated NPs: 34.74 µg/mg) and 14 d (non-seeded NPs: 20.6 µg/mg; repopulated NPs: 46.28 µg/mg) (Mercuri *et al.*, 2013). Recently, the same group performed a screening of different conditions to decellularise bovine NPs. The authors increased treatment time as well as amount of detergents and concentration of DNAse used. They found that 1.2 % of Triton X-100 treatment for 72 h combined with ultrasonication was the optimal procedure for bovine samples. An ethanol wash prior to decellularisation was used to guarantee total detergent absorption. This protocol removed 93 % of DNA, while retaining around 30 % of GAGs and high collagen levels (decellularised NP: 13.87 HYP/mg sample dry weight; native NPs: 8.63 µg HYP/mg

Table 2. Summary of the different strategies used for IVD decellularisation and key outcomes reported for the optimal procedures. *Treatment time for detergent solutions.

	Physical			Chemical						Enzymatic		Others	Results	Key outcomes		Reference
Animal	Sonication	Freeze-thaw	Treatment time*	EDTA	Triton X-100	Triton X-200	SDC	NaN3	SDS	Dnase	Rnase			ECM retention	DNA removal	
NP tissue decellularisedlularisation																
Porcine	-	-	19 h, 39 h, 72 h	0.10 %	0.15 %	-	0.25 %	0.02 %	-	720 mU/mL	720 mU/mL	EtOH	-	GAGs: 49 % loss Collagen: 75.24 µg/mg (decellularised) and 36.20 µg/mg (native)	98.44 %	Mercuri *et al.* (2011)
					0.10 %		0.15 %									
					0.05 %		0.05 %									
					0.60 %		1.00 %									
	3×; 10 min		72 h		0.15 %		0.25 %									
					0.60 %		1.00 %						Optimal			
Porcine	3×; 10 min	-	72 h	0.10 %	0.60 %	-	1.00 %	0.02 %	-	720 mU/mL	720 mU/mL	Peracetic acid (0.1 %)	Optimal	Not determined		Mercuri *et al.* (2013)
Bovine	6×	-	72 h	0.20 %	0.64 %	-	1.00 %	0.02 %	-	720 mU/mL	720 mU/mL	-	-	GAGs: 69.68 % loss Collagen: 13.87 µg/mg (decellularised) and 8.63 µg/mg (native)	92.77 %	Fernandez *et al.* (2016)
	12×				0.6 %											
	6×		6 d													
	12×															
	6×		72 h							1,440 mU/mL	1,440 mU/mL					
	3×			0.40 %	1.20 %		2.00 %	0.04 %		720 mU/mL	720 mU/mL					
				0.20 %	0.60 %		1.00 %	0.02 %				EtOH (30 min)				
												EtOH 60 min				
												EtOH 45 min				
							-					-				
				2.21 mmol/L	-		-	-	0.40 %							
				0.40 %	1.20 %		-	0.02 %	-				Optimal			

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	Physical			Chemical						Enzymatic		Others	Results	Key outcomes		Reference
Animal	**Sonication**	**Freeze-thaw**	**Treatment time***	**EDTA**	**Triton X-100**	**Triton X-200**	**SDC**	**NaN3**	**SDS**	**Dnase**	**Rnase**			**ECM retention**	**DNA removal**	
NP cell-derived matrix decellularisedlularisation																
Rabbit	-	-	1 h	-	2 %	-	-	-	-	-	-	-	-	GAGs: ~ 0.1 µg (decellularised) Collagen: ~ 10 µg (decellularised)	~ 100 %	Yuan *et al.* (2013)
			-		3 %											
			-									SDC 0.5 %				
			1 h									SDC 1 %				
			-									SDC 2 %				
			1 h 30 min			0.14 %	-					50 mmol/L SB-10 + 0.6 mmol/L SB-16	Optimal			
			1 h 30 min									125 mmol/L SB-10 + 0.6 mmol/L SB-16	-			
Whole disc decellularisedlularisation																
Bovine	-	6× (1 h 37 °C; 1 h liquid nitrogen)	6 h	1×	-	-	-	-	0.10 %	-	-	-	-	GAGs: 557.46 µg/mg decellularised NPs; 574.74 µg/mg fresh NPs; 233.42 µg/mg decellularised AFs; 277.54 µg/mg native AFs	71 % of NP cells and 76 % of AF cells removal	Chan *et al.* (2013)
			24 h													
			48 h										Optimal	Collagen: qualitative		
Bovine	3×; 10 min	3× (2 h − 80 °C; 22 h RT)	72 h	0.20 %	1.20 %	-	-	0.02 %	-	720 mU/mL	720 mU/mL	Distilled water, EtOH (30 min), peracetic acid (0.1 %)	Optimal	GAGs: AF: 56.84 % loss NP: 45 % loss Collagen: qualitative	AF: 73 % NP: 84 %	Hensley *et al.* (2018)

Table 2. Summary of the different strategies used for IVD decellularisation and key outcomes reported for the optimal procedures. *Treatment time for detergent solutions.

	Physical			Chemical						Enzymatic		Others	Results	Key outcomes		Reference
Animal	Sonication	Freeze-thaw	Treatment time*	EDTA	Triton X-100	Triton X-200	SDC	NaN3	SDS	Dnase	Rnase			ECM retention	DNA removal	
Human IVD decellularisedlularisation																
Human	-	6× (4 h 20 °C; 30 min 37 °C)	72 h	0.04 %	3 %	-	-	-	2 %	-	-	Trypsin (0.2-5 %), EtOH (20 min)	Optimal	GAGs: ~ 500 μg/mL (decellularised) and ~ 1,000 μg/mL (native) Collagen: no significant differences	No significant decrease	Huang *et al.* (2016)
AF decellularisedlularisation																
Porcine	-	5× (22 h − 80 °C; 2 h 37 °C)	48 h	-	-	-	-	-	0.10 %	50 U/mL	1 U/mL	APR (10 KIU/mL)	Optimal	GAGs: 15.9 % loss Collagen: 96.72 μg/mg (decellularised) and 98.65 μg/mg (native)	86 %	Wu *et al.* (2014)
Porcine	-	-	72 h	0.10 %	3 %	-	-	-	-	0.2 mg/mL	0.2 μg/mL	APR (10 KIU/mL)	Optimal	GAGs: ~ 40 μg/mg (decellularised) and ~ 50 μg/mg (native)	No presence (qualitative analysis)	Xu *et al.* (2014)
		3× (3 h − 80 °C; 4 h RT)			-				0.50 %				-			
		-		0.20 %					-		20 μg/mL	Trypsin 0.5 %		Collagen: not significant differences		
Porcine	-	22 h − 80 °C; 2 h 37 °C	24 h	0.10 %	-	-	-	-	0.10 %	50 U/mL	1 U/mL	APR (10 KIU/mL)	-	GAGs: 114.77 μg/mg (decellularised) and 121.63 μg/mg (native) Collagen: 151.73 μg/mg (decellularised) and 153.63 μg/mg (native)	85.7 %	Wu *et al.* (2017)
		22 h liquid nitrogen; 2 h 37 °C			1 %				-							
			48 h		-				0.10 %				Optimal			
			72 h										-			
Rabbit	-	-	24 h	-	1 %	-	-	-	-	50 U/mL	1 U/mL	Trypsin (0.2-5 %)	Optimal	Not determined		Liu *et al.* (2019)

sample dry weight). Mechanical features resembled those of native NPs. It is still necessary to discover whether a similar protocol without sonication would be less disruptive to the ECM structure (Fernandez *et al.*, 2016). Indeed, ECM architecture of bovine NP was disrupted following decellularisation, when ultrasonication was applied (unpublished data).

NP-cell-derived matrix decellularisation

Yuan *et al.* (2013) focused on the decellularisation of *in-vitro*-derived rabbit NP matrices (deposited onto collagen microsphere templates), which were used to instruct human MSC differentiation towards NP-like lineages. A combinatorial protocol of Triton X-200 with SB-10 and SB-16 avoided fast transitions of detergents to water, causing less structural damage than using them separately. Zwitterionic and anionic detergents removed most cell components while retaining around 0.1 μg/100 μL of solubilised GAGs from 30 microspheres and 10 μg of collagen per 100 μL of sample digest, by generating smaller surfactant micelles that were able to easily penetrate into the tissue. Higher detergent concentrations might have had reduced decellularisation effectiveness since emulsifying micelles were too large to penetrate into the collagen microspheres (Yuan *et al.*, 2013). The obtained scaffolds were further characterised by proteomics, demonstrating partial preservation of the ECM microenvironment (Yuan *et al.*, 2018).

AF decellularisation

Concerning the development of acellular AFs, Huang's lab used a combination of repeated freeze-thawing followed by incubation in 0.1 % SDS for 48 h to decellularise porcine tissue (Wu *et al.*, 2014). A reduction of 86 % in DNA content and 16 % in GAGs was achieved in decellularised AF-based scaffolds when compared to fresh ones. No significant differences were seen in collagen content (fresh AF: 98.65 μg/mg; decellularised AF: 96.72 μg/mg). Although stiffness and Young's modulus have exhibited a tendency to decrease after decellularisation, these differences were not statistically significant (Wu *et al.*, 2014). In contrast, Xu *et al.* (2014) compared different AF decellularisation protocols and demonstrated that 3 % Triton X-100 treatment for 72 h was better than a freeze-thawing combination with a 0.5 % SDS treatment or even a trypsin-based enzymatic method. In this study, the Triton X-100-based protocol enabled the maintenance of biomechanical properties without affecting tissue structure and, also, retained the highest GAG content (~ 40 μg GAG/mg dry weight) (Xu *et al.*, 2014). 3 years later, Huang's group validated the previous results but reducing the treatment time to 24 h, given that extension of the decellularisation time had a greater effect on collagen (fresh AF: 120.94 μg/mg; decellularised AF for 24 h: 109.72 μg/mg; decellularised AF for 48 h: 94.18 μg/mg; decellularised AF for 72 h: 89.80 μg/mg) and GAGs (fresh AF: 96.09 μg/mg; decellularised AF for 24 h: 82.77 μg/mg; decellularised AF for 48 h: 47.49 μg/mg; decellularised AF for 72 h: 14.44 μg/mg) content (Wu *et al.*, 2017). Finally, Liu *et al*. (2019) created a hydrogel by combining rabbit decellularised AF, chitosan and genipin as crosslinker. The tissue was decellularised using a similar protocol to that of Huang's group after tissue digestion with trypsin. bFGF incorporation promoted expression of ECM genes and corresponding proteins in the supernatants of seeded rabbit AF stem cells (Liu *et al.*, 2019).

Whole disc decellularisation

Chan *et al.* (2013) reported, for the first time, decellularisation of an entire bovine IVD (including the endplates). They tried different SDS washing temperatures and freeze-thawing cycles to preserve GAG and collagen content. With 6 freeze-thaw cycles followed by 48 h washing with SDS 0.1 % at 4 °C, the authors succeeded in removing over 70 % of the cells in both the AF and NP. The protocol was improved by increasing the number of freeze-thaw cycles, which completely abolished metabolic activity of the remaining cells. After decellularisation, both NP and AF maintained a GAG content similar to native tissue (fresh NP: 574.74 μg/mg; decellularised NP: 557.46 μg/mg; fresh AF: 277.54 μg/mg; decellularised AF: 233.42 μg/mg). Mechanical properties were also preserved. This enabled NP cell penetration after 7 d in culture (Chan *et al.*, 2013).

5 years later, Mercuri's group also tried to decellularise a complete disc xenograft. To obtain large (C1-C4) acellular bovine scaffolds, they used a longer Triton-based protocol [adapted from the one for NP (Fernandez *et al.*, 2016)]. However, unlike Chan *et al.* (2013), they did not include the cartilaginous endplates. No significant differences were observed in swelling pressure or in toe-region modulus between decellularised and native tissues. However, decellularised IVDs showed a decrease of linear-region moduli, peak stress and equilibrium moduli (Hensley *et al.*, 2018).

Human IVD decellularisation

In 2016, Schulze-Tanzil's lab was able to decellularise human IVDs from elder individuals undergoing spinal fusion or disc replacement. They adopted a protocol based on the combination of freeze-thawing, trypsin digestion and chemical detergents (2 % SDS and 3 % Triton X-100). Although decellularised IVDs contained almost half the GAGs content, as compared to native tissues, matrix architecture was maintained within the decellularised IVD cylindrical punches. Although cell removal was gauged by lack of nuclear and H&E staining, no significant differences were observed in total DNA content by the CyQuant Assay. In addition, despite the larger numbers of human IVD cells found in repopulated scaffolds, only differentiated MSCs were capable of increasing collagen and GAG content (Huang *et al.*, 2016).

Injectable strategies for decellularised matrix administration

In recent years, the use of ECM-based scaffolds has advanced with the development of injectable and biocompatible hydrogels (Hussey *et al.*, 2018).

Hydrogels are defined as highly hydrated polymer materials that are able to preserve their structural integrity by physical and chemical crosslinks between chains (Saldin *et al.*, 2017). The development of hydrogels from decellularised tissue is guided by the presence of biochemical factors and proteins of decellularised tissue through a collagen-based self-assembly process (Saldin *et al.*, 2017). Injectable ECM-based hydrogels can be formed mainly using two different methods. The first approach consists of grinding or milling the ECM into a fine powder, followed by resuspension in a solvent prior to injection. The second method consist of digesting enzymatically the ECM in an acidic solution and subsequently neutralising the pH and salt concentration to mimic *in vivo* physiological conditions. After digestion, the ECM can form a hydrogel by thermal crosslinking (Spang and Christman, 2018). Pepsin is the most used enzyme for tissue solubilisation, since it digests most protein structures (Hulmes, 2008) and hydrolyses collagen (León-López *et al.*, 2019). However, dispase can be an alternative for soft tissues (Saldin *et al.*, 2017).

In the disc field, hydrogels provide biochemical and biological signals to drive NP repair and regeneration and constitute a promising cell delivery system for minimally invasive strategies to treat IVD degeneration.

Illien-Junger *et al.* (2016) were able to develop decellularised injectable bovine NP fragments by using a protocol based on SDC. Prior to treatment, all samples went through a process of freeze-thawing, lyophilisation and grinding to increase surface area, facilitating fragment suspension. To test its injectability, the hydrated ECM suspension was transferred into a dual-barrel syringe and injected through a 25-G needle into an injured IVD. Apart from the optimal protocol using 2 % deoxycholate and DNAse, other treatments tested included additional decellularisation steps with 2 % SDS and 0.1 % Triton X-100. These alternatives produced looser scaffolds with thicker fibres and resulted in increased DNA levels with minimal GAG and collagen content. Interestingly, several cell-seeded constructs were immersed in low-melting-point agarose to create a protective shell that avoided swelling and dissociation. No cytotoxicity was observed, neither with human NP cells nor MSCs, after 21 d in culture (Illien-Junger *et al.*, 2016).

Lin *et al.* (2016) were also able to develop ECM microparticles from decellularised rabbit IVD by grinding the tissues. Following homogenisation, the obtained microparticles were passed through a sieve and their size was confined to smaller than 200 μm. Acellular IVD derived-microparticles injected using a 27-G needle prevented disc degeneration in a rabbit model, by increasing water level and disc height as well as ECM integrity and content (Lin *et al.*, 2016).

Lin and co-workers optimised porcine NP decellularisation using an SDS-based method that could remove up to 95.1 % of DNA and still maintain tissue microstructure and ECM components, particularly collagen (decellularised NP: 174.8 μg/g; native NP: 90.3 μg/g). However, GAGs decreased after decellularisation (decellularised NP: 19.33 μg/mg; native NP: 22.84 μg/mg). Moreover, mass spectrometry revealed the presence of important signalling molecules (*e.g.* lactadherin, metallopeptidase inhibitor 1 and alpha-1-antitrypsin) in NP-ECM scaffolds, which are involved in several cell activities. Particularly, TGF-β1, a protein associated with NP cells differentiation, was also detected. After repopulation with MSCs, NP-related genes (*COL2A1*, *ACAN* and *SOX9*) were upregulated in the NP-ECM scaffolds when compared to the controls as well as *TGF-βR2* at an early culture stage (3 d). Finally, IF showed higher synthesis of NP-cell-related proteins (ACAN and SOX9) in the repopulated scaffolds. These results suggested that NP-ECM scaffolds were able to induce MSC differentiation towards NP-like cells through the activation of TFG-β1 signalling pathway (Xu *et al.*, 2019). After reseeding, decellularised NPs were cut into small pieces to allow the passage through a 25-G needle. Following resuspension, ECM fragments were injected into a rabbit model of disc degeneration. Following 4 and 8 weeks of injection, NP structure and IVD disc height were preserved, when compared to a degenerated IVD group. Although proteoglycans were partially lost, as shown by a reduction of safranin O staining, the typical ECM network structure was still maintained at 8 weeks upon injection. Overall, reseeded scaffolds were able to delay disc degeneration *in vivo* (Xu *et al.*, 2019).

Wachs *et al.* (2017) developed, for the first time, a NP-based hydrogel from porcine tissue. The protocol was based on the combination of SB-10, Triton X-200 and SB-16 detergents and similar to that used by Yuan *et al.* (2013) for *in-vitro*-derived matrices. Instead of injecting a resuspension of lyophilised particles as in Illien-Junger *et al.* (2016), dried scaffolds were digested in an acidic solution and then neutralised with sodium hydroxide. The newly formed hydrogel retained native tissue architecture and was used to culture human NP cells that were able to acquire an elongated morphology and increased their GAG content over time, specifically from around 100 ng/mg on day 7 to 250 ng/mg on day 21 (Wachs *et al.*, 2017).

Yu and colleagues created an injectable and thermosensitive decellularised NP-based hydrogel from bovine tissue, suitable for minimally invasive applications. Following decellularisation with a combination of freeze-drying and SDS 1 % treatments, tissues were lyophilised, ground to a powder and digested in an acidic solution, using a similar method to that of Wachs *et al*. (2017). Afterwards, digested NPs were turned into hydrogels at 37 °C. The hydrogels were not cytotoxic and were well tolerated (Yu *et al.*, 2020).

Zhou *et al.* (2018) decellularised porcine NPs using Wachs' protocol (Wachs *et al.*, 2017). As Illien-Junger *et al.* (2016) and Lin *et al.* (2016) had done earlier, they fragmented the decellularised samples. Since decellularisation treatments can affect ECM ultrastructure, with a decrease in protein content, particularly GAGs, Zhou and colleagues decided to supplement the acellular scaffold with chondroitin sulphate (20 mg/mL) to obtain a GAG/HYP ratio similar to that of the native tissue. Human or rabbit ADSCs were encapsulated and this gel crosslinked using 0.02 % genipin (higher genipin concentrations were cytotoxic). The hydrogel was able to induce NP-like differentiation *in vitro* and partially recover the degenerated NP *in vivo*, in a rabbit IVD degeneration model (Zhou *et al.*, 2018). Finally, Peng and colleagues developed an injectable genipin-crosslinked decellularised AF hydrogel (g-DAF-G) from bovine tissue that was able to direct human BM-MSCs differentiation towards an AF-cell-like phenotype *in vitro*. AF decellularisation was achieved by using a combination of freeze-drying cycles, Triton X-100 (2 %) and SDS (1 %) detergents and sterile water. After lyophilisation, decellularised AF samples were digested in 0.01 mol/L HCl containing 1.5 mg/mL pepsin under moderate agitation for 48 h to create a decellularised AF-based hydrogel (DAF-Gs). Finally, hydrogels were crosslinked using genipin, forming g-DAF-G. The storage modulus (G') of g-DAF-G was superior to that of DAF-G and increased with higher concentration of genipin (DAF-G: 465.51 Pa; 0.01 % genipin: 2.57 MPa; 0.02 % genipin: 3.29 MP; 0.04 %genipin: 4.34 MPa). Therefore, g-DAF-G showed improved biomechanical properties when compared to DAF-G (Peng *et al.*, 2020).

Apart from being used for hydrogel formation, solubilised decellularised matrices can also be used as coating of 2D substrates by adsorbing ECM proteins onto a tissue culture surface, as has been performed for other tissues (Agmon and Christman, 2016; DeQuach *et al.*, 2010; Lee *et al.*, 2014). Despite that they no longer retain native tissue architecture, coated plates will provide biochemical signals that can be sensed by the seeded cells, which will change their behaviour accordingly.

Sterilisation of decellularised IVD matrices

Considering that implant-associated infections are one of the major issues halting the widespread use of biomaterials in the clinics (Campoccia *et al.*, 2006; Li and Webster, 2018), the optimisation of the sterilisation methods for ECM-based scaffolds is crucial before their clinical application.

Acidic solutions or solvents, heat treatment, ethylene oxide exposure, iodine and irradiation (gamma or electron beam), represent some of the methods available for scaffold sterilisation. Freeze-drying and supercritical carbon dioxide are also currently being tested (Dai *et al.*, 2016). A combination of different techniques can be required to achieve complete removal of viral or bacterial contaminants from biomaterials. Sterilisation conditions should be tightly controlled and post-sterilisation effects evaluated individually. Problems that might arise from matrices' sterilisation include insufficient cell penetration upon repopulation, incomplete microorganism inactivation, cell toxicity and loss of integrity (Crapo *et al.*, 2011; Dai *et al.*, 2016). Therefore, sterilisation can compromise efficiency of biomaterials. Several approved and standardised methods of sterilisation (*e.g.* heat, pressure, irradiation, chemical agents, supercritical carbon dioxide and ionised gas plasma) can induce degradation of ECM components, thus modifying its physiological and biomechanical properties (Fidalgo *et al.*, 2018).

One option that has been used for IVD scaffold culture in sterile conditions is the addition of antibiotics or antifungal solutions such as sodium azide or a combination of penicillin and streptomycin (Lin *et al.*, 2016; Mercuri *et al.*, 2013; Yuan *et al.*, 2013). Peracetic acid (0.1 % or 0.01 %) might also be used in combination with an antibiotic infusion (Mercuri *et al.*, 2013; Zhou *et al.*, 2018). 70 % ethanol is another alternative (Huang *et al.*, 2016; McGuire *et al.*, 2017; Mercuri *et al.*, 2011) although the final objective of at least some of the authors does not seem to be sterilisation but only tissue dehydration (Fernandez *et al.*, 2016; Huang *et al.*, 2016). In addition, it can denature proteins, dehydrate ECM and affect cell-ECM interactions (Poornejad *et al.*, 2015).

Xu *et al.* (2019) used gamma irradiation to sterilise porcine NP scaffolds. Nevertheless, at least for porcine renal decellularised matrices, Poornejad *et al.* (2015) showed that 0.2 % peracetic acid in 1 mol/L NaCl solution presented the best results in terms of GAG content and ECM structure preservation, rather than gamma-irradiation, ethanol alone or even peracetic acid in 4 % of alcohol. In this study, gamma-irradiation was the most damaging sterilisation method since it caused modification of tissue microstructure and considerable reduction of ECM components (collagen and GAGs) as well as increased tissue porosity and altered mechanical properties. Significant decrease of cell adhesion and proliferation after scaffold repopulation were also observed (Poornejad *et al.*, 2015). Badylak's group demonstrated that a high dose of gamma-irradiation (30 kGy) prevented hydrogel formation from ECM of several tissues (porcine intestinal submucosa, liver and urinary bladder, bovine bone), when compared to the supercritical CO_2 method (White *et al.*, 2018). Finally, Peng *et al.* (2020) adopted a combination of

Table 3. Current procedures for IVD recellularisation.

IVD tissue origin	Cell source	Seeding method	Assays									Key outcomes	Reference
			Cell number	Cell viability	DNA content	GAG content	Collagen content	Water content	Tissue organisation	Gene expression	Others		
Bovine (NP)	Bovine NP cells	Seeding onto tissue surface	DAPI staining	Calcein/ PKH26 staining	-	-	-	-	-	-	-	Day 7: cells viable and migration into the scaffolds with limited penetration.	Chan *et al.* (2013)
	Human BM-MSCs, human NP cells	Cell-seeded constructs	-	Calcein-AM/ ethidium homodimer-1 assay	-	Alcian blue and toluidine blue	Picrosirius red staining	-	-	-	-	Day 21: cells viable. GAGs: decrease in scaffolds repopulated with hNP cells.	Illien-Junger *et al.* (2016)
	Human AMSCs	Drop-wise addition onto the tissue surface or cell injection	-	Calcein-AM/ ethidium homodimer-3 assay	-	-	-	-	-	-	-	Cells viable for up to 14 d. No cell migration and penetration.	Fernandez *et al.* (2016)
	Rat ADSCs	Seeding onto hydrogel	-	Live/dead assay	-	-	-	-	-	*Acan*, *Sox9*, *Col2*, *Col1*	Cell proliferation (CCK8)	Day 5: cells viable. Higher expression of *Col2*, *Acan* and *Sox9*. No differences in *Col1*.	Yu *et al.* (2020)
Rabbit (NP)	Rabbit BM-MSCs, human BM-MSCs	Dropping onto microspheres	Trypan blue assay	Calcein-AM/ ethidium homodimer-1 assay	Hoechst	DMMB assay, alcian blue staining	HYP assay	-	H&E	*Acan*, *Sox9*, *Col1*, *Col2*, *Gpc3*, *Krt19*, *Ca12*, *Pax1*, *Foxf1*	IHC (COLI, COLII, K19, TGFβ, TGFβR1)	Cells viable for 18 d in culture with migration into the microspheres. GAGs: more in scaffolds re-seeded with hMSCs. Increased *Col2* (> 10-fold), *Gpc3* (~ 6-fold) and *Foxf1* (~ 8-fold) expression in re-seeded microspheres with hMSCs.	Yuan *et al.* (2013)
	Human dermal fibroblasts	Dropping onto microspheres	DAPI staining	Live/dead assay	-	Alcian blue staining	-	-	H&E	*Col1*, *Col2*, *Acan*, *Sox9*, *Krt19*, *Gpc3*, *Ca12*, *Foxf1*, *Pax1*	IHC (COLII, K19)	Day 18: cells viable with migration into the microspheres. Decreased *Col1* and increased *Col2* expression when cells were culture in NPC-derived matrix.	Yuan *et al.* (2018)

Table 3. Current procedures for IVD recellularisation.

IVD tissue origin	Cell source	Seeding method	Assays									Key outcomes	Reference
			Cell number	Cell viability	DNA content	GAG content	Collagen content	Water content	Tissue organisation	Gene expression	Others		
Porcine (NP)	Human ADSCs	Drop-wise	MTS proliferation assay	Calcein-AM/ ethidium homodimer-1 assay kit	-	Alcian blue staining	-	-	-	-	-	Day 7: cells viable and increased cell migration and proliferation.	Mercuri *et al.* (2011)
	Human ADSCs	Seeding onto hydrogel	-	Live/dead assay	PicoGreen	DMMB assay, alcian blue staining	HYP assay	Wet-dry weight	H&E, Movat's pentachrome staining	*Acan*, *Sox9*, *Col2*, *Col3*, *Timp1*, *Pax1*, *Ibsp*	MMPs and TIMPs antibody arrays, dynamic mechanical analysis	Day 14: cells viable. Mixed cell phenotype between NP cell and chondrocyte. Higher *Col2* expression at day 7 and 14 (9-fold *vs.* 2-fold increase, respectively). GAGs: more synthesis at day 14 (non-seeded NPs: 20.6 μg/mg; repopulated NPs: 46.28 μg/mg).	Mercuri *et al.* (2013)
Porcine (NP)	Human NP cells	Cells mixed with NP gel	-	Calcein-AM/ ethidium homodimer-1 assay	-	Blyscan assay	Soluble and insoluble collagen assays	-	-	-	-	Day 21: cells viable with a NP-like morphology and increased GAG content.	Wachs *et al.* (2017)
	Human and rabbit ADSCs	Cells mixed with NP gel	DAPI staining	-	Hoechst	Alcian blue staining, blyscan assay	Picrosirius red staining, HYP assay	Wet-dry weight	-	*Acan*, *Sox9*, *Col1*, *Col2*, *Krt19*, *Pax1*, *Gdf10*	IHC (COLII), metabolic activity (CCK8), cytotoxicity (lactate dehydrogenase assay)	Day 14: higher cell proliferation and gene expression level (*Acan*, *Col2*, *Sox9*, *Krt19* and *Pax1*). Increase of GAGs and decrease of collagen.	Zhou *et al.* (2018)
	Human MSCs	Dropping onto scaffold	DAPI staining	-	DNeasy	Alcian blue staining, blyscan assay	HYP assay	Wet-dry weight	H&E	*Col1*, *Col2*, *Acan*, *Sox9*, *Ncam1*, *Mmp13*	IHC (COLI, COLII), metabolic activity (CCK8) IF (ACAN, SOX9)	Day 14: higher cell proliferation. Upregulation of *Col2*, *Acan*, *Col1a1*, *Ncam-1* and *Sox9* and downregulation of *Mmp13*. Day 14: higher ACAN and SOX9 production by reseeded cells.	Xu *et al.* (2019)

Table 3. Current procedures for IVD recellularisation. IF: immunofluorescence ; IHC: immonohystochemistry.

IVD tissue origin	Cell source	Seeding method	Assays									Key outcomes	Reference
			Cell number	Cell viability	DNA content	GAG content	Collagen content	Water content	Tissue organisation	Gene expression	Others		
Rabbit (IVD)	Rabbit BM-MSCs, human IVD cells	Multiple seeding onto tissue surface	DAPI staining	Calcein-AM/ ethidium homodimer-1 assay	DNeasy	Alcian blue staining	HYP assay	Wet-dry weight	H&E	*Col1*, *Col2*, *Sox9*, *Gpc3*, *Foxf1*, *Acan*, *Ca12*, *Timp1*, *Timp2*, *Tgfβ*, *Tgf-βr*	Metabolic activity (CCK8), IHC (COLI, COLII, ACAN, CD8, MAC387)	Day 21: cells viable and cell migration into the scaffolds. Increased *Col2* (more than 12-fold increase), *Sox9* (14 times greater), *Foxf1* (118-fold increase), *Ca12* (5-fold increase), *Acan* and *Gpc3* gene expression.	Lin *et al.* (2016)
Human (IVD)	Human BM-MSCs, human IVD cells	Seeding onto tissue surface	-	Fluorescein diacetate/ ethidium bromide assay	CyQUANT	DMMB assay	HYP assay	-	-	-	-	Day 14: cells viable with limited migration. Higher DNA content in scaffolds seeded with hIVD cells. Increased GAG and collagen content in scaffolds seeded with hBM-MSCs.	Huang *et al.* (2016)
Bovine (AF)	Human BM-MSCs	Seeding onto hydrogel	-	Live/dead assay	-	-	-	-	-	*Col1A1*, *Col5A1*, *Ibsp*, *Fbln1*, *Tnmd*	Cell compatibility (CCK8), cytotoxicity (lactate dehydrogenase assay)	Day 21: cells viable and higher gene expression level of *Col1A1*, *Col5A1*, *Fbln1*, *Ibsp*, and *Tnmd*.	Peng *et al.* (2020)
Porcine (AF)	Rabbit AF cells	Drop-wise addition onto the tissue surface (with turnover)	-	Calcein-AM/ ethidium homodimer-1 assay	-	-	-	-	H&E	-	-	Day 7: cell infiltration into the mid-horizontal plane of scaffolds. No dead cells observed.	Xu *et al.* (2014)
Rabbit (AF)	Rabbit AF stem cells	Seeding onto hydrogel	DAPI staining	-	Hoechst	DMMB assay	COLI and COLII ELISA kit	-	-	*Col1*, *Col2*, *Acan*	Metabolic activity (CCK8)	Day 14: upregulation of Col1, Col2 and Acan. GAGs: higher release in the hydrogel media with bFGF (4.43 ng/µg) than in those without (2.28 ng/µg), as well as col1 (with bFGF: 4.68 ng/µg; without bFGF: 3.56 ng/µg), col2 (with bFGF: 11.95 ng/µg; without bFGF: 10.42 ng/µg) and acan (with bFGF: 1,022.23 pg/µg and without bFGF: 790.95 pg/µg).	Liu *et al.* (2019)

95 % alcohol fumigation and ultraviolet irradiation to sterilise lyophilised decellularised AF tissues.

All in all, it is crucial to choose an adequate sterilisation method to maximise the properties and *in vivo* performance of biomaterials (Destefani *et al.*, 2017).

Recellularisation of decellularised IVD scaffolds

After decellularisation, acellular scaffolds can be repopulated with specific cell sources to reconstitute a healthy ECM and enhance the regenerative process. Choosing the appropriate cell source for recellularisation is a complex issue that needs to be extensively studied.

Native IVD cells are widely used to recellularise IVD-based scaffolds, since they already present a chondrocyte-like phenotype and have shown positive outcomes (Chan *et al.*, 2013; Ganey *et al.*, 2003; Gruber *et al.*, 2002). However, other cell types and different repopulation methods have been widely investigated. As summarised in Table 3, successful recellularisation of IVD scaffolds [either injectable (Illien-Junger *et al.*, 2016; Lin *et al.*, 2016; Peng *et al.*, 2020; Wachs *et al.*, 2017; Xu *et al.*, 2019; Yu *et al.*, 2020; Zhou *et al.*, 2018) or not] has already been reported with bovine (Chan *et al.*, 2013) and human (Illien-Junger *et al.*, 2016; Wachs *et al.*, 2017) NP cells as well as with rabbit (Liu *et al.*, 2019) [either stem or not (Xu *et al.*, 2014)] and human (Huang *et al.*, 2016) AF cells.

At first glance, healthy human IVD cells seem to be the ideal cell source. Nevertheless, they represent only a small population in the disc and their isolation is a complex process due to ethical issues in using healthy young volunteers and a high risk of tissue disruption. Alternatively, IVD cells can be collected from patient's undergoing spinal surgeries. However, their degenerative phenotype can negatively impact the subsequent regenerative cascade in the context of a therapeutic approach. Nevertheless, IVD cells' behaviour will better mimic the response within a diseased microenvironment, being ideal for developing *in vitro* models of disc degeneration.

MSCs have started to be widely used because of their relatively ease of isolation and expansion, ability to differentiate into native disc-like cells, immunomodulatory properties and ability to produce their own ECM, inducing disc repair. MSCs are a more readily available option than IVD cells (Le Maitre *et al.*, 2009; Lin *et al.*, 2016; Wei *et al.*, 2009; Zhou *et al.*, 2018) and have started to be used in clinical trials to treat LBP (Kumar *et al.*, 2017; Noriega *et al.*, 2017). Moreover, MSCs have long-term self-renewal capability, are reservoirs of growth factors and cytokines and can contribute to the restoration of the disc matrix (Wang *et al.*, 2015; Watanabe *et al.*, 2010; Yang *et al.*, 2008; Zhang *et al.*, 2005). MSCs used for IVD regeneration studies are mainly bone-marrow- or adipose-tissue-derived as they can be obtained through minimally invasive procedures (Huang *et al.*, 2016; Illien-Junger *et al.*, 2016; Lin *et al.*, 2016; Mercuri *et al.*, 2011; Mercuri *et al.*, 2013; Peng *et al.*, 2020; Yu *et al.*, 2020; Yuan *et al.*, 2013; Zhou *et al.*, 2018). However, stem cells from other sources such as amniotic fluid (Fernandez *et al.*, 2016) or synovial tissue (Pei *et al.*, 2012; Shoukry *et al.*, 2013) also exhibit promising results. As previously discussed, it is important to choose the appropriate cell type for recellularisation but the use of autologous, allogenic or xenogenic cells must be carefully considered to reduce the chance of having scaffold rejection by the host.

Several methods have been investigated to achieve successful recellularisation of ECM-based scaffolds (Fig. 3). Seeding cell suspension over the biomaterial by simple dropping is the most used approach. However, cells tend to form a monolayer on the surface of dense scaffolds, rendering problematic their penetration and migration (Chan *et al.*, 2013; Fernandez *et al.*, 2016; Huang *et al.*, 2016). Also, particular decellularisation detergents, such as SDS, affect GAG content of the native tissue, which leads to decreased water retention and consequently reduced cell adhesion to the matrix (Gilbert *et al.*, 2006; Huang *et al.*, 2016). Several techniques can be adopted to improve cell penetration and migration into the scaffolds, such as: rotatory cultures (Huang *et al.*, 2016), scaffold turnover (Xu *et al.*, 2014), repetitive cell seedings with 1 h intervals (Lin *et al.*, 2016) and cell injection (Fernandez *et al.*, 2016). Finally, cell repopulation can be enhanced by pre-incubating acellular scaffolds with FBS or BSA. These solutions can diminish the cytotoxicity caused by the decellularisation reagents. Particularly, Mercuri *et al.* (2011) immersed the decellularised scaffold in culture medium with serum (50 %), 24 h prior to cell seeding and observed a relative 2.4-fold increase in cell number from ~ 2.5×10^4 cells on day 3 to ~ 6.1×10^4 cells on day 7 and cell migration into the scaffold after 7 d of culture. Nevertheless, with the same approach, Fernandez *et al.* (2016) did not observe cell migration within the scaffold. In another study, Schulze-Tanzil's group preconditioned the decellularised matrices in 5 % BSA for 24 h and FBS for additional 24 h. Although most of the seeded cells (either MSCs or IVD cells) survived, they only colonised the scaffold surface (Huang *et al.*, 2016). It is important to consider that these differences may also reflect different cell sources or decellularisation methods used, since each particular approach can have a different impact on the physicochemical properties of the ECM, affecting cell adhesion and migration. After choosing the recellularisation method, it is also mandatory to determine the cell seeding density, which may depend on scaffold volume, cell type, culture duration and purpose of the experiment.

After reseeding, the success of the recellularisation procedure should be estimated. In most studies, the authors evaluate cell number, proliferation, cell viability, DNA and water content, tissue organisation, GAG and collagen composition. This information is also reviewed in Table 2. Overall, recellularised scaffolds are a valuable tool that can be optimised and refined to develop innovative therapies for IVD degeneration. In the future, high-throughput proteomics or single-cell transcriptomics could help to maximise the understanding of all the dynamic biological processes and different cell populations involved in the process of IVD regeneration.

Controlling the immune response against decellularised IVD matrices

The main cause of implant failure is hyper immunoreactivity towards the graft or its degradation products. The most common antigens that trigger such an inflammatory response are DNA and Gal (Badylak and Gilbert, 2008; Cheng *et al.*, 2014). Their elimination can extend xenograft survival. Non-self-antigen (from transplants, bacteria or viruses) recognition initiates an immune response mediated by MHC class I and II (Boccafoschi *et al.*, 2017; Chen *et al.*, 2017; Warrington *et al.*, 2011).Therefore, controlling non-self acute and chronic immune response (through adjusting both pro- and anti-inflammatory cues) is crucial for a successful implantation (Boccafoschi *et al.*, 2017). Optimisation of the decellularisation process is key to avoid dampening the bioactivity of native ECM while minimising residual immunological agents. This prevents disease transmission, reduces inflammation and immune response towards the scaffold, decreasing rejection after implantation (Badylak *et al.*, 2011; Cheng *et al.*, 2014). Given that ECM proteins are among the most conserved proteins in evolution, with high levels of sequence homology, decellularisation should be enough for explants to be well tolerated (Hutter *et al.*, 2000; Moroni and Mirabella, 2014; Ozbek *et al.*, 2010; van der Rest and Garrone, 1991). In fact, due to being considered anti-immunogenic, decellularised matrices have been proposed not only for autografts (within the same individual) and allografts (from one individual to another of the same species with a different genotype) but also for xenografts (from another species) (Boccafoschi *et al.*, 2017). Although dense matrices hinder complete cell removal, most commercially available decellularised materials do contain DNA traces without compromising their clinical efficacy (Cheng *et al.*, 2014; Derwin *et al.*, 2006; Gilbert *et al.*, 2009; Zheng *et al.*, 2005), thus, demonstrating that the DNA remnants may exist below a threshold that triggers a harsh immune response (Badylak and Gilbert, 2008; Cheng *et al.*, 2014). Gal epitopes are usually found in most species but not in humans.

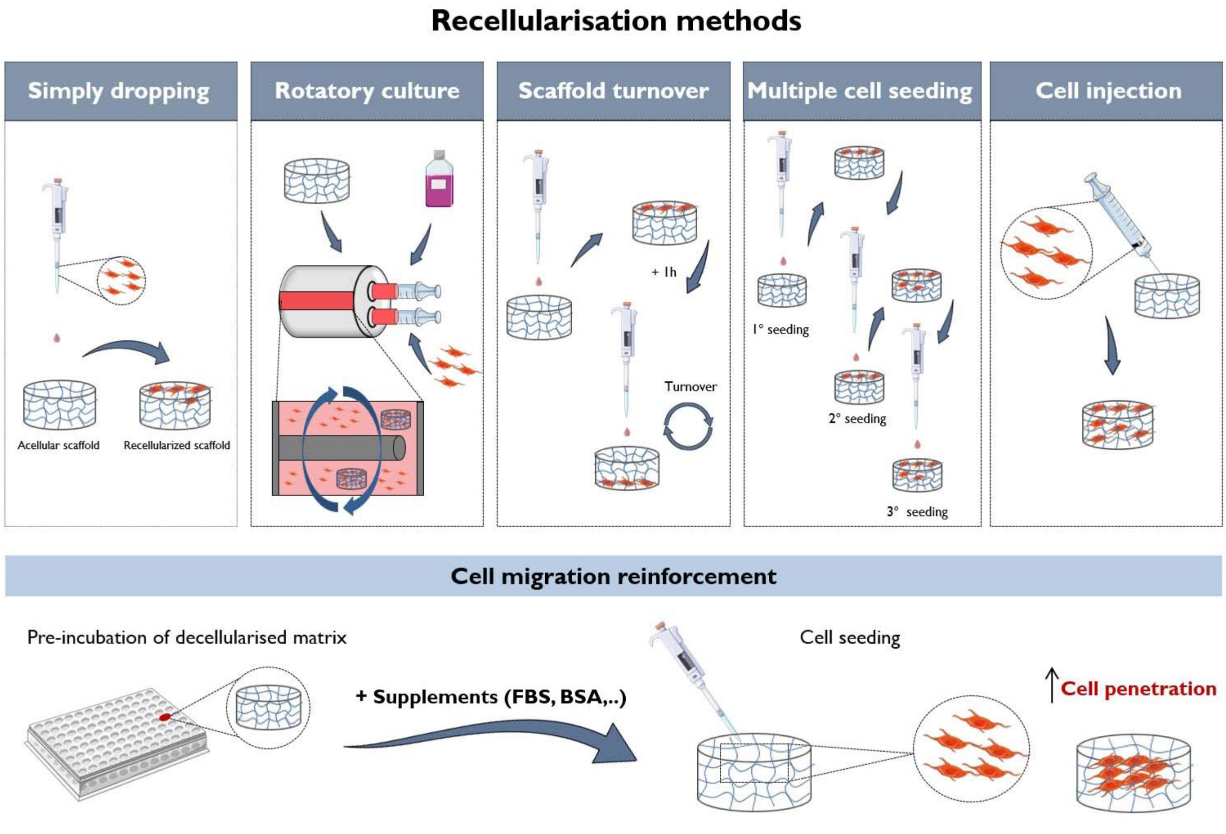

Fig. 3. Scaffold repopulation strategies. IVD scaffold repopulation, either with IVD cells or MSCs from different origins can be performed by drop-wise addition onto the surface of the decellularised scaffold. To improve recellularisation efficiency other strategies can be used such as mechanical agitation, scaffold turnover, multiple seedings or cell injection into the scaffold. To further promote cell penetration, decellularised matrices can be pre-incubated with protein-rich solutions such as FBS or BSA.

Because humans are constantly exposed to intestinal bacteria that carry Gal epitopes, they produce large amounts of anti-Gal antibodies (Badylak and Gilbert, 2008; Cheng *et al.*, 2014). In that context, porcine bioprosthetic heart valves have shown to induce a xenograft-specific immune response with high levels of cytotoxic IgM antibodies against a-Gal and have failed in some patients (Cheng *et al.*, 2014; Konakci *et al.*, 2005). Although organs from Gal-knockout pigs have been rejected due to other antigens (Chen *et al.*, 2005), graft treatment with a-galactosidase has been able to remove Gal epitopes, minimising an adverse host immune reaction (Cheng *et al.*, 2014; Stone *et al.*, 2007; Stone *et al.*, 1998). Research is still limited and further studies are needed to improve the safety and efficacy of decellularised material (Cheng *et al.*, 2014).

In the disc field, only two works describe a-Gal assessment after decellularisation and in both cases there seems to be a removal (Mercuri *et al.*, 2011) or at least a significant reduction of the a-Gal epitope in the decellularised scaffolds (native AFs: less than 10 ng/mL; decellularised AFs: less than 5 ng/mL) (Wu *et al.*, 2017). Even if a residual amount remains, it evokes minimal to no immune response *in vivo* (Lin *et al.*, 2016). Finally, it should be borne in mind that ECM fragments that result from degradation can also trigger inflammation (Molinos *et al.*, 2015). This issue has not been given due consideration in most reports. Mechanisms responsible for macrophage switch from an M1 to an M2 profile should also be further studied *in vivo* to promote tissue remodelling and consequently improve scaffold biocompatibility (Moroni and Mirabella, 2014).

In vivo behaviour of decellularised ECMs

Biocompatibility of ECM-based scaffolds cannot be addressed only by using *in vitro* tests since they lack the complex biology and physiology of a whole organism. For that, *in vivo* assessments are needed to evaluate host responses to the scaffolds (Aamodt and Grainger, 2016). Mercuri *et al.* (2011) evaluated biocompatibility of the porcine-derived material in a rat model (subdermal pockets). Most non-crosslinked NP decellularised scaffolds were degraded completely after 4 weeks whereas crosslinking delayed degradation. In all the cases an inflammatory response was observed. Since DNA was removed in their previous work (Mercuri *et al.*, 2011), the authors hypothesised that this reaction may be triggered by a delayed degradation of the crosslinked material and a possible interaction between GAGs, present in the scaffold, and pro-inflammatory cytokines (Mercuri *et al.*, 2013).

Lin *et al.* (2016) assessed immunological response to decellularised rabbit IVD-based scaffolds *in vivo* in a rabbit model, in comparison to native tissue grafts. Subcutaneous decellularised implants presented minimal signs of inflammation 1 month post-implantation, while native scaffolds presented high levels of cell infiltration, namely of neutrophils, blood vessel formation and additional signs of inflammation (Lin *et al.*, 2016). A similar effect was observed when the same model was used with decellularised porcine xenografts seeded with MSCs (Xu *et al.*, 2019).

Yu *et al.* (2020) evaluated the *in vivo* biocompatibility of a bovine NP hydrogel compared to a synthetic material (poly-ε-caprolactone) by subcutaneous implantation in a rat model. Following 2 and 4 weeks of NP hydrogel implantation, H&E staining showed a smaller neutrophil and giant cell number, evidencing mild inflammation and reduced foreign body reaction *in vivo* (Yu *et al.*, 2020). Also, the immunocompatibility of porcine decellularised AF scaffolds has been under studied. Using Wistar rats (box incision in rat tail), the authors have shown cell infiltration and tissue remodelling in the implanted animals, as evidenced by an increase in collagen and GAG content (Wu *et al.*, 2017). More recently, Peng and colleagues have explored the ability of bovine AF-derived hydrogels to repair AF defects *in vivo*. They injected AF pre-hydrogel solutions, crosslinked or not with genipin (g-DAF-G and DAF-G, respectively), that were able to form a hydrogel *in situ* and to fill in AF defects. Moreover, both hydrogels induced disc cell migration and ECM production, demonstrating their regenerative potential *in vivo* (Peng *et al.*, 2020).

Although most works did not observe cytotoxicity *in vitro* (except as a response to material crosslinking) (Borem *et al.*, 2017; Fernandez *et al.*, 2016), *in vivo* cell recruitment and repopulation is required for biointegration of the decellularised matrix and should be given due consideration.

Experiments using human tissue will be the ultimate frontier before clinical trials. Human *ex vivo* organ cultures are already underway to serve other purposes (Gawri *et al.*, 2011; Walter *et al.*, 2014) and should be used not only to test the effect of decellularised tissue grafts but also to assess the host response, at least to some extent.

Limitations/precautions of using decellularised matrices

Donor profile

The development of successful ECM-based scaffolds depends on a wide range of factors that need to be considered before their use in the clinics. The selection of tissue donor profile, including animal source and age, for instance, can have a dramatic impact on the regenerative process. Concerning IVD source, on one hand, the use of human degenerated tissues can negatively influence tissue repair, leading to implantation failure. On the other hand, human cadaveric IVDs, although supposedly healthy, are scarce and difficult to obtain due to ethical restrictions. Therefore, animal tissues can be a good alternative to overcome this issue. Baboons would be ideal, as they are large and conduct forces through their spine similarly to humans to which they are closely related (Lauerman *et al.*, 1992). However, they are also limited in number, raise ethical concerns and constitute a potential source of zoonoses (Mafuyai *et al.*, 2013). Porcine tissues are also good candidates and are already available on the market, as replacement heart valves and for wound management solutions (Tsuchiya *et al.*, 2014). Bovine discs are large, easily available, have a similar NP aspect ratio and an identical ECM composition (Alini *et al.*, 2008; Demers *et al.*, 2004; O'Connell *et al.*, 2007; Oshima *et al.*, 1993; Roberts *et al.*, 2008).

The donor age can also affect biomechanical properties and composition of ECM-based scaffolds (Cramer and Badylak, 2019). With ageing, native ECM undergoes several biochemical and structural changes, which can influence cell response and tissue remodelling when using biomaterials derived from these tissues (Cramer and Badylak, 2019).

Neonatal-derived scaffolds have an enhanced pro-regenerative potential, as already reported in the heart, abdominal wall muscle and kidney (Nakayama *et al.*, 2011; Sicari *et al.*, 2012; Silva *et al.*, 2016). In the disc field, pro-regenerative proteins (collagen type XII and XIV) are uniquely expressed in prenatal IVD microenvironments (Caldeira *et al.*, 2017). Moreover, foetal discs are also characterised by a different topography, when compared to young and adult tissues (Caldeira *et al.*, 2018). Other age-associated structural differences of bovine discs can be observed in Fig. 1.

Legislative issues

Tissue engineering derivatives do not fall into the classification of drugs, transplants nor artificial tissues. As reviewed elsewhere (Boccafoschi *et al.*, 2017), both the FDA and the European Commission proposed guidelines with a unified approach to regulate tissue-engineering products. Likewise, safety issues regarding xenotransplantation of cells and tissues should also be addressed. Characterisation of animal source, facilities and maintenance as well as of xenotransplantation products, selection of adequate preclinical models and recipient monitoring should be considered, as documented by the FDA and the European Union (Boccafoschi *et al.*, 2017).

Conclusions and future challenges

The present review summarised the latest advances in IVD decellularisation. Recently, decellularised ECM-based scaffolds have gained significant attention for tissue remodelling with a regenerative purpose, given the success in cell removal and maintenance of most ECM properties with biological implications. Significant progress has been achieved in the last decade due to several exhaustive studies using a panoply of methods either alone or in combination, a wide range of reagents, several cell types and distinct animal sources. But there is still room for improvement, for instance by reducing treatment time and using milder detergents. Although recent progress is encouraging, several aspects need to be considered before commercialisation and clinical application, such as the following.

- Absence of a standardised protocol for decellularisation and for evaluating its efficacy, which renders the comparison of methods difficult. The ideal protocol should be scalable and effective, independent of donor species, age or pathological condition, and a final scaffold sterilisation step must be contemplated.
- Lack of uniformity of the optimal cell type, cell seeding density and cell repopulation method required for effective recellularisation. Appropriate selection of cell source will certainly determine the success of the therapy *in vivo*. Moreover, given the dense nature of the disc, cell infiltration into decellularised disc matrices can be hindered. The use of dynamic conditions could improve cell migration towards the inner scaffolds or, in alternative, decellularised matrix-based hydrogels (with increased porosity) or powders incorporated in different gels could be used to increase uniform cell distribution.
- Donor age (of the animals selected for tissue decellularisation). Despite being an often-neglected aspect, IVD matrisome is profoundly affected by age (Caldeira *et al.*, 2017; 2018). Therefore, novel solutions using foetal tissues that mimic a healthy pre-natal landscape should be pursued for IVD regeneration (Fiordalisi *et al.*, 2020).
- Limited *in vivo* validation. To verify clinical potential, biocompatibility of decellularised IVD-based scaffolds should be pursued preferentially using chondrodystrophic dogs, given that small rodents maintain notochordal cells throughout adulthood and do not reproduce human-disc size nor loading (Daly *et al.*, 2016; Novais *et al.*, 2020).

In conclusion, decellularised ECM-based scaffolds have a great potential to be translated into clinical applications for IVD repair and regeneration. Still, many challenges need to be solved and clinical trials must be conducted before these scaffolds can be launched on the market. Natural biomaterials are already revolutionising the tissue engineering field and their use for IVD could bring a new hope for LBP treatments.

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Discussion with Reviewer

Reviewer: Often, sterilisation methods impact the macromolecular structure of a biomaterial and, therefore, its performance. Have different sterilisation methods of decellularised ECM been characterised in terms of effects on bioactivity, mechanical and degradation properties? Do the authors anticipate any specific challenges, in sterilisation methods, that will need to be overcome for clinical translation of this technology?

Authors: Sterilisation of IVD-based scaffolds remains an underexplored field. Although different techniques have been under study to effectively remove any tissue contaminants, as described in the section "Sterilisation of decellularised IVD matrices", the authors of the revised works have not exhaustively investigated the impact of these methods on scaffold bioactivity, degradation, composition or biomechanics. As described for other tissue-derived matrices, most of the methods used for scaffolds sterilisation are disruptive and can affect ECM structure and biomechanical properties, which are essential for the success of tissue regeneration. Nowadays, there is no ideal option for effective and minimally destructive sterilisation, however research is advancing with significant progress. The major challenge that needs to be overcome in IVD sterilisation is the development of a less destructive method but at the same time efficient enough to face clinical requests. Recently, supercritical carbon dioxide sterilisation has started to emerge as a promising strategy for terminal sterilisation, with no signs of negative effects regarding biomaterial composition (molecular weight, components content) and properties (biological, mechanical and physicochemical) (Ribeiro *et al.*, 2020, additional reference). In the future, this technology should be further explored for tissue sterilisation, including the IVD.

Additional Reference

Ribeiro N, Soares GC, Santos-Rosales V, Concheiro A, Alvarez-Lorenzo C, García-González CA, Oliveira AL (2020) A new era for sterilization based on supercritical CO2 technology. J Biomed Mater Res B Appl Biomater 108: 399-428.

Editor's note: The Guest Editor responsible for this paper was Zhen Li.