

Faculdade de Engenharia da Universidade do Porto



Biological Characterization of a Decellularized Nucleus Pulposus (NP)-Based Hydrogel

Inês Sofia Barreto de Sousa

Dissertation for the Master's Degree in Bioengineering

Molecular Bioengineering

Supervisor: Joana Caldeira

Co-Supervisor: Raquel Gonçalves

Prof Mário Barbosa

September 2022

© Inês Sousa, September 2022

This page was intentionally left in blank.

ACKNOWLEDGEMENTS

Em primeiro lugar, quero agradecer à minha orientadora, Joana Caldeira, e à minha co-orientadora, Raquel Gonçalves, pela disponibilidade, apoio e aprendizagem que me proporcionaram desde o início deste projeto.

Gostaria também de agradecer ao professor Mário Barbosa por me ter deixado fazer parte deste grupo e aos meus colegas do i3s, pelo companheirismo e por me mostrarem que nunca estive sozinha.

Por fim, mas não menos importante, agradecer à minha família e amigos, pelo apoio incondicional, por acreditarem em mim e por me fazerem acreditar também.

This work was supported by Fundação para a Ciência e a Tecnologia (FCT) through FETBIO project (FCT-2020-PTDC/BTM-MAT/0438/2020) and (UIDB/04293/2020) and FEEdIT (EXPL/BTM-ORG/0880/2021). It was also financed by QREN and a grant (20-165) from EUROSPINE/ON Foundation.

ABSTRACT

Intervertebral disc (IVD) is a fibrocartilaginous tissue, constituted by three structures: the annulus fibrosus (AF), the nucleus pulposus (NP), and the cartilaginous endplates (CEPs) [2]. Its degeneration is the leading cause of Low Back Pain (LBP) [3]. This condition affects over 80% of the people, encompassing all age groups, from children to the elderly and its incidence is rising, as the population ages [4].

As current therapies to manage LBP (e.g., pharmacological treatment treatments, microdissectomies and spinal fusions) are unable to provide long-term solutions and to restore disc function, there is a huge necessity to develop novel strategies to prevent or reverse disc degeneration and treat LBP. Hydrogels based on decellularized NPs (dNPs) constitute a promising approach, since they mimic the native microenvironment and can be injected in a minimally invasive way [5].

As so, the aim of this work was to produce a stable, biocompatible and easily injectable hydrogel from dNPs.

Decellularization of young bovine NPs using a SDS-based protocol was effective in removing over 98.31% of DNA while partially preserving GAGs (48.75%) and collagen (82.07%), with no significant traces of SDS being kept in the material after processing. Hydrogels were then produced, according to a previously optimized protocol, and showed to be mechanically stable at 37°C, for at least one week, and to be easily injected through needles, whose size ranged from 21G to 30G.

To assess their biological behavior, cells were cultured either in contact with extracts derived from the hydrogel or in direct contact with this material. Results demonstrated that the developed NP dECM-based hydrogels did not display high levels of cytotoxicity for bovine NP cells which were able to survive and proliferate in the hydrogels, retaining a normal metabolic activity in both cases.

Finally, envisioning further product development in an industrial context, a scale-up decellularization procedure was successfully validated in both fetal and young bovine IVDs. Indeed, the scale-up system, composed by a magnetic agitator and a vacuum pump, demonstrated promising results, since it achieved an optimal compromise between cell removal (high %DNA removal) and retention of matrix components (highest %sGAG preservation within the tissue), comparable to that obtained using the small-scale more time-consuming bench agitation system.

In conclusion, this work showed the development of a stable in-situ gelling biomaterial from NP decellularized matrices, with promising mechanical and biological features. Indeed, although many studies need to be performed, this is a notable step-forward in the development of a hydrogel capable of preventing or reversing disc degeneration and the associated Low Back Pain.

LIST OF CONTENTS

Acknowledgements.....	iv
Abstract.....	v
Chapter I: Introduction	1
Intervertebral Disc Structure	1
AF Structure	2
NP Structure.....	3
CEPs Structure	3
IVD Composition	5
AF Composition.....	6
NP Composition	6
CEPs Composition	7
Aging and Degeneration.....	8
Cellular Changes.....	9
Matrix Alterations	9
Biomechanical Changes	10
Cell Senescence	10
Proinflammatory Environment	11
Innervation	11
Low Back Pain and Available Treatments	12
IVD Regenerative Strategies.....	16
Biomaterials	16
Cells	18
Biologically Active Molecules.....	20
Hydrogels	22
Decellularized Scaffolds	24
dECM-based Hydrogels for NP Regeneration	27
Chapter II: Materials and Methods	32
Production of Decellularized Tissues	32

Biochemical Analysis.....	33
Assessment of Toxic Residues in Decellularized Tissues.....	35
Hydrogel Development and Biomechanical Characterization	35
Hydrogel Features.....	36
Biological Behavior of the Produced Hydrogel	37
Statistical Analysis.....	39
Chapter III: Results and Discussion.....	41
Decellularization Efficiency	41
SDS Quantification	44
Hydrogel Formation.....	45
Injectability.....	49
Swelling Behavior and Hydrogel Degradation	51
Evaluation of Young Bovine NP Cells.....	53
Cytotoxicity	54
Cell Proliferation	57
Optimization of Young Bovine IVD Decellularization System.....	59
Decellularization of Fetal Bovine IVDs.....	63
Chapter III: Conclusions and Future Perspectives	67
References:	69

LIST OF ABBREVIATIONS

<i>AB</i>	Alcian Blue
<i>ADAMTs</i>	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
<i>ADSCs</i>	Adipose-derived Stem Cells
<i>AF</i>	Annulus Fibrosus
<i>AGE</i>	Advanced Glycation End-Product
<i>AS</i>	Agitation System
<i>BDNF</i>	Brain-derived Neurotrophic Factor
<i>BSA</i>	Bovine Serum Albumin
<i>CEPs</i>	Cartilaginous Endplates
<i>CS</i>	Chondroitin Sulfate
<i>CS2</i>	Chondroitin Sulfate-rich Domain 2
<i>DAPI</i>	4',6-Diamidino-2-Phenylindole
<i>dECM</i>	Decellularized Extracellular Matrix
<i>DMEM</i>	Dulbecco's Modified Eagle Medium
<i>dNP</i>	Decellularized Nucleus Pulposus
<i>EDTA</i>	Ethylenediaminetetraacetic Acid
<i>FDA</i>	Food and Drug Administration
<i>GAG</i>	Glycosaminoglycans
<i>HA</i>	Hyaluronic Acid
<i>H&E</i>	Hematoxylin and Eosin
<i>IGD</i>	Interglobular Domain
<i>IL-1β</i>	Interleukin 1 β
<i>IVD</i>	Intervertebral Disc
<i>IVDD</i>	Intervertebral Disc Degeneration
<i>ISO</i>	International Organization for Standardization
<i>KS</i>	Keratan Sulfate
<i>LBP</i>	Low Back Pain
<i>LDH</i>	Lactate Dehydrogenase
<i>LVR</i>	Linear Viscoelastic Region
<i>MBAS</i>	Methylene Blue Active Substances
<i>MMP</i>	Matrix Metalloproteinase
<i>MSCs</i>	Mesenchymal Stem Cells

<i>NCs</i>	Notochordal Cells
<i>NGF</i>	Nerve Growth Factor
<i>NP</i>	Nucleus Pulposus
<i>NSAIDs</i>	Non-Steroidal Anti-Inflammatory Drugs
<i>PBS</i>	Phosphate-buffered Saline
<i>PEDF</i>	Pigment Epithelium-Derived Factor
<i>PEG</i>	Polyethylene Glycol
<i>PGA</i>	Polyglycolic Acid
<i>PGE 2</i>	Prostaglandin 2
<i>PLA</i>	Polylactic Acid
<i>PLGA</i>	Polylactic-co-Glycolic Acid
<i>PSR</i>	PicroSirius Red
<i>RGD</i>	Arginine-Glycine-Aspartate Sequence
<i>RT</i>	Room Temperature
<i>SA-βgal</i>	Senescence-Associated β -Galactosidase
<i>Scx</i>	Scleraxis
<i>SDC</i>	Sodium Deoxycholate
<i>SDS</i>	Sodium Dodecyl Sulfate
<i>sGAG</i>	Sulfated Glycosaminoglycans
<i>SLRPs</i>	Small Leucine-rich Proteoglycans
<i>Sox9</i>	SRY-Box Containing Gene 9
<i>SSP</i>	Small-Scale Protocol
<i>SUS</i>	Scale-Up System
<i>TIMP</i>	Tissue Inhibitor Metalloproteinases
<i>TNF-α</i>	Tumor Necrosis Factor α
<i>TSP1</i>	Thrombospondin 1
<i>VAS</i>	Vacuum and Agitation System
<i>YLD</i>	Years Lived with Disability

GLOSSARY OF KEY TERMS

<i>Acute Low Back Pain</i>	A pain that persists for less than four weeks.
<i>Anisotropy</i>	A characteristic of materials, that exhibit different properties in a direction-dependent manner.
<i>Axial Back Pain</i>	Pain caused by mechanical factors.
<i>Chronic Low Back Pain</i>	A pain that persists for more than twelve weeks.
<i>Compressive Forces</i>	Forces applied to a material that tend to reduce its original size.
<i>Crosslinking</i>	Formation of a bond or a sequence of bonds that link polymer chains.
<i>Delamination</i>	Separation of a material into layers.
<i>Epidemiology</i>	Study of the distribution, causes and possible risk factors of events (e. g., diseases) in a specific population.
<i>Genetic Polymorphism</i>	Occurrence of two or more variant forms of a specific DNA sequence in a population.
<i>Glycosaminoglycans</i>	Negatively charged polysaccharide compounds.
<i>Glycosylation</i>	Process catalyzed by glycosyltransferase enzymes that covalently attach sugars to proteins and lipids.
<i>Hyaline Cartilage</i>	Most common type of cartilage, mainly found in ears, nose, trachea and joints.
<i>Hydraulic Permeability</i>	Property that describes resistance to fluid flow.
<i>Hydroxylation</i>	Process catalyzed by hydroxylase enzymes that replace a hydrogen by a hydroxyl group.
<i>Hypertrophic Chondrocytes</i>	Specialized cells, that mediate endochondral ossification during development and are involved in the osteoarthritis progression.
<i>Hypoxia</i>	State in which oxygen is present in low levels (typically, in the range of 1-5%).
<i>Incidence</i>	Proportion of people that develop a specific condition at a determined time.
<i>Matrix Metalloproteinase</i>	Zinc-dependent endopeptidases that degrade specific proteins in the extracellular matrix.
<i>Prevalence</i>	Proportion of people with a specific condition at a determined time.
<i>Proteoglycans</i>	Macromolecules that consist of a core protein linked to one or more GAG chains.
<i>p-value</i>	Number that describes how likely it is that any observed difference between groups is due to chance.

<i>Nociceptors</i>	Peripheral sensory neurons involved in pain perception.
<i>Normoxia</i>	State in which oxygen is present in regular levels (typically, in the range of 10-21%).
<i>Notochord</i>	Rod-like embryonic structure, that plays an important part in the formation of various structures, such as nucleus pulposus.
<i>Sclerotome</i>	Embryonic tissue.
<i>Senescence</i>	Biological process, in which cells stop dividing and acquire a specific secretory phenotype.
<i>Shear Stress</i>	Stress resulting from forces applied parallel to the surface.
<i>Translamellar Cross-Bridges</i>	Radial structures that connect two or more non-consecutive lamellae in the annulus fibrosus.
<i>Tensile Forces</i>	Stretching forces acting on a material.
<i>Viscoelastic Material</i>	Materials that exhibit viscous and elastic features, under loading.
<i>Years Lived with Disability</i>	Number of years of healthy life lost due to disability in a population.

LIST OF FIGURES

Figure 1: IVD Structures (top and lateral view)	1
Figure 2: Differences between inner and outer AF	2
Figure 3: Differences between a healthy and a degenerated IVD.....	8
Figure 4: Total number of YLD cases, by age and sex	12
Figure 5: Classification of LBP	13
Figure 6: Ongoing clinical trials for LBP treatment, based on stem cell therapies	18
Figure 7: Hydrogel formation.....	29
Figure 8: Decellularization Efficiency	42
Figure 9: SDS Quantification.....	44
Figure 10: Gelation kinetics via turbidimetric assay.	45
Figure 11: dECM-based hydrogel development and biomechanical characterization	47
Figure 12: Gelation kinetics via rheological analysis	48
Figure 13: Assessment of the dNPs (solubilized and in particles) injectability.....	49
Figure 14: Swelling percentage and degradation rate of NP dECM-based hydrogels.....	51
Figure 15: NP cell isolation.	53
Figure 16: Schematic representation of the two assays (extraction and direct contact) that were conducted for assessing hydrogel toxicity.	54
Figure 17: Cytotoxic analysis of the extracts	55
Figure 18: Metabolic activity and cytotoxicity analysis for direct contact assay	56
Figure 19: Cell proliferation assessed by DAPI and H&E, PSR and AB staining.	57
Figure 20: DNA Quantification	58
Figure 21: Decellularization Systems for a Scale-Up Approach.....	59
Figure 22: Comparison of two decellularization systems for young IVDs	60
Figure 23: Quantitative analysis of the two decellularization systems tested for young IVDs	61
Figure 24: Comparison of two decellularization systems for fetal IVDs	64
Figure 25: Quantitative analysis of the two decellularization systems used for fetal IVDs.	65

LIST OF TABLES

Table 1: List of ECM components present in a healthy IVD.....	5
Table 2: Conservative and surgical approaches used to alleviate LBP.	14
Table 3: Characteristics of natural and synthetic polymers and their applications in IVD regeneration. ...	17
Table 4: Summary of the bioactive molecules used for IVDD treatment.....	20
Table 5: Decellularization agents and mechanisms of action.....	25
Table 6: Summary of the decellularization protocols used by distinct groups, regarding different tissue sources.....	26
Table 7: Main advantages of using hydrogels and decellularized tissues for NP regeneration.....	27
Table 8: Summary of the protocols used for the creation of dNP-based hydrogels.....	30

CHAPTER I

INTRODUCTION

INTERVERTEBRAL DISC STRUCTURE

The intervertebral disc (IVD) is an avascular tissue located between two vertebral bodies of the spine [2], with low cell density, and reduced metabolic activity [6]. The main functions of the IVD are to facilitate the spine's motion, act as a shock absorber, and prevent the vertebrae from grinding together [7].

IVDs consist of three main structures: an annulus fibrosus (AF), a nucleus pulposus (NP), and two cartilaginous endplates (CEPs) [2], shown in **Figure 1**.

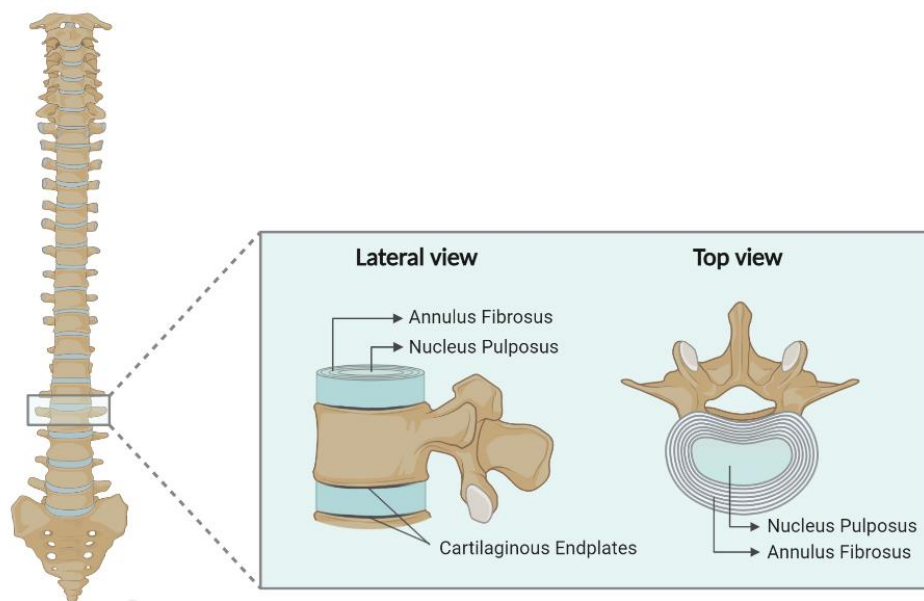


Figure 1: IVD Structures (top and lateral view). Herein, it is represented the three main structures that constitute an IVD: an AF, an NP and two CEPs. Briefly, the NP is a gelatinous tissue that support compressive forces and is surrounded by a fibrous tissue, called AF, that prevents its collapse. In turn, the CEPs cover both structures previously referred and separate the IVD from the cortical bone. In addition, it also appears to act as a permeable barrier (created with BioRender.com). AF, annulus fibrosus. CEPs, cartilaginous endplates. IVD, intervertebral disc. NP, nucleus pulposus.

AF STRUCTURE

AF is a highly fibrous tissue, originated from mesenchymal tissue [8], that is composed of 15 to 25 lamellae [9], with a cell density ranging from 3000 cells/mm³ to 9000 cells/mm³ [10]. During embryonic development, sclerotomal cells migrate and condense around the notochord, giving rise to this tissue [11]. AF has two distinct regions: the outer AF and the inner AF (also known as the transitional zone).

The outer region consists of elongated fibroblast-like cells and, mainly, collagen type I, organized in a concentric lamellar structure [2], and connected directly to the cortical bone of the vertebrae [12]. In adjacent lamellae, collagen fibers assume different directions [13], which provide anisotropic (i.e., direction-dependent) mechanical properties [12]. There is also a network of translamellar cross-bridges, linking fibers from adjacent lamella [10].

The inner AF, in turn, shares some features with both outer AF and NP. Thus, unlike the outer section, it possesses more spaced layers [14] and a higher amount of collagen type II and proteoglycans, produced by chondrogenic cells [2].

Undoubtedly, there are substantial differences between the two regions. Although all AF cells derive from the same progenitors (Scx⁺/Sox9⁺), their phenotype differs from the outer to the inner region, giving rise to a heterogeneous tissue (see **Figure 2**) [10].

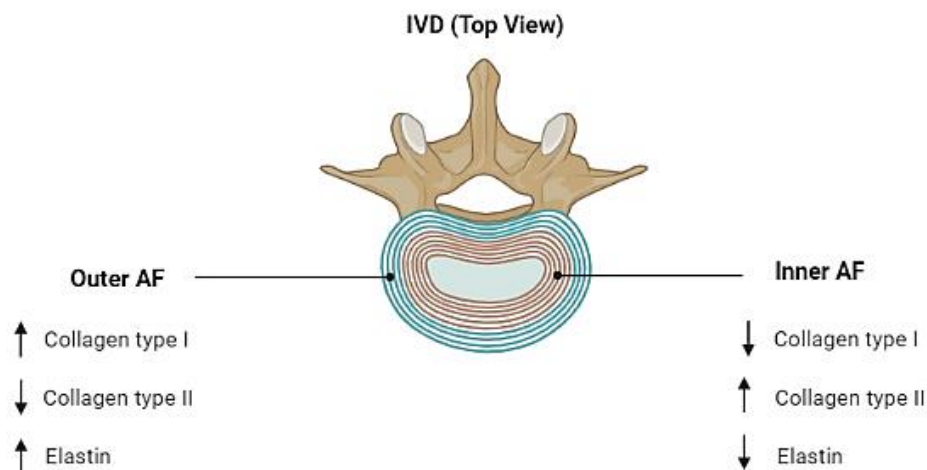


Figure 2: Differences between inner and outer AF. In the AF tissue, the two regions display different features. Thus, while the inner region is constituted by a higher collagen type II content, the outer is richer in collagen type I and elastin. This image was created with BioRender.com. AF, annulus fibrosus.

Furthermore, distinct mechanical stresses can also be noted, with the outer AF experiencing higher tensile loads and the inner AF greater compressive loads [12].

Some of the main AF functions are preventing the NP collapse, when subjected to compressive forces [15], and anchoring the NP to the CEPs through elastin fibers [14].

NP STRUCTURE

The NP derives from the notochord and corresponds to a gel-like tissue with a high content of proteoglycans and water. Additionally, it is also constituted by collagen type II randomly organized, and elastic fibers with a radial [8, 16] and axial orientation [8]. This structure supports compressive forces [15] and is described as a viscoelastic material since it behaves both as a liquid and as a solid [12].

The origin and marker profile of the NP cell population remains a controversial topic. Studies showed that, during embryogenesis, the NP is predominantly composed of notochordal cells (NCs). Their phenotype is characterized by the presence of an elevated number of vacuoles, an organization into interconnected cell clusters, and strong cell-cell interaction, such as gap junctions, cadherins, and desmosomes [17]. However, in humans, its abundance gradually decreases from birth, and, hence, they are replaced by chondrocyte-like cells [8], with a density of 4×10^6 cells/cm³ [18]. In contrast to NC, these are smaller, with a low number of intracellular vacuoles [14], rounded [14, 17], and organized into a non-clustering population [8]. In fact, although a substantial decrease in the number of human NCs from birth is observed, numerous studies demonstrated that a subpopulation of cells with notochordal origin remains in the disc. Richardson *et al* analyzed the expression of four markers: carbonic anhydrase-12 (NP cell marker), CD24, galectin-3, and brachyury (NC markers). They found a small population of cells in the NP that express all these four markers, which seems to validate the idea that the NP is constituted by a heterogeneous population of cells, including a sub-population with notochordal features [19].

CEPs STRUCTURE

CEPs cover both the NP and inner AF [15], and separate the IVD from adjacent vertebrae [14], avoiding a direct application of pressure to the bone [12]. They have a thickness of around 0.5 to 1mm in the periphery and are usually thinner in the center [9, 14].

During embryonic development, CEPs consist of two components: hyaline cartilage, which is maintained throughout development; and an osseous component [20]. Over time, its thickness and diameter decrease and it becomes calcified [8].

This structure functions as a selective permeability barrier, allowing the passage of uncharged and small solutes, and limiting the diffusion of anions or larger solutes [14, 21]. It was observed that its hydraulic permeability is higher than that measured in the NP and AF, indicating that CEPs' central function may be related to the transport of nutrients, fluids, and waste products [12]. The cells that constitute them are round, such as the articular chondrocytes [14].

IVD COMPOSITION

The IVD is mainly composed of proteoglycans, water, and collagen [8] (see **Table 1**). Their percentage varies within the IVD and is responsible for the different mechanical properties found in the IVD.

Table 1: List of ECM components present in a healthy IVD.

IVD Component	Type	Localization
Fibrillar Collagen	Type I	AF (mostly), NP
	Type II	AF, NP (mostly)
	Type III	AF
	Type V	AF
	Type XI	AF, NP
Fibril-Associated Collagen	Type IX	AF, NP
	Type XII	AF, NP (only in fetus)
	Type XIV	AF, NP (only in fetus)
Pericellular Collagen	Type VI	AF, NP
Aggregating Proteoglycans	Aggrecan	AF, NP
	Versican	AF
Small Leucine-rich Proteoglycans (SLRPs)	Decorin	AF (outer region), NP
	Biglycan	AF (outer region), NP
	Fibromodulin	AF, NP
	Lumican	AF, NP
Pericellular Proteoglycans	Perlecan	AF, NP

AF, annulus fibrosus. NP, nucleus pulposus. SLRPs, small leucine-rich proteoglycans.

In the IVD, collagen molecules can be grouped into three categories: fibrillar (type I, II, III, V, and XI), fibril-associated (type IX, XII and XIV) and pericellular collagens (type VI). Nevertheless, the most abundant are type I and II, present, mainly, in the AF and NP, respectively [22]. Proteoglycans, in turn, are divided into three groups: aggregating proteoglycans (aggrecan and versican, for example), pericellular proteoglycans (perlecan), and SLRPS (decorin, biglycan, fibromodulin, and lumican, for example), which are involved in the interaction with proteins, such as fibrillar collagen, fibronectin, and elastin, as well as with cytokines and growth factors [23]. The most abundant proteoglycan is aggrecan, which binds to hyaluronic acid (HA), forming aggregates [24].

In addition to collagen fibers and proteoglycans, healthy IVDs are also constituted by other components, such as fibronectin in a small amount [13].

AF COMPOSITION

Apart from collagen type I, the AF contains also a small amount of other components, such as: (1) collagen type III and V, that associate with collagen type I, originating heterotypic fibrils [2]; (2) collagen type VI, present in translamellar cross-bridges, as well as aggrecan, versican, and collagen type I [25]; (3) collagen type IX, XI, and types XII and XIV that seem to reside on the surface of collagen type I [13, 22]; (4) and elastin, that constitutes up to 2% of AF dry weight. The latter component can also be found between lamellae and is possibly involved in the recovery of the structure after deformation under radial loads and protection from delamination [2]. The AF is composed of proteoglycans and water, with a percentage of 5% and 70% of the wet weight, respectively [16].

NP COMPOSITION

The NP is constituted mostly of collagen type II, but also of collagen type I, VI, IX, and XI in lower quantities [22]. In fetuses, collagen type XII and XIV are also found in the NP, however, their abundance decreases, and their expression is suppressed in young discs [26]. Collagen type II is involved in the formation of a fibrillar network that allows the entrapment of proteoglycans [22]. Its posttranslational modifications, such as the hydroxylation of proline and lysine residues and the glycosylation of hydroxylysine residues, have a particular impact on thermal stability and degradability. Thereby, a higher level of proline hydroxylation and glycosylation can, respectively, increase the thermal stability and the resistance to mammalian collagenase. Glycosylation may also contribute to a reduced lateral growth [27] and mediate interactions with proteoglycans, which leads to higher water content [24]. Collagen type IX is a fibril-associated collagen that is crosslinked with collagen type II [2]. Compared to hyaline cartilage, it lacks the NC4 domain, whose effect on the disc is not known yet [8]. Furthermore, type XI forms heterotypic fibrils with collagen type II [2]. The NP is also composed of proteoglycans and water, with a percentage of 15% and 80% of the wet weight, respectively [16]. As referred before, the most abundant proteoglycan is aggrecan, which reaches its maximum content in the NP during late adolescence [24]. This component has two different chains: chondroitin sulfate (CS) and keratan sulfate (KS); however, their content changes over time. Thus, in fetal discs, aggrecan is mainly composed of CS chains whereas, in young juvenile discs, is composed of both CS and KS [8].

Aggrecan is included in a family of hyaluronan-binding proteoglycans and it is constituted by a globular domain (G1 region) that interacts with HA and promotes the formation of aggregates [24]. Aggrecan is cleaved by a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs), mainly, ADAMT-1, 4, 5, 9, and 15 [28], in two regions: interglobular domain (IGD) and chondroitin

sulfate-rich domain 2 (CS2). The cleavage leads to the formation of fragments unable to retain water. This process occurs naturally in the healthy IVD and, therefore, their turnover involves a balance between aggrecanases and tissue inhibitor metalloproteinases (TIMPs) [29]. When the equilibrium is disrupted, a progressive fragmentation of aggrecan is observed and a degenerated state emerges. The main function of aggrecan is to provide resistance to compression. Due to its high negatively charged density, it allows the water to enter into the disc by osmosis until the swelling potential is counterbalanced by tensile forces in the collagen network [16, 24]. On the one hand, when a compressive load is applied, water is displaced and the aggrecan concentration and, consequently, its swelling potential increase. On the other hand, when the compressive load is removed, the water enters into the disc and the equilibrium is re-established [24].

Studies also revealed that aggrecan is partly responsible for the aneural and avascular environment of the IVD, since it has some inhibitory effects in neurite initiation and outgrowth [30], as well as in endothelial cell adhesion and migration [31]. Furthermore, due to its negative charge, this proteoglycan may use calcium as counterions, limiting their free availability and, consequently, inhibiting the calcification of the disc [29].

CEPs COMPOSITION

The CEPs are mainly composed of water and proteoglycans. These compounds are usually embedded in a collagen type II network, whose fibers are parallel to the vertebral surface [32]. The balance between these constituents is important to guarantee their ability to support compressive loads [14].

Nevertheless, their distribution across this structure is not uniform, varying in a direction-dependent manner. Therefore, while proteoglycans and water content decrease towards the bone, the amount of collagen increases. In turn, when moving to the center of the CEPs, the opposite occurs. Thus, areas closer to the AF display a higher collagen content, whereas zones adjacent to the NP present higher concentrations of proteoglycans and water [33].

AGING AND DEGENERATION

IVD degeneration (IVDD) and aging are closely related and share some features. Indeed, the first one is a consequence of the second, but not only. Degenerated discs can also occur in younger people due to genetic polymorphisms (e. g., in collagen type IX and aggrecan genes [8, 16]), injury [34], and lifestyle (obesity and smoking, for example), among other factors.

In **Figure 3**, it is schematically represented the major alterations that occur in diseased states.

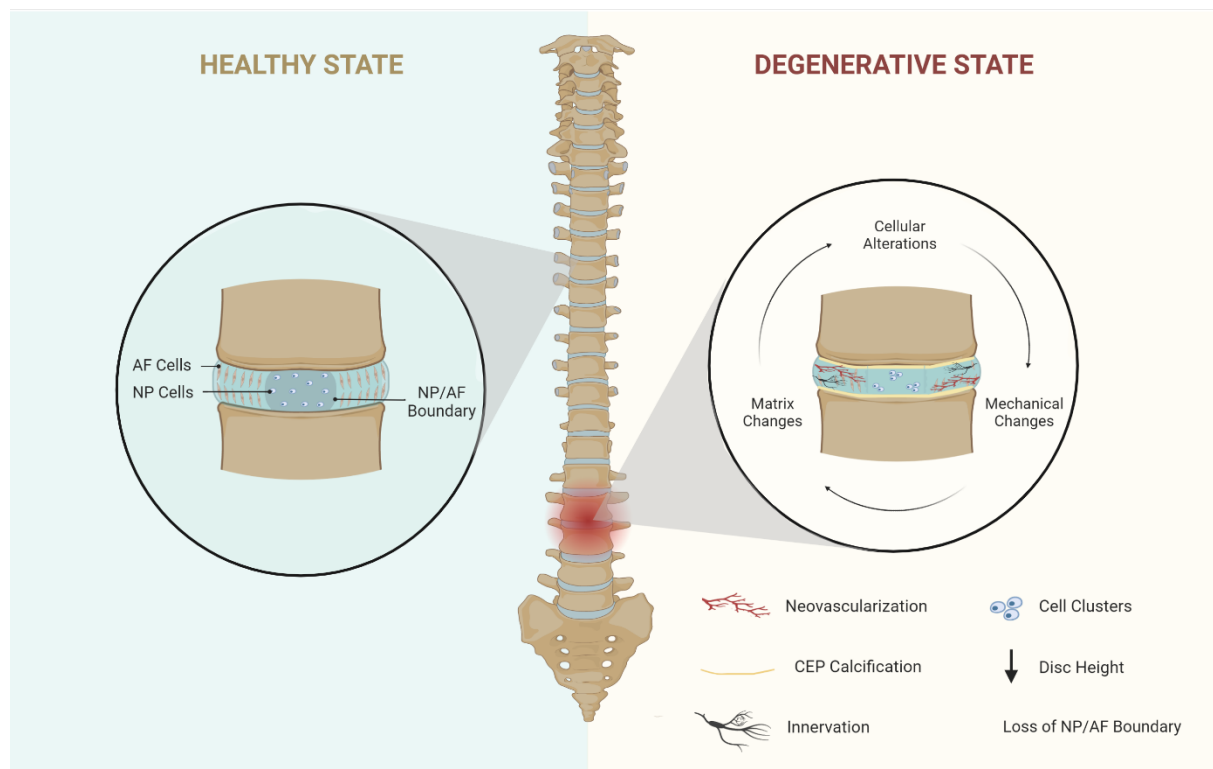


Figure 3: Differences between a healthy and a degenerated IVD. During degeneration, the main alterations occur at the cellular, matrix and biomechanical level. Briefly, NP cells appear organized in clusters and start producing a higher amount of collagen type I, turning the NP into a stiffer tissue. As for the matrix changes, a progressive fragmentation of aggrecan is observed. Consequently, a lower proteoglycan content leads to a decrease in intradiscal pressure and, therefore, to an impaired compressive loading behavior (created with *Biorender.com*). AF, annulus fibrosus. CEP, cartilaginous endplates. IVD, intervertebral disc. NP, nucleus pulposus.

CELLULAR CHANGES

Over time, several changes are observed in the IVD, and can lead to a degenerated state or promote its progression (see **Figure 3**). As the disc grows, the vascularization decreases, hindering the nutrient supply [8]. This creates a microenvironment, characterized by oxygen and nutrient deprivation and low pH, unfavorable to NCs viability [2]. Due to these changes, alterations regarding NP cell populations can be detected. In fact, during IVDD, NP cells seem to be organized as clusters, which is not characteristic of them [2]. Although their appearance seems to be a distinctive feature of disc degeneration, some studies have investigated their role in IVDD and suggested that the formation of cell clusters may correspond to an (unsuccessful) attempt to repair the damaged sites [35, 36].

MATRIX ALTERATIONS

Along with cellular changes, there are modifications at the matrix level. One alteration relies on collagen crosslinking. A study revealed a substantial decrease of pyridinoline crosslinks and an increased level of nonenzymatic glycosylated crosslinks, such as pentosidine, with disc aging and degeneration [37]. Pentosidine is an advanced glycation end-product (AGE), derived from a natural reaction occurring between sugars and basic amino acid residues, present in collagen (e. g., lysine and arginine residues) [8]. Due to a low turnover rate, collagen fibers are more susceptible to AGEs accumulation, with aging [38]. These alterations have effects on the biomechanical properties of the disc, increasing its stiffness and contributing to the loss of its integrity [37].

Additionally, there is also a variation in aggrecan composition. Indeed, while the abundance and size of KS chains increase with aging, the number and length of CS chains become smaller [39]. Oxygen deprivation seems to be responsible for this alteration since the KS synthesis does not involve an oxidation step, whereas CS synthesis does [29]. Furthermore, changes in HA are also described. In this case, there are two processes that contribute to HA degradation: enzymatic and non-enzymatic mechanisms. While in the former, HA is cleaved by hyaluronidase enzymes, in the latter, degradation is triggered by proinflammatory agents, such as IL-1 β [40, 41]. In fact, all these phenomena lead to a progressive fragmentation of aggrecan. This process decreases the number of aggregates [8], leading to a lower osmotic gradient and, consequently, to a reduced water content in the NP [15].

Moreover, in the NP, collagen type II is substituted by collagen type I, while in the outer AF, higher amounts of collagen type II can be found [2]. Consequently, the AF acquires a more disorganized lamellar structure and the boundary between IVD compartments becomes less evident

[26]. Chondrocyte-like cells produce collagen type I and reduced amounts of proteoglycans and collagen type II [42], which can explain these matrix changes. It has also been observed an increased level of fibronectin in degenerated and aged discs [26], that showed higher proteolytic fragmentation. These fragments can stimulate a lower synthesis of aggrecan and an increased production of matrix metalloproteinases (MMPs) [16], contributing to a higher degree of degeneration [8]. Additionally, with aging, an increased expression of prolargin has been also found and its presence may be responsible for the reduced fibers' density [26].

Furthermore, in CEPs, studies showed a high expression of collagen type X. This collagen type is related to the presence of hypertrophic chondrocytes and, consequently, is involved in tissue calcification.

BIOMECHANICAL CHANGES

Regarding these modifications, it is expected an alteration in IVD biomechanics. In the NP, a lower proteoglycan content leads to a decrease in intradiscal pressure and, consequently, to a water drop. A decline in intradiscal pressure has several effects on the disc: reduced disc height and axial compliance; greater risk of CEPs fractures, due to a deficient stress distribution; and increased shear stresses [42]. Furthermore, degenerated NPs showed a higher level of collagen type I crosslinks, which results in an increased stiffness [12]. Multiple studies have evaluated the mechanical alterations in the degeneration of the IVDs and concluded that there is an abnormal distribution of the elastic modulus in the disc, with this parameter being higher than in healthy discs [43]. Indeed, changes in biomechanical properties are a result of matrix and cellular modifications, however, it must be considered that these altered biomechanics also influences cell activity and phenotype, contributing to a higher level of IVDD [42].

CELL SENESENCE

With disc aging, the prevalence of NP cells with senescent features increases. The higher expression of senescence-associated β -galactosidase (SA- β gal), the release of pro-inflammatory cytokines and chemokines are observed. Several factors can lead to this phenomenon, and oxidative stress is considered the main cause of such alterations in aging discs. Studies revealed that NP cells treated with hydrogen peroxide stimulated the production of MMPs and pro-inflammatory cytokines and reduced the expression of collagen and aggrecan. Additionally, the treatment with H_2O_2 showed an increase in the fragmentation of both collagen and aggrecan. There is also an association between genotoxic stress and senescence. Hyperosmolarity is a recurrent

stimulus at which cells are exposed and appears to induce DNA damage, which causes cell senescence.

PROINFLAMMATORY ENVIRONMENT

Regarding inflammatory stress, it was observed a higher expression of pro-inflammatory cytokines. Among the inflammatory molecules involved in IVDD, there are two that should be highlighted as important key mediators: TNF- α and IL-1 β . These compounds appear to be involved not only in collagen II decrease, but also in the upregulation of ADAMTS-4 and -5 and MMPs-1, -2, -3, -4, -13, and -14. Furthermore, along with other pro-inflammatory molecules (IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, IFN- γ , chemokines, and prostaglandin [PGE] 2), they may also contribute to senescence, autophagy, and apoptosis of disc cells. Finally, both TNF- α and IL-1 β seems to stimulate nerve ingrowth, through the production of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and substance P, and therefore, induce pain [44].

This inflammatory response is carried out not only by disc cells but also by the immune cells that infiltrate in the IVD [45].

INNERVATION

The mechanism underlying innervation is not completely understood yet; however, investigators believe that there is a positive correlation between higher levels of neurotrophins and increased innervation in the IVD [46]. In fact, it was demonstrated that NP cells, in response to mechanical injury, strain, and pro-inflammatory cytokines, enhance the production of molecules, such as NGF. These may lead to nerve ingrowth, and, consequently, to a larger distribution of nociceptors nerve endings within the disc [47].

Furthermore, in degenerated IVDs, a lower aggrecan content may disrupt the inhibitory effect on vascularization and innervation, allowing the ingrowth of these structures [46]. Recently, investigators suggested that re-vascularization and re-innervation occur only when a structural disruption is observed, such as the appearance of fissures in AF, that allow the ingrowth due to the stimulation of cell migration by collagen surfaces [48].

LOW BACK PAIN AND AVAILABLE TREATMENTS

Low Back Pain (LBP) is a common health problem, that affects over 80% of the people worldwide [49], and constitutes the leading cause of years lived with disability (YLDs) since 1990. According to the *European Guidelines for Prevention in Low Back Pain*, this problem can be described as “a pain and discomfort, localized below the costal margin and above the gluteal folds, with or without leg pain” [50].

This medical condition has a great socioeconomic impact on both developed and developing countries [51]. In fact, it is correlated with a high economic burden (approximately, \$134.5 billion, only in the United States [52]) and a poor quality of life.

According to a recent study, in 2019, the prevalence of LBP was approximately 6972.5 cases per 100 000 population, whereas its incidence was estimated to be around 2748.9 cases per 100 000 [53]. Moreover, the number of YLD cases differed substantially across age and sex, being higher in women and at the age group of 45-54 years old (see **Figure 4**).

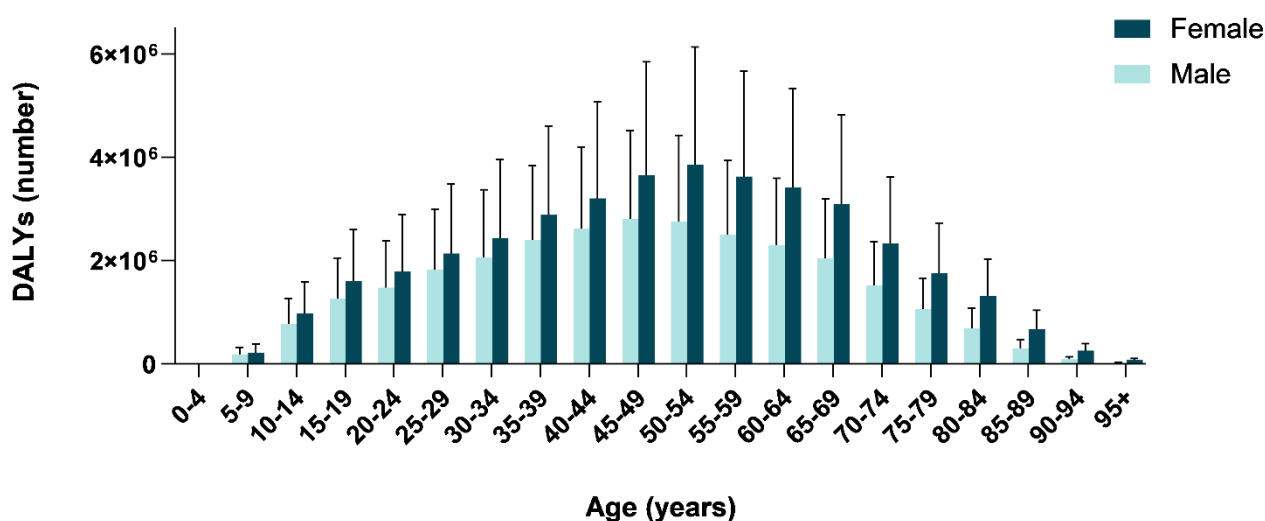


Figure 4: Total number of YLD cases, by age and sex. This graph was generated according to data available in the *Global Burden of Disease Study 2019* (<https://vizhub.healthdata.org/gbd-compare/>). YLD, years lived with disability.

LBP can be classified into specific and nonspecific, whether pain’s origin is a known factor or not, respectively [54]. Specific LBP constitutes only 15% of the total number of LBP cases and is associated with a wide variety of medical conditions (see **Figure 5**); however, IVDD appears to be one of the leading causes of this problem [3].

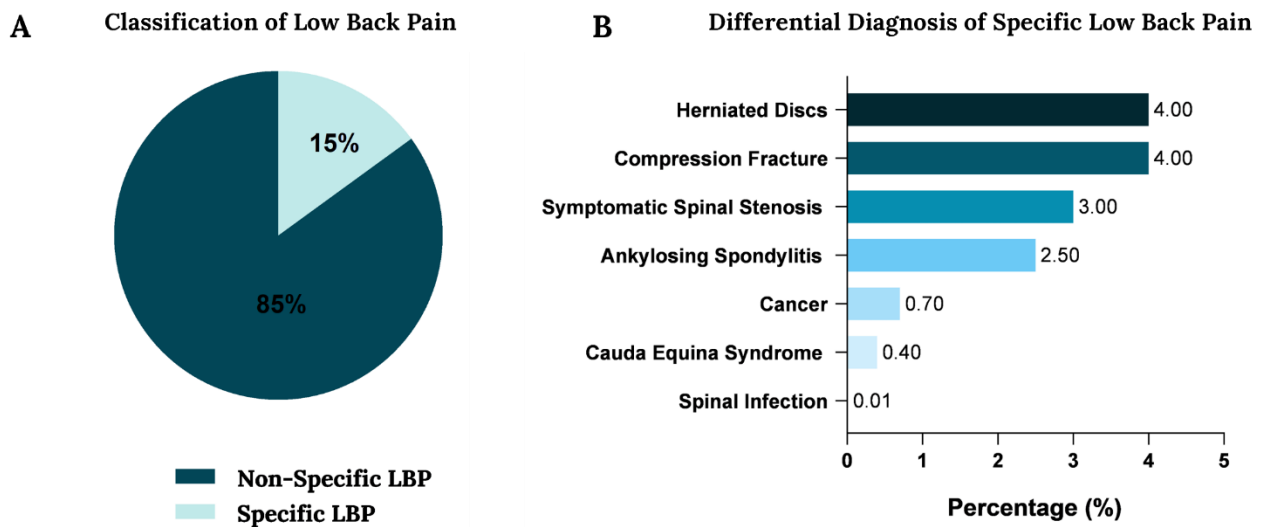


Figure 5: Classification of LBP. LBP can be categorized as specific or non-specific (A). Several medical conditions can give rise to specific LBP (B) (created with *BioRender.com*). IVDD, intervertebral disc degeneration. LBP, low back pain.

Indeed, IVDD can give rise to various outcomes, such as herniated discs, which compress roots or spinal cord, inducing nerve damage, edema and inflammation [55], as well as radicular back pain [56] (which is caused by nerve root compression/irritation). Discogenic LBP (which is described as an axial pain, produced by a degenerative disc) is also a consequence of IVDD, and it seems to be a result of three phenomena: inflammation, innervation, and hypermotility [57].

Briefly, inflammation has been described as a crucial contributor to discogenic LBP. Studies revealed that inflammatory mediators not only play an important role in IVDD initiation, but also have a huge impact on its progression. As for IVD innervation, studies have demonstrated that, upon disc degeneration, the abundance of nerve fibers increases, giving rise to LBP. Finally, hypermobility should not be overlooked. This medical condition can be defined as an abnormal range of joints movements [58] that occur due to an unusual laxity of ligaments. Indeed, it is one of the major factors that induces LBP and is a result of biochemical alterations and, subsequently, biomechanical changes [59].

Currently, some strategies are being used to alleviate LBP, including conservative approaches (e.g., physical therapy and pharmacological treatment), and surgical procedures (e.g., microdiscectomy, spinal fusion and prostheses replacement) [60] (see **Table 2**).

Table 2: Conservative and surgical approaches used to alleviate LBP.

Type	Name	Description	References
Conservative Strategies	Physical Therapy	It is a first-line treatment, that includes: • Massages. • Manual Therapy. • Stretching. • Strengthening exercises.	[61]
	Pharmacological Treatment	Recommended when first-line therapy is ineffective. NSAIDs are the most prescribed medicines to manage LBP. Other drugs include: • Skeletal muscle relaxants. • Opioids. • Tricyclic antidepressants.	[62]
	Acupuncture	It consists in the insertion of fine needles at specific sites in the body, that stimulate sensory nerves under the skin. This leads to the production of endorphins, and therefore to pain relief. While there is no scientific evidence that shows the benefits of acupuncture in acute LBP, its clinical effect in chronic LBP patients is very clear.	[63-65]
	Transcutaneous Electrical Nerve Stimulation	It involves the use of mild electrical impulses, to alleviate pain in a target area. It has been shown to be a useful adjuvant method in certain LBP patients, in order to reduce medication intake.	[66-68]
	Epidural Steroid Injections	Epidural steroid injections are a more invasive strategy, that act in radicular pain, by reducing nerve irritation and inflammation. They are usually prescribed as an adjunct, when pharmacological and physical treatments are no longer effective on their own. The clinical effects of these therapy are temporary and allow LBP patients to return to their daily activities.	[69]
Surgical Options	Microdiscectomy	Microdiscectomy is considered the gold standard among the surgical options. Briefly, it consists in removing the herniated disc that is compressing nerve roots and, consequently, causing pain to the patient. This strategy exhibits good clinical outcomes when it comes to pain relief.	[70, 71]
	Spinal Fusion	It consists in the fusion of two adjacent vertebrae, to limit excess spinal motion and alleviate pain. Spinal fusion surgery includes a wide variety of techniques, such as: • Anterior or posterior interbody fusion (posterior approach is more commonly used). • Posterolateral fusion. • Extreme lateral interbody fusion. • Axial lumbar interbody fusion.	[72]
	Prostheses Replacement	It is a recent technology, whose main purpose consists of spine stabilization. In this case, IVD is replaced by an artificial prosthesis that restore biomechanical properties of the injured tissue as well as spine's motion. However, its long-term benefits are not well known.	[60]

IVD, intervertebral disc. LBP, low back pain. NSAIDs, non-steroidal anti-inflammatory drugs.

Although available therapies are, to some extent, successful at managing symptomatology, all of them have limitations. While conservative approaches are not suitable for severe LBP treatment and may be associated with serious side-effects (e. g., potential risk of cardiovascular disease with the use of non-steroidal anti-inflammatory drugs [NSAIDs]) [73], spine surgeries can induce biomechanical alterations in the IVD at long-term [74]. Furthermore, none of them can really stop the progressive nature of the disease. Therefore, restore and/or reverse degeneration remains the major unmet need in the treatment of IVDD.

IVD REGENERATIVE STRATEGIES

As current therapies do not reverse degeneration nor restore a functional IVD, it is essential to develop new strategies. For disc regeneration, several approaches are being developed with the help of tissue engineering, that use biomaterials, cells, and biologically active molecules in order to create biomaterials capable of promoting a favorable biological response from the tissue [75].

BIOMATERIALS

Biomaterials correspond to an important part of the tissue engineering field, whose main purpose is to provide a biological and biomechanical support that stabilizes tissue defects, promotes cell adhesion, and stimulates regeneration [76].

When creating a responsive biomaterial, some features must be assessed, as they can affect the clinical outcome. Firstly, the scaffold must be biocompatible, in order to stimulate cell adhesion, as well as cell migration and proliferation. In addition, the immune response should not negatively affect tissue remodeling [77].

Secondly, this material should be biodegradable. In fact, within the tissue, cells must be able to modulate the ECM, *i. e.*, they must synthesize and degrade its components at a normal rate [78]. Therefore, when a regenerative biomaterial is inserted into the body, its degradation rate should be in line with the rate at which cells produce neo-tissue. When this balance is disturbed, one of two phenomena may occur. Since the biomaterial works as a scaffold for the cells, a higher degradation rate may lead to a mechanical instability, incapable of restoring the native function of the tissue. In turn, when the biomaterial degrades at a lower rate, a foreign body reaction may be elicited, resulting in encapsulation, that limits cell interaction [79].

Finally, the material must provide mechanical properties similar to the native tissue and present a microarchitecture, that enables cell infiltration and nutrient diffusion [77]. Nevertheless, studies revealed that an increased porosity greatly impacts the mechanical behavior of a scaffold, thus reducing its strength [80]. Therefore, as these two parameters are critical for the creation of a successful scaffold, a compromise should be achieved.

Regarding the IVD, the degenerated state is described as a microenvironment with a high rate of degradation and altered mechanical properties. Thus, an ideal biomaterial should maintain disc height, allow motion and provide mechanical properties similar to the native tissue, to resist mechanical loading [6]. For this, the use of hydrogels and other biomaterials appears to be a promising substitute to the current strategies [81]. Herein, the materials can be divided primarily into two categories: natural and synthetic (see **Table 3**).

Table 3: Characteristics of natural and synthetic polymers and their applications in IVD regeneration.

Type	Polymer	Description	Applications	References
Natural Biomaterials	Alginate	It is a natural polysaccharide obtained from seaweed, constituted by two polymers: guluronic acid and mannuronic acid.	- Cell carrier biomaterial (essentially for mesenchymal stem cells [MSCs]). - Cell-free scaffold.	[82-85]
	Chitosan	Polysaccharide, derived from chitin, used in medical applications.	- Injectable cell carrier, essentially for NP cells and MSCs. - Functionalized thermo-sponge.	[86-90]
	Silk Fibroin	Protein secreted by silkworms and spiders, with excellent low inflammatory and biocompatible features.	- Porous scaffold. - Hydrogel.	[91-93]
	HA	It consists of a non-sulfated glycosaminoglycan (GAG), with exceptional viscoelasticity and high biocompatibility.	- Injectable cell carrier biomaterial (essentially for adipose-derived stem cells [ADSCs] and NP cells). - Hydrogel for irregular defects filling. - Cell-free implant for NP regeneration.	[94-99]
	Collagen	Most abundant protein in mammals and is composed of three chains, that give rise to the characteristic triple helix structure.	- Injectable cell carrier biomaterial (essentially for ADSCs and NP cells). - Highly dense collagen type I scaffolds, for NP regeneration. - Cell-free hydrogel for NP regeneration.	[86, 95, 100-104]
Synthetic Biomaterials	Polylactic Acid (PLA)	Synthetic biodegradable polyester from lactic acid.	Injectable cell carrier scaffold (essentially for MSCs).	[105, 106]
	Polyglycolic Acid (PGA)	It is a biodegradable and biocompatible thermoplastic polymer. It gives rise to PLGA, when copolymerized with PLA.	Cell-free implant.	[107, 108]
	Poly(lactic-co-Glycolic Acid) (PLGA)	Approved FDA polymer, biocompatible and biodegradable.	- Microspheres seeded with ADSCs. - Porous cell-free scaffold for NP regeneration. - Hydrogel for annular defects.	[109-112]
	Polyethylene Glycol (PEG)	Biocompatible, nontoxic, and hydrophilic polyether, widely used in biomedical applications. It is produced using raw materials from petroleum refining or derived from natural gas or coal.	- Injectable cell carrier biomaterial (essentially for ADSCs and NP cells). - Cell-free hydrogel.	[95, 113-115]

FDA, Food and Drug Administration. HA, hyaluronic acid. NP, nucleus pulposus.

Each of these groups has its benefits and limitations. Briefly, natural polymers are known for their biocompatibility, biodegradability, and safety [116, 117], however, there is batch-to-batch variability, as well as some concerns related to their mechanical properties [117]. On the other hand, large-scale production is easier to obtain using synthetic polymers. Nevertheless, it must be ensured that all contaminants are removed, to guarantee their safety, when implanted into the body [116, 118].

CELLS

Cell-therapies have received increasing attention over the last years, due to their ability to regenerate and restore the normal function of diseased tissues. For this effect, several cell types are being used, including native NP cells. The main advantages regarding their use are related to their ability to survive in the harsh IVD microenvironment and to produce ECM components, characteristic of this tissue. However, it also has some limitations. In fact, upon harvesting, the cell culture yield is relatively low. Furthermore, they are not able to change the disease progression when administered alone into the damaged site.

Nevertheless, for cell-therapies, the use of stem cells stands out, not only because of their regenerative potential, but also due to their immunomodulatory behavior and ability to differentiate into various phenotypes [119].

According to *ClinicalTrials.gov*, more than 6500 clinical trials use stem cells as promising therapeutic tools. Of these, thirteen are being carried out for LBP treatment (see **Figure 6**).

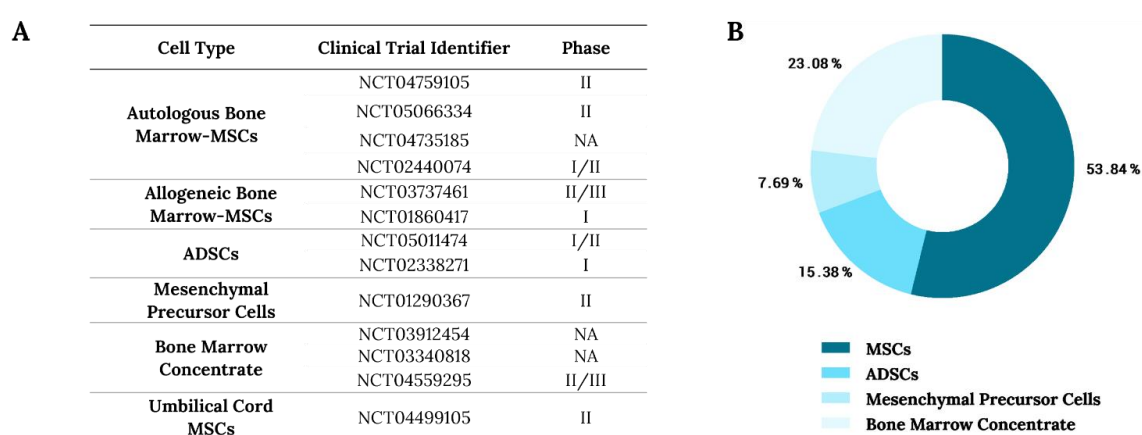


Figure 6: Ongoing clinical trials for LBP treatment, based on stem cell therapies. Herein, it is summarized the stem cell types that are under investigation (A), as well as the percentage of treatments that use each of them (B). According to this, it is easily confirmed that the use of MSCs as therapeutic agents accounts for more than half of the total clinical trials (approximately, 54%). This graph was generated taking into account the data provided by the *ClinicalTrial.gov* website. ADSCs, adipose-derived stem cells. MSCs, mesenchymal stem cells. NA, not applicable.

As observed in **Figure 6**, different stem cell types with distinct origins are under investigation for IVD regeneration. In the past years, several studies have assessed the use of MSCs as potential therapeutic agents. Curiously, it was found that the interaction between MSCs and endogenous NP cells leads to a synergistic effect, characterized by a substantial increase in proteoglycans production. Indeed, MSCs not only differentiated into a NP-like phenotype, but they also created a favorable microenvironment for the regeneration of local cells, through the secretion of cytokines and growth factors [120].

An alternative to MSCs is the use of ADSCs, that can be easily expanded. Various studies showed that they acquire a chondrocyte-like phenotype, in the presence of soluble compounds secreted by NP cells. Furthermore, they upregulate the production of collagen type II and aggrecan [120], and control apoptosis as well as the production of MMPs [121]. These findings make ADSCs implantation a promising cell therapy for IVD regeneration.

Regarding their source, cells can be divided into three groups: autologous (patient's own), allogeneic (human donor), and xenogeneic (animal origin) [122]. Autologous cells are the most suitable option, as they do not trigger an exacerbated immune response. Nevertheless, the time required for cell expansion is too high, limiting their use [122]. In addition, these cells can be in a diseased state, negatively aggravating the clinical condition of the patient [123]. Allogeneic cells, in turn, are readily available, however they may elicit an unfavorable immune reaction. To overcome this problem, allogeneic transplantations are usually combined with immunosuppressors intake [124]. Finally, xenogeneic cells have been extensively investigated for multiple purposes. Yet, their use still requires the development of induced tolerance and genetic modifications, to guarantee their safety [125].

As for the implantation method, cells can be directly administered into the target site or cultured on a scaffold.

The injection of cell suspensions has demonstrated low efficacy *in vivo*, not only due to the lack of cell retention within the tissue and migration to off-target sites but also because of the low survival rate in such a harsh environment. Thus, when designing a therapy of this kind, some issues should be addressed. Firstly, the optimal number of administered cells needs to be evaluated, to avoid severe effects derived from higher doses (e.g., risk of micro-embolism and tumorigenesis). Secondly, the delivery system should be adequate and minimize undesired cell-material interactions, as well as reduce the impact of shear forces on cell membranes. Finally, assessing their viability right before injection is essential, as the presence of a small number of dead cells may affect the viable portion, through the release of cytotoxic compounds [126].

The second strategy involves the combination of cells with biomaterials. This approach greatly improves the efficacy of these therapies, as it provides molecular cues that support the proliferation

and survival of the cells. Additionally, biomaterials are also important in controlling cell fate, thus guaranteeing differentiation into the desired phenotype [127, 128].

BIOLOGICALLY ACTIVE MOLECULES

Biological molecules play an important part in the modulation of tissue maintenance, through the recruitment and stimulation of endogenous cells [129]. For IVDD regeneration, multiple growth factors have been studied, including platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and growth and differentiation factor (GDF). Additionally, some members of the transforming growth factor (TGF), and bone morphogenetic protein (BMP) families have also been widely used [130].

Table 4: Summary of the bioactive molecules used for IVDD treatment.

Bioactive Molecule	Function	Effect on IVD cells	Reference
PDGF	Stimulates growth and survival of some cell types, such as mesenchymal cells and controls tissue homeostasis.	It retards IVDD, as it prevents apoptosis of AF cells and promotes ECM production.	[131-133]
IGF-1	It is an important hormone, that promotes tissue growth. Its synthesis is controlled by the production of growth hormone.	It has an anti-apoptotic effect on AF cells.	[133, 134]
GDF-5	Participates in bone and cartilage formation, through the regulation of chondrogenic differentiation.	Chujo <i>et al</i> reported an increase in ECM production and IVD cells proliferation <i>in vitro</i> . Concerning <i>in vivo</i> studies, it was observed a disc height improvement.	[135-137]
TGF-1β	Regulates the function of immune cells. In addition, it controls bone remodeling and stimulates the production of collagen.	Stimulates NP cells to produce collagen type II and aggrecan.	[138-140]
TGF-3β	Has an anti-fibrotic function in certain conditions.	Promotes ADSCs differentiation into NP-like cells.	[141, 142]
BMP-7	Involved in skeletal repair and regeneration, as well as in calcium regulation and bone homeostasis.	Studies suggested that this molecule has a significant effect on disc height. Additionally, it increases proteoglycans production by NP and AF cells.	[143, 144]

AF, annulus fibrosus. BMP, bone morphogenetic protein. GDF, growth and differentiation factor. IGF, insulin-like growth factor. IVD, intervertebral disc. IVDD, intervertebral disc degeneration. PDGF, platelet-derived growth factor. NP, nucleus pulposus. TGF, transforming growth factor.

Although several growth factors have beneficial effects on IVD cells, it is noteworthy that they present brief periods of biological activity, due to their poor stability. Thus, to produce an effective outcome, these therapies require repeated administrations, which may increase their cost [145]. To overcome this problem, it is crucial to guarantee a sustained release of growth factors into the target site. In this sense, the development of gene therapies emerges as a potential solution. In fact, the main purpose of these strategies is to genetically alter IVD cells, in order to increase the production of specific growth factors within the disc [129]. Although it is a promising area, the alteration of genetic material still faces huge ethical problems, which can be seen as important obstacles in the development of new therapies.

HYDROGELS

Hydrogels are a subtype of biomaterials that consists of a highly hydrated 3D network, constituted by hydrophilic chains, that can be crosslinked physically or chemically. While physical crosslinking refers to reversible interactions that are formed, chemical crosslinking is related to the formation of permanent and strong interactions and ensures greater stability under physiological conditions [146]. The main goal of hydrogels is to mimic the ECM microenvironment and promote biological interactions, contributing to cell viability [147].

According to the nature of polymers, they can be divided into two groups: hydrogels based on natural polymers, such as collagen, alginate, chitosan, hyaluronan and decellularized tissues, and based on synthetic polymers, like polyethylene glycol (PEG) and poly(ϵ -caprolactone) (PCL) [116]. Each of these groups has its benefits and limitations, as previously mentioned.

Hydrogels' versatility makes them a great option for use in the biomedical area. Thus, to design these materials, it is important to ensure that their characteristics are suitable for a specific application. Among others, degradation rate, gelation time, biological performance, and mechanical properties are some parameters that should be considered [148]. Degradation rate has an impact on biomechanics [148] and will be discussed later. Biological performance can be improved by functionalizing hydrogels with adhesive ligands, like the arginine-glycine-aspartate sequence (RGD), fibronectin, and laminin [147]. Finally, since hydrogel mechanical properties affect cell response, they should be, approximately, the same as the native tissue [147]. These features can be tuned by changing the type of material, molecular weight, degree of crosslinking, chemistry and functionalization [118].

Hydrogels can be used in both AF and NP repair. For the AF, IVDD can lead to the appearance of fissures as a result of high mechanical stress experienced by the AF. Tears have to be treated to avoid herniation of the injured disc. Therefore, the use of hydrogels with *in situ* gelation may be a solution for this problem, since they fill the fissure created [149]. Several hydrogel formulations, like FibGen and BioGlue, have been developed. FibGen is composed of fibrinogen (which forms a primary fibrin network after being injected), and genipin, (which functions as a crosslinker of the network formed and allows the integration of the hydrogel in the tissue). BioGlue, in turn, is a glue composed of bovine serum albumin (BSA) and glutaraldehyde [150].

Hydrogels are also used for NP repair, due to several reasons. One of them is the fact that it resembles its gel-like nature. Another is related to the ability to be injected. Indeed, considering that NP is surrounded by AF and other tissues, such as muscles, it is extremely difficult to reach its anatomical location without causing any damage to the adjacent structures. Therefore, the use of hydrogels seems to be the best option to work around this problem, since they can be injected

locally via AF or transpedicularly (via CEPs), in a minimally invasive way. These hydrogels can be divided into two groups of materials: mechanically competent and/or biologically competent. While the first ones are designed to support compressive loads and, hence, their main function is to mimic mechanical properties of the native NP conferring mechanical stability; the second ones aim to provide a scaffold for cell growth, differentiation, survival, and migration, as well as for matrix production [6]. The development of a material with both characteristics is the ideal approach. In fact, a study has shown differences between acellular and cellular approaches, using a synthetic Laponite crosslinked with a poly(N-isopropylacrylamide) and dimethylacetamide copolymer (L-pNIPAM-co-DMAc) hydrogel and MSCs. It revealed that the material itself provides mechanical stability to NP and that MSCs can differentiate into NP cells and synthesize matrix. Thus, the combination of these two components gives rise to a strategy capable of restoring the mechanical properties of the NP tissue and at the same time functioning as a cell delivery system. Thus, it improves hydrogel integration in the tissue and contributes to matrix remodeling [151]. Indeed, synthetic polymers have been extensively studied, however, their *in vivo* application remains challenging, due to a low biological activity. The combination of these polymers with a variety of molecules (e.g., proteins and growth factors) may improve it, but does not resemble ECM complexity of the IVDs. Therefore, the interest in using decellularized tissues as an approach to mimic healthy IVDs has increased [152].

DECELLULARIZED SCAFFOLDS

Decellularization is a process through which cellular components are removed from the tissue, reducing the immune response, while maintaining ECM components [153]. In the last years, decellularized ECM (dECM)-based scaffolds have received significant attention in the biomaterials field, due to their low immunogenicity, and ability to mimic the microenvironment and architecture of the native tissue. In fact, the use of these materials seems to be a promising solution to IVD regeneration. Nevertheless, it is known that several factors may affect the properties of decellularized scaffolds and, subsequently, cell response, such as the decellularization protocol adopted [154].

There are multiple methods used in tissue decellularization, including mechanical/physical, chemical, and enzymatic techniques (see **Table 5**). Briefly, agitation, sonication, pressure, and freeze-thaw cycles are included in the mechanical group. In the chemical methods, several compounds are used, such as detergents (e.g., sodium dodecyl sulfate [SDS], Triton-X-100, and sodium deoxycholate [SDC]), acids, bases, and hypertonic and hypotonic solutions [155]. Lastly, in terms of enzymatic methods, proteases, like trypsin, or nucleases can also be used [155].

Detergents have an impact on tissue ultrastructure, since they disrupt and dissociate proteins from the ECM [156]. In turn, both acids and bases reduce the mechanical properties, due to collagen removal by the former and disruption of the collagen crosslinks by the later [156]. Finally, hypertonic and hypotonic solutions interfere with the interaction between DNA and proteins [157] and help to remove cell residues from the tissue [156]. Although enzymatic treatment provides a higher specificity related to cell removal, it is not efficient alone [156]. It is important to choose the most suitable method, as several features (e.g., ECM molecules and cell density) change from tissue to tissue. It should also be noted that different techniques cause different alterations in the ECM, from increased stiffness, as a result of the use of alcohol that induces collagen crosslinking, to matrix components degradation, resulting from the use of alkaline or acidic solutions. Thus, all of these consequences should be taken into account, to minimize their effects in terms of the interaction between cells and biomaterials [158], and a compromise between decellularization and retention of ECM components should be attempted [1].

Apart from this, other characteristics have also an influence on decellularization efficiency, such as tissue source. Indeed, a study showed that, when a specific decellularization protocol was applied to different species, the efficacy of cell removal and ECM preservation were not the same [154]. This can be easily explained by the differences found in ECM composition among species. In the case of IVD, Monaco *et al* compared three different animals (ovine, bovine, and porcine) and observed that bovine IVDs were the ones that maintained the highest collagen concentration [159]. Due to these discrepancies, an adjustment of the decellularization protocol may be required.

Table 5: Decellularization agents and mechanisms of action.

Method	Agent	Mechanism of Action	References
Mechanical/ Physical	Agitation	Facilitates and increases sample exposure to the solution.	[160]
	Sonication	Promotes cell lysis, through exposure to high frequencies of sound waves.	[161]
	Freeze-Thaw Cycles	It is a common precursor step to decellularization, that disrupts cell membranes, through the production of ice crystals.	[162, 163]
	Vacuum	Allows the solution to penetrate the inner parts of the tissue and can reduce the time needed for decellularization.	[162, 163]
	High Hydrostatic Pressure	Promotes cell death when a high pressure is applied. To achieve an optimal outcome (<i>i. e.</i> , allow cell disruption while maintaining protein stability), the chosen pressure should range from 150 MPa to 500 MPa.	[163]
	Supercritical carbon dioxide (CO ₂)	When CO ₂ reaches its supercritical point (above critical temperature and pressure), it can penetrate the target material, originating cell debris that must then be removed.	[164]
Chemical	Ionic, Non-Ionic and Zwitterionic Detergents	Solubilize cytoplasmatic and nuclear membranes.	[156]
	Acids and Bases	Denature proteins and solubilize cellular components.	[156]
	Hypertonic/Hypotonic Solutions	These solutions interfere with DNA and protein interactions. They also help to remove cell residues from the tissue.	[165, 166]
Enzymatic	Nuclease	Cleaves DNA and RNA strands.	[167]
	Trypsin	Cleaves the bonds established between ECM components.	[163, 168]
	Dispase	Breaks down specific ECM components, such as fibronectin and collagen IV.	[166]

ECM, extracellular matrix.

To date, several researchers have tried to design the ideal protocol for NP decellularization, through the combination of various decellularization agents (see **Table 6**), however, a standard procedure has not yet been developed, which hinders comparisons between different studies.

Table 6: Summary of the decellularization protocols used by distinct groups, regarding different tissue sources.

Animal	Decellularization Agents	Reference
New Zealand White Rabbit	• 0.25% trypsin, for 24h, at 37°C. • 50 U/mL DNase and 1 U/mL RNase, for 12 h. • 1% Triton X-100, for 24h. • PBS washes.	[169]
	• Decellularization solution (Tris Buffer, with 0.2% ethylenediaminetetraacetic acid (EDTA), 0.3% Triton X-100, 0.5% sodium deoxycholic acid, and 1% phenylmethanesulfonylfluoride), for 12h. • DNase and RNase solution, for 30 min.	[170]
Bovine	• Five freeze/thaw cycles. • 1% SDS, for 24h, at 0°C to 4°C.	[171, 172]
	• 2% SDC, for 1h, at 37°C, under agitation. • DNase solution, for 1h, at 37°C, under agitation. • PBS washes.	[173]
	• Five freeze/thaw cycles. • 2% Triton X-100. • 1% SDS. • 200 U/mL DNase solution.	[174]
	• Three ultrasonication cycles (40kHz), for 10 min. • Decellularization solution (0.4% EDTA, 1.2% Triton X-100, and 0.02% sodium azide), for three days, at 150rpm and room temperature (RT). • 720 mU/mL DNase and 720 mU/mL RNase solution, for 48h, at 37°C.	[175]
	• SB-10 detergent, for 5.75h. • Triton X-200/SB-16, for 6h. • 75 U/mL DNase and RNase, for 36h. All steps were performed under agitation (50rpm), at RT.	[1]
Porcine	• 5 freeze/thaw cycles. • 0.5% SDS, for 8h. • DNase, for 2h. • PBS wash, for 12h.	[176]
	• 50 mM Tris Buffer, containing 1% deoxycholic acid, 0.6% Triton X-100, 0.1% EDTA, and 0.02% sodium azide, for 72h. • DNase/RNase, for 48h.	[177, 178]

PBS, phosphate-buffered saline. SDC, sodium deoxycholate. SDS, sodium dodecyl sulfate.

Despite these challenges, decellularized matrices are useful tools for tissue engineering as well as for the development of multiple therapies, such as LBP treatment.

dECM-BASED HYDROGELS FOR NP REGENERATION

The use of dECM-based hydrogels for the NP compiles the advantages of both hydrogels and decellularized tissues, referred in **Table 7**. Hence, it is a great strategy to prevent the appearance of LBP since it mimics the native composition of the IVD, prevents innervation, and can function as a cell carrier system. Additionally, as the matrix is processed into a hydrogel, it can be injected locally in a minimally invasive way, preventing damages to the AF [5, 179].

Table 7: Main advantages of using hydrogels and decellularized tissues for NP regeneration.

Type of Material	Advantages	Reference
Hydrogels	Highly hydrated materials, similar to the NP nature.	[180]
	Designed to be injected in a minimally invasive manner, thus avoiding damages in the adjacent tissues.	
	Tunable mechanical properties.	
	Efficient delivery system for cells and/or biologically active molecules (e. g., growth factors).	
	Ability to retain their cargo in the injured site.	
dECM	Can be used for filling irregular defects.	[181]
	Low immunogenicity.	
	Preservation of the native composition and microarchitecture.	
	Presence of biological cues that stimulate adhesion, proliferation, differentiation, migration and survival of the cells.	

dECM, decellularized extracellular matrix. NP, nucleus pulposus.

It is well-known that decellularization agents can cause alterations in tissue constituents [160]. Therefore, although dECM-based materials mimic the native tissue composition, some mechanical strength and stability are lost, due to solubilization and formation of the hydrogel. As cells sense tissue stiffness and adjust their behavior [182], it is crucial to avoid any modifications of the mechanical behavior. In this sense, dECM can be crosslinked with biocompatible agents, such as riboflavin and genipin, to improve its properties, and missing components can be added. A study by Zhou et al proposed a hydrogel composed of decellularized NP (dNP), CS (to counterbalance the loss of proteoglycans during the decellularization process), and genipin, that functions as a crosslinker turning the hydrogel mechanically more stable and with a lower degradation rate [1]. Indeed, the incorporation of CS was not only important to maintain the osmotic pressure in the NP, but also necessary to keep an avascular and aneural microenvironment, since it has been

demonstrated that GAGs have an inhibitory effect in the vascularization and neurite outgrowth [5]. Results showed good mechanical properties and an ability to induce the differentiation of ADSCs into cells with an NP phenotype [1].

Other studies have shown the ability of dECM-based hydrogels to promote stem cells differentiation into a chondrocyte-like phenotype similar to NP cells [120]. Xu *et al* developed a dECM-based biomaterial loaded with MSCs. The scaffold was cut and injected together with cells into the NP. Results showed disc height restoration, upregulation of collagen type II, accumulation of GAGs, and differentiation of the MSCs into NP cells. However, a high degradation rate of the scaffold was observed *in vivo*. This issue can be overcome through material crosslinking [183]. Mercuri *et al*, in turn, seeded ADSCs on dNP-based hydrogels and assessed their biological behavior. A quantitative polymerase chain reaction (PCR) analysis was performed and showed that ADSCs differentiated towards a NP-like phenotype. This ensures the efficacy of the hydrogel to be used as a cell carrier, in order to guide a correct ADSC differentiation.

Many experiments use decellularized bovine or porcine IVDs, due to their availability, as well as similar composition and mechanical properties to human discs. Another important factor that may contribute to their vast use is linked with the abundant number of dECM-based materials from porcine and bovine animals already on the market (e.g., *CorMatrix ECM*, for cardiovascular use, and *ACell MatriStem™*, for wound healing). In fact, these successful examples show the potential (and safety) of these animals in the regeneration field [184], turning them into attractive tissue sources. Nevertheless, a recent study investigated the use of decellularized Wharton's jelly matrix derived from the human umbilical cord. Wharton's jelly is composed of high amounts of hyaluronan and sulfated proteoglycans, components present in the NP [185]. This study evaluated and confirmed the ability of the material to restore the chondrocyte-like cell phenotype of human degenerated disc cells. Although it still requires further investigation, Wharton's jelly matrix may be a promising strategy for IVD regeneration [186].

Finally, the use of fetuses, many times discarded as animal byproducts from slaughterhouses, should not be overlooked but seen as an opportunity for circular economy and to create added value from material that would be otherwise incinerated. In fact, fetal IVDs are expected to have a huge pro-regenerative potential, due to the presence of unique ECM components, including collagen type XII and XIV, that have a proven role in the regeneration of other tissues like limbs and cornea, and whose expression is suppressed in young discs [26]. Furthermore, fetuses also contain collagen fibers with fewer cross-links (responsible for the fast turnover and remodeling of these proteins) [187], a high GAG content (promotes cell proliferation and migration) [188], and an upregulated expression of thrombospondin 1 (TSP1) and pigment epithelium-derived factor (PEDF) [189, 190]. These two molecules display anti-angiogenic functions, important in maintaining the avascular nature of the IVD. Additionally, PEDF is also described as a neurotrophic and neuroprotective factor,

that controls neuron fate. Bearing this in mind, the use of decellularized fetal tissue is a promising area that should be further explored.

Production of a dECM-based hydrogel involves the self-assembly of collagen and can be seen as a three-step process: production of a powder, solubilization of dECM components, and neutralization. The process is, briefly, described in **Figure 7**.

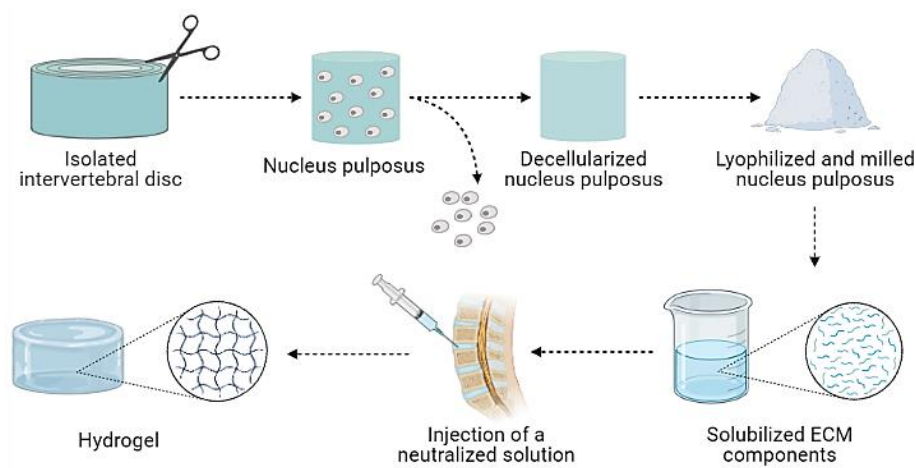


Figure 7: Hydrogel formation. After decellularization, tissue is lyophilized to increase its stability and shelf-life and milled into a powder to facilitate subsequent solubilization. Then, the pre-gel solution is neutralized to physiologic salt concentration and pH. Finally, the produced solution is injected locally, where gelation occurs at 37°C (created with *BioRender.com*). ECM, extracellular matrix.

After decellularization, the ECM is, usually, lyophilized. This process is characterized by the removal of solvents (water, for example) through sublimation, after freezing and placing the samples under vacuum [191]. Next, lyophilized dECM is milled into a powder. The particles that constitute the powder should have a small size and, consequently, an increased surface area to increase solubility [154].

Then, dECM components are solubilized. Enzymatic digestion is, usually, the chosen method for obtaining a solution composed of protein monomeric constituents. Pepsin is the most used enzyme. It corresponds to an endopeptidase secreted in the stomach that allows the maintenance of collagen ultrastructure since it does not cleave sites that are important for the stabilization of the three-helical structure [192]. The pH at which pepsin has highest activity ranges from 1,5 to 2,5 [193]. Therefore, it is important to create an acidic environment. As so, hydrochloric acid or acetic acid can be added to activate the enzyme. When a homogeneous solution (without any particles in suspension) is obtained, solubilization step can be finished.

The duration of this process varies from application to application and can range from 24h to 96h. For dNP-based hydrogels, there is not a standard protocol. As such, different procedures have been attempted by different authors (see **Table 8**).

Table 8: Summary of the protocols used for the creation of dNP-based hydrogels.

Animal	ECM Concentration (mg/mL)	Acidic Solution	Temperature (°C)	Digestion Time (h)	Agitation	Reference
Bovine	12	Pepsin in a hydrochloric acid (HCl) solution (0.25% w/v, pH = 2)	20	72	-	[171, 172]
	10	1.5 mg/mL pepsin in a 0.01 M HCl solution	25	24-48	Moderate	[174]
Porcine	5-20	1 mg/mL pepsin in a 3% acetic acid OR 0.01 M HCl solution	RT	32-64	-	[5]
	20	1 mg/ml of pepsin in a 0.05 M HCl solution	RT	44	300 rpm	[194]

ECM, extracellular matrix. RT, room temperature.

At the end of solubilization, a solution constituted by collagen monomers [195] and other proteins present in the ECM that may play a role in collagen polymerization is obtained. Regarding collagen type I, studies revealed that the presence of collagen type V functions as a nucleation site for its fibril formation [195]. In turn, it was shown that collagen type XI stimulates fibril reconstitution of collagen type II.

Additionally, although collagen type IX is not a crucial protein for fibril formation, its presence increases the efficiency of the process and, for that, may play a role in the stabilization of the fibrils produced [196].

The final step involves the neutralization of the solution. The parameters considered are pH, ionic strength, and temperature. Thus, after digestion, the resulting solution is neutralized to physiologic salt concentration and pH. The standard conditions for these parameters are PBS 1X and 7.4, respectively [195]. This procedure is done at 4°C to avoid premature crosslinking [197]. Finally, to obtain a hydrogel, the temperature must rise to 37°C, the physiologic temperature. This is an important aspect when it comes to the development of an injectable biomaterial. In this case, the material is expected to be a solution when injected and to become a hydrogel *in situ* [195].

An alternative technique for ECM solubilization involves a dispase and a urea extraction step. Dispace is a protease that cleaves collagen IV, collagen I, and fibronectin and, hence, its use

solubilizes ECM. Urea, in turn, increases ECM solubility and cleaves non-covalent bonding ECM proteins. The final solution gels at 37°C or when pH decreases to 4 [195]. Nevertheless, this approach still needs to be optimized and the effect of dispase on protein integrity has to be assessed [198].

As mentioned before, material concentration, pH, ionic strength, and gelation temperature are parameters that need to be controlled, to obtain a hydrogel. A study showed that variations in, at least, one of these features can result in modifications regarding gelation kinetics. It was observed that an increase in salt concentration significantly impacted on mechanical properties and, hence, it was associated with a lower gelation rate and storage modulus [199]. Indeed, storage modulus is related to the energy stored in an elastic manner [200]. Therefore, its decrease leads to a hydrogel with less rigidity. Besides, it was also evaluated the impact of material concentration in mechanical behavior and it was concluded that it increases storage modulus, giving rise to a hydrogel with higher stiffness [199]. Regarding the temperature at which gelation occurs, it was concluded that, when stored at 4°C or at RT, the pre-gel solution did not form a hydrogel. The self-assembly only took place when the solution was incubated at 37°C. Finally, an increase in pH gave rise to a stiffer material, in the case of collagen gels.

CHAPTER II

MATERIALS AND METHODS

PRODUCTION OF DECELLULARIZED TISSUES

a) NP Dissection and Isolation

Young and fetal bovine tails were obtained from the local slaughterhouse. For the dissection, excess tissues were removed, by using a sterile scalpel and a tweezer, to access and isolate the IVD, leaving the endplate to be used as a frame for cutting the tissue.

After dissection, discs were separated from the vertebral body using a scalpel and thin sections were obtained with the help of a slicer.

Finally, NPs were separated from the AF and cut into several cylinders, using a 4 mm biopsy punch.

b) Decellularization Process

NP samples were decellularized, using two different systems: small-scale protocol (SSP) and a scale-up system (SUS). Briefly, while the first experimental setup was solely constituted by a magnetic stirrer under agitation (approximately, 165 rpm), the second, in addition to the magnetic stirrer, had a vacuum pump connected to a desiccator, inside which a flask with the samples was placed under agitation.

The decellularization process was based on a previously optimized protocol (Fiordalisi *et al*, unpublished). Succinctly, NPs were immersed in Hypotonic Buffer A (pH 7.8, 10 mM Tris, 0.1% EDTA) for 18 hours, followed by three washes with PBS (pH 7.4). Then, samples were treated with 0.1% sodium dodecyl sulfate (SDS) solution (pH 7.8, 10mM Tris) for 1 hour, and washed three times with Hypotonic Buffer B solution (pH 7.8, 10mM Tris). Next, NPs were placed in a DNase treatment solution (pH 7.8, 20 mM Tris, 2mM MgCl₂, 50 U/mL DNase), for 3h, at 37°C and under orbital agitation (165 rpm). Lastly, samples were washed three times in PBS (pH 7.4).

BIOCHEMICAL ANALYSIS

a) Hematoxylin and Eosin, Alcian Blue and PicroSirius Red Stainings

Two NP samples for each condition (a native and a decellularized) were fixed in 1 mL of formalin, at 4°C, overnight. After that, samples were placed in PBS inside a histology cassette, until they were processed. Next, tissue samples were embedded in paraffin, sectioned at a 5 µm thickness, and stained with: a) Hematoxylin and Eosin (H&E), b) PicroSirius Red (PSR), c) Alcian Blue (AB). For nucleic acid evaluation, 4',6-diamidino-2-phenylindole (DAPI) was also used. Briefly, hematoxylin stains nucleic acids (such as DNA), giving them a blue-purple color (Fischer Ah Fau - Jacobson *et al.*), and eosin stains cytoplasm and ECM components (e.g., collagen fibers). AB, in turn, stains sGAGs as well as other negatively charged compounds present in the samples (Kubaski *et al.*, 2017). Finally, DAPI is a blue fluorescence compound that penetrates cell membranes and strongly binds to regions within the DNA with a high adenines and thymines content (AT regions) (Tarnowski *et al.*).

For H&E, PSR and AB-stained samples, histological images were acquired with a 10X, and 20X magnification, using an Axioskop 2 Plus microscope (ZEISS). For DAPI-stained samples, images were acquired with a 10X and 20X magnification, using an Axiovert 200M inverted fluorescence microscope (ZEISS).

b) Collagen Type II Staining by Immunohistochemistry

Collagen type II present in native and decellularized tissues was evaluated by immunohistochemistry, using Novolink™ Polymer Detection Kit (Leica Biosystems).

Briefly, slides were first dewaxed and rehydrated, followed by antigen retrieval and an endogenous peroxidase and protein block. Then, sections were incubated with the primary antibody (SC-52658, Santa Cruz Biotechnology) at a 1:100 ratio, overnight, at 4°C. Slides were then incubated in post-primary solution and Novolink™ Polymer, for 30 minutes at RT. A final incubation of 5 minutes with peroxidase-substrate DAB solution in the dark allowed the revelation of the bound antibody, followed by a hematoxylin staining. In the end, slides were mounted with Entellan and imaged using an Axiovert 200M inverted fluorescence microscope (ZEISS).

c) DNA Quantification

DNA content of native, dNPs and of hydrogels cultured with cells was assessed by using the *Preparing Lysates-Mini Kit* (K182001, Invitrogen) for DNA extraction and the *Quant-iT PicoGreen dsDNA kit* (Invitrogen) for DNA quantification, following manufacturer's instructions.

Briefly, DNA extraction involved the digestion of small pieces of lyophilized tissue in a digestion buffer for 4h, at 55°C. Next, the collected supernatant was treated with RNase A and a buffer/ethanol mix. After that, two washing steps were performed to remove any impurities and, finally, two elution steps were carried out to recover the DNA.

Regarding DNA quantification, all the samples were prepared, as well as two standard curves: a low-range (6,25 pg/mL to 25 ng/mL) and a high-range (1 ng/mL to 2 µg/mL) calibration curve. Fluorescence was measured with excitation at 480 nm and emission at 520 nm using a black plate reader. Quantification was performed in triplicates.

d) sGAG Quantification

Sulfated GAG (sGAG) content of native and dNPs was assessed by using the *Blyscan* Sulfated Glycosaminoglycan assay (Biocolor) that uses the 1,9-dimethylmethylen blue (DMMB) dye. Briefly, NP samples were cut in small pieces, digested with 1 mL of papain extraction reagent (pH=6.4, 0.1 mg/mL papain enzyme, 0.1M Sodium Acetate, 0.01M EDTA, 0.005M L-cysteine), at 65°C, for 18h, and, finally, centrifuged, for 10 min, at 10,000g. Supernatants were collected to new Eppendorfs before pursuing with the assay. To determine GAG content, 100 µL for dNP extracts were used, as well as two different volumes of native tissue (10µL and 20µL). A standard curve was also prepared with concentrations ranging from 3 µg/mL to 50 µg/mL. The absorbance was read at 656 nm. Quantification was performed in triplicates.

e) Collagen Quantification

Collagen content of native and dNPs was assessed by using the *Sircol™* Soluble Collagen Assay (Biocolor).

For soluble collagen isolation, 10 mg of NP samples were cut into small pieces and digested in a 5 mg/mL solution of pepsin dissolved in 0.5M acetic acid, overnight. The solution was then neutralized and 200 µL of the *Isolation & Concentration Reagent* was added to each sample, which allowed the precipitation of collagen molecules after a 10-minute centrifugation. Finally, the precipitate was rinsed with *Acid-Salt Wash Reagent*.

To quantify this protein, the prepared samples were incubated with 1 mL Sircol dye reagent at room temperature for 30 min, under gentle agitation. A standard curve was also prepared with concentrations ranging from 0 μg to 50 μg . The collagen–dye complex was precipitated by centrifugation at 10,000 rpm for 10 min and the supernatant was carefully removed. The pellets were then dissolved in 1 mL alkaline reagent and the absorbance was recorded at 556 nm.

ASSESSMENT OF TOXIC RESIDUES IN DECELLULARIZED TISSUES

a) SDS Quantification

To determine the presence of SDS traces in decellularized samples, a methylene blue active substances (MBAS) assay was carried out.

Briefly, for the standard curve, SDS was prepared at different concentrations: 0%, 0.03%, 0.06%, and 0.125%. As for sample preparation, 1 mg of cryomilled powder from each experimental group was added to 1 ml of distilled water, and vortexed for 1 minute. Then, the solution was centrifuged, and the supernatant was collected to an Eppendorf. Next, 250 μL of each sample/standard curve solution were mixed with 250 μL of methylene blue solution (250 $\mu\text{g}/\text{ml}$) and 1 mL of chloroform was added. After 10 minutes, a clear phase separation was observed. Finally, 200 μL of the bottom phase was pipetted to a 96-well plate and the absorbance was read at 665 nm. Quantification was performed in triplicates.

HYDROGEL DEVELOPMENT AND BIOMECHANICAL CHARACTERIZATION

a) Hydrogel Production

Hydrogels were produced in sterile conditions, according to an optimized protocol. Briefly, after decellularization, NPs were lyophilized for 72h and milled into a fine powder. Then, 40 mg of ECM were added to a 5 mg/mL solution of pepsin dissolved in 3% acetic acid, and digested for 72h, at RT. Afterwards, the solution was neutralized to pH 7.4 with sodium hydroxide (NaOH) and HCl and buffered with PBS 10X. The neutralization process was performed at 4°C, to prevent the solution from crosslinking. Pre-gel solution was stored at 4°C, for up to one week.

b) Rheology

The viscoelastic properties of the samples were analyzed using a *Kinexus* rheometer with *rSpace* software. Briefly, 50 μL of pre-gel solution was placed on an 8mm parallel plate separated by a 1 mm gap and pre-heated at 37°C.

To determine the linear viscoelastic region (LVR), frequency and amplitude strain sweep tests were performed. As for the gelation kinetics, a dynamic time sweep was run by applying a small amplitude oscillatory strain of 0.06% and a frequency of 1 Hz, for 30 minutes, at 37°C. Experiments were performed in triplicates.

c) Turbidity Analysis

Pre-gel solutions were preserved at 4°C for up to 1 week and 50 μL were loaded into a 96-well plate. Absorbance was measured every 2 minutes for 120 minutes, at 405 nm, in *BioTek* Microplate Spectrophotometer preheated at 37°C, using *Gen5 Data Collection and Analysis Software*. Experiments were performed in triplicates.

HYDROGEL FEATURES

a) Injectability

To assess the injectability potential, two different solutions were tested. The first consisted of a 500 μL suspension, constituted by 5 mg of NP particles with different sizes (<1 mm, <250 μm , and <150 μm) in PBS 1X. The second one corresponded to the pre-gel solution.

Briefly, samples were ejected through three different needle sizes (21G, 25G and 30G) and the time was recorded. PBS 1X was used as a control. Experiments were performed in triplicates.

b) Swelling and Degradation Behavior

To analyze the swelling and degradation behavior, 50 μL of pre-gel solution were incubated at 37°C, for 20 to 30 minutes in a 12 well-plate, to allow crosslinking. After gel formation, each hydrogel was weighted (initial weight, W_i) and placed in a 12 well-plate with 6 mL of PBS 1X. The plate was incubated at 37°C, for 1h, 24h, 72h and 7 days. Hydrogels were carefully removed from

the plate, and their weight was recorder before and after lyophilization (swollen weight, W_s , and dry weight, W_d , respectively).

Swelling and degradation ratios were obtained following these equations:

$$\text{Swelling Ratio: Swelling (\%)} = \frac{W_s - W_d}{W_d} \times 100\%$$

$$\text{Degradation Ratio: Degradation (\%)} = \frac{W_d - W_i}{W_i} \times 100\%$$

BIOLOGICAL BEHAVIOR OF THE PRODUCED HYDROGEL

a) Bovine NP Cells Isolation

Young bovine tails were dissected, as previously described. NPs were then separated from the AF, collected and finely minced into small pieces. Meanwhile, a digestion media, containing Dulbecco's Modified Eagle Medium (DMEM) low glucose (10-014-CV, Corning™), 0.2 mg/mL collagenase type I (C0130, Sigma Aldrich), 5% penicillin/streptomycin (P/S), 10% Fungizone and 1.5% NaCl/KCl, was prepared. Next, the small pieces were transferred into 15mL falcons with the adequate volume of digestion medium (1-2g of NP per 10 mL) and one sterile magnet inside. Tissue was incubated overnight at 37°C under agitation.

After digestion, the solution was filtered through a 40/70 μm cell strainer and centrifuged at 400 g for 15 minutes. The resulting pellet was resuspended in culture media, containing DMEM low glucose, 5% FBS, 1% P/S, 0.5% Fungizone and 1.5% NaCl/KCl, and cultured at 37°C, under normoxic conditions. Three different donors were used in the following tests.

b) Hydrogel Extracts Preparation

To prepare the hydrogel extracts following the standard ISO 10993-12, 50 μL of pre-gel solution were added to a 96-well plate, which was incubated at 37°C to allow hydrogel formation.

Then, 200 μL of DMEM culture medium were added to each well and the plate was stirred at 37 °C for 72 hours. After the incubation, the gel still retained its form. The culture medium was collected, and four different concentrations were prepared (100%, 75%, 50%, and 25%).

c) Extract Exposure Method

After 24 hours in culture, the medium was removed from the wells and 200 µl of test samples (i.e., extracts at different concentrations) were added to bovine NP cells. Cells treated with 70% ethanol were used as a cytotoxic control and cells cultured with regular medium served as negative controls. The cell culture plates were incubated for the following 7 days in standard culture conditions. Viability and cytotoxicity were assessed at day 1, 3 and 7. Experiments were performed in triplicates.

d) Direct Contact Assay

50 µL of pre-gel solution was added to a 96-well plate, which was incubated at 37°C to allow hydrogel formation.

Then, 10 000 bovine NP cells were cultured on top of the hydrogels and 200 µL of DMEM culture medium were added to each well. Cells treated with 70% ethanol were used as a cytotoxic control and cells cultured with regular medium served as negative controls. The plate was incubated for the next 7 days in standard culture conditions. Viability and cytotoxicity were assessed at day 1, 3 and 7. Experiment was conducted under normoxic conditions and triplicates were performed.

e) Metabolic Activity of the NP Cells

Metabolic activity was calculated using the resazurin-based assay. At different timepoints (1, 3, and 7 days), the culture medium was removed from the plates and fresh medium with 10% of resazurin (Sigma-Aldrich) was added. Cells were incubated for 3 hours, after which 200 µL per well were transferred to a black 96 well plate and measured using a fluorimeter (Synergy Mx, BioTek) at 530/590 nm. Experiments were performed in triplicates.

Metabolic activity was obtained following this equation:

$$\text{Metabolic Activity (\%)} = \frac{Abs_{treated} - Abs_{background}}{Abs_{untreated} - Abs_{background}} \times 100\%$$

in which $Abs_{treated}$ corresponds to cells treated with the extract, $Abs_{untreated}$ consists in the absorbance measured for the negative control (cells cultured with regular medium), and, finally, $Abs_{background}$ corresponds to culture medium with resazurin.

f) Cytotoxicity

Cytotoxicity was evaluated using *CytoTox 96® Non-Radioactive Cytotoxicity Assay* kit (Promega), which measures lactate dehydrogenase (LDH) present in the medium. Culture supernatants were collected at day 1 and 7 and LDH was quantified after a coupled enzymatic assay, in which tetrazolium salt is converted into a red formazan product. A blank control with fresh medium, and a positive control of cell death were also included. Data was normalized by the control. Experiments were performed in triplicates.

Cytotoxicity was obtained following this equation:

$$\text{Cytotoxicity (\%)} = \frac{Abs_{exp} - Abs_{medium}}{Abs_{LDH\ PC}} \times 100\%$$

in which Abs_{exp} corresponds to experimental samples, in which the cytotoxicity is being measured, Abs_{medium} consists in the absorbance measured for the culture medium used for cell culture, and, finally, $Abs_{LDH\ PC}$ corresponds to the absorbance of the positive control for cell death (which is provided by the kit).

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software. First, Shapiro-Wilk test was performed to assess if data is normally distributed in a population. For the quantification data (DNA and sGAGs), paired students t tests were performed. For SDS quantification, an unpaired students t-test was carried out. For injectability, cytotoxicity and metabolic activity, a one-way ANOVA was performed and, finally, a two-way ANOVA was followed for the swelling ratio and degradation rate analysis.

All tests were performed using a 95% confidence interval.

This page was intentionally left in blank.

CHAPTER III

RESULTS AND DISCUSSION

DECELLULARIZATION EFFICIENCY

Decellularization is defined as a process that consists in the removal of cells while preserving maximal matrix components and ultrastructure. However, if not fully optimized, this process may lead to matrix disruption up to some extent and might leave cell traces within the tissue [201]. Therefore, a compromise between cell removal and retention of ECM components must be sought [1].

To ensure decellularization was well succeeded, the presence of cells was assessed by qualitative (H&E and DAPI staining) and quantitative methods (DNA quantification using *PicoGreen*). Concerning DAPI staining, a significant reduction of fluorescence intensity was visible in the decellularized NP, when compared to the native tissue. Moreover, no intact cell nuclei were detected in the H&E staining (presence of larger empty spots in the matrix without stained material in the middle) (see **Figure 8B**), confirming an efficient removal of cellular and nuclear components. In line with this, DNA quantification revealed a significant DNA content decrease (approximately, 98.31%) (see **Figure 8C**). In fact, DNA concentration within the decellularized tissue was significantly lower ($p = 0.0184$) than that of the native NP (5.87 ± 6.44 ng/mg vs 347.71 ± 85.71 ng/mg). According to the literature, to avoid an adverse immune response from the host, decellularized tissues must have less than 50 ng dsDNA per mg of ECM dry weight [166]. By analyzing the data obtained, it can be concluded that this criterion was fulfilled, as the DNA concentration present in the dNP was below the established limit.

The preservation of ECM components was also examined. Indeed, as NP is mainly constituted by collagen type II and sGAGs, its content within the decellularized matrix was assessed [2]. In this sense, both native and decellularized samples were stained with Alcian Blue (AB) (which detects GAGs and other negatively charged compounds) [202] and PSR (which identifies collagen fibers, regardless of their type) [203] – **Figure 8B**. Furthermore, collagen type II was also detected by immunohistochemistry staining (**Figure 8B**).

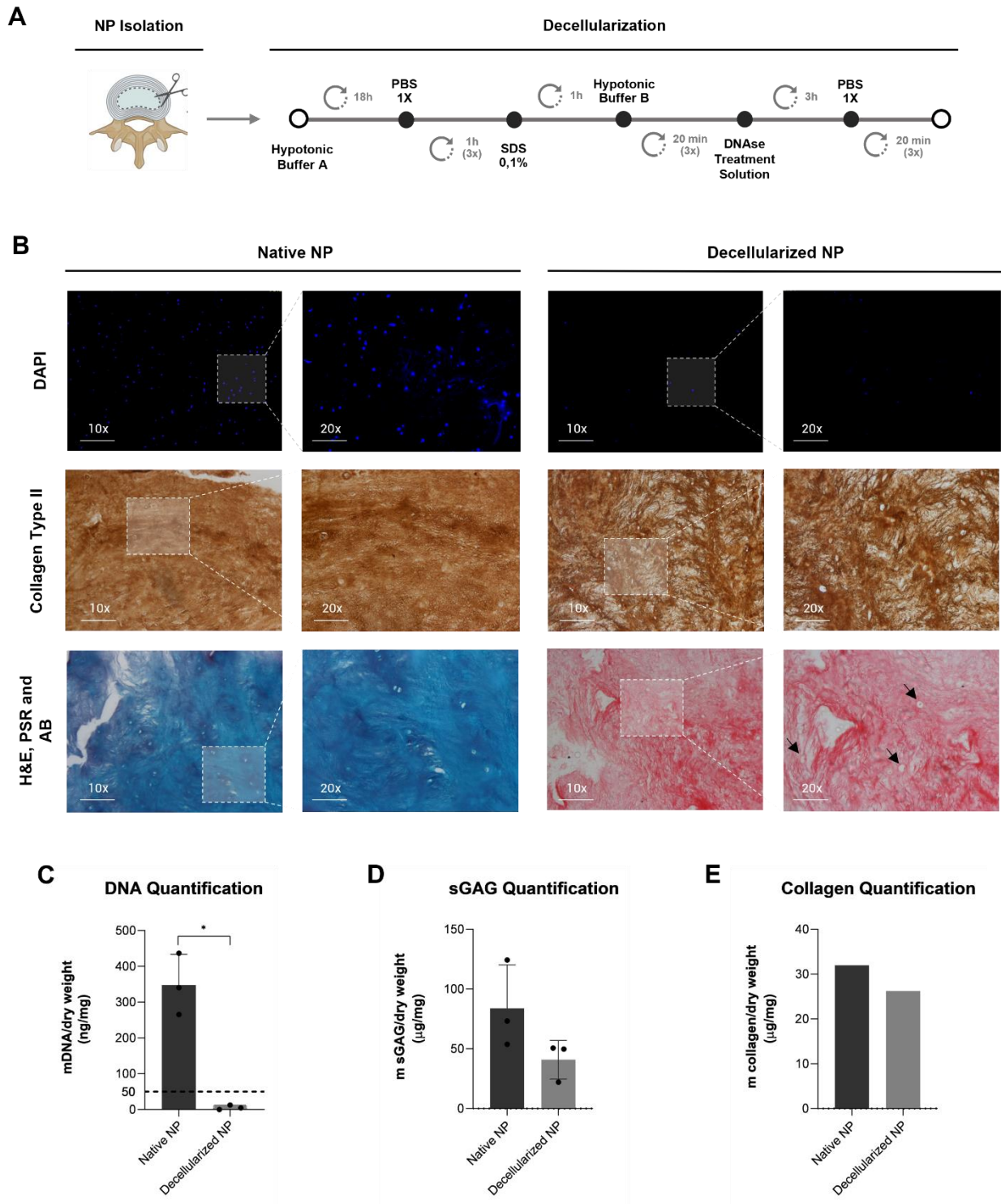


Figure 8: Decellularization Efficiency. Decellularization was performed under agitation, following a protocol, previously optimized by our group (A). To evaluate its efficiency, the presence of cells in the decellularized tissue was assessed qualitatively, by DAPI and H&E staining. Moreover, collagen was identified by immunohistochemistry and PSR staining. GAGs, in turn, were stained with AB (B). A quantitative analysis was also carried out, regarding DNA (C), sGAGs (D), and collagen (E). All images were acquired with a magnification of 10X and 20X. Dashed line represents the DNA concentration below which decellularization is considered efficient: 50 ng/mg. Arrows are pointing to spaces previously occupied by cells. A paired t-test was carried out for DNA and sGAG quantification (N=3). * $p < 0.05$. AB, Alcian Blue. DAPI, 4',6-diamidino-2-phenylindole. H&E, hematoxylin and eosin. IVD, intervertebral disc. NP, nucleus pulposus. PBS, phosphate-buffered saline. PSR, PicroSirius Red. sGAG, sulfated glycosaminoglycans. SDS, sodium dodecyl sulfate.

Histological data showed a strong reduction of blue staining when comparing native samples with the decellularized tissue (see **Figure 8B**), thus suggesting a significant loss of proteoglycans during the decellularization process. Indeed, quantitative data showed a decrease of 51.25% in the amount of sGAGs (see **Figure 8D**), corroborating histological analysis. In fact, using the Blyscan Assay kit, we observed that while the native tissue exhibited a total content of $83.85 \pm 36.38 \mu\text{g}/\text{mg}$, the decellularized group showed a total mass of $40.88 \pm 16.23 \mu\text{g}/\text{mg}$. No significant differences were found between the two samples. This decrease may be explained by the use of SDS as decellularization reagent. Indeed, studies have reported that this anionic detergent has an impact on GAG removal. Fernández-Pérez *et al* demonstrated that the use of SDS resulted in lower GAG retention than other methods (Triton and freeze-thaw) [204]. Since this compound plays a crucial role in the IVD microenvironment, different strategies have been developed to overcome GAG loss: culture of IVD cells in the hydrogel for ECM production [205], and GAG supplementation to the hydrogel (e.g., chondroitin sulfate) [206]. In fact, it is expected that native NP cells are able to restore GAG content *in situ* once sensing healthy regenerative microenvironmental cues provided by the hydrogel.

Regarding collagen content, images showed its presence by the red PSR staining in decellularized tissues. In the case of collagen type II (present in high amounts in the NP tissue), both native and decellularized samples were analyzed. In fact, a strong staining was found in the two groups, confirming the retention of this protein. Lastly, collagen was also quantified using the *Sircol*[™] Soluble Collagen Assay (see **Figure 8E**). Indeed, a slight decrease in collagen content of about 17.93% was observed (31.95 and $26.22 \mu\text{g}/\text{mg}$ in the native and dNP, respectively). Nevertheless, the differences were minimal, demonstrating a highly preserved collagen network.

SDS QUANTIFICATION

This decellularization protocol involves the use of multiple chemical products, such as SDS. In fact, it has been proven that, when a high concentration of detergent residues is still found within the tissue, a severe immune response from the host cells is triggered, affecting the material's ability to promote regeneration [207]. In order to avoid the creation of a harmful biomaterial, SDS residues were quantified in the decellularized samples, our samples, through a MBAS assay (see **Figure 9**). In this case, distilled water and the SDS solution used during decellularization served as a negative and positive control, respectively.

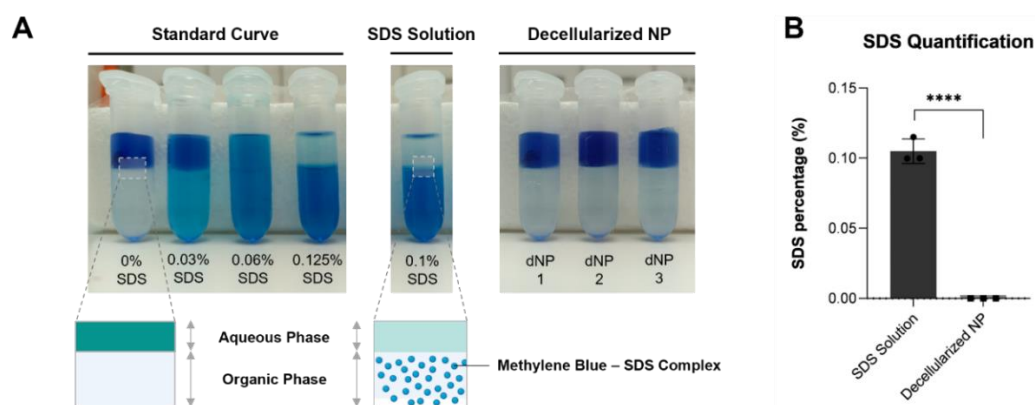


Figure 9: SDS Quantification. A MBAS assay was performed in order to detect SDS in decellularized samples. Briefly, in the presence of this detergent, a colorimetric complex is formed through the conjugation of SDS with methylene blue. These compounds can be extracted to an organic phase containing chloroform and the absorbance measured at 665 nm (A). Herein, the solution in contact with the dNPs was quantified and compared to the SDS solution used during the decellularization process (B). An unpaired t-test was carried out for SDS quantification (N=3). **** $p < 0.0001$. dNP, decellularized nucleus pulposus. NP, nucleus pulposus. SDS, sodium dodecyl sulfate.

By observing **Figure 9A**, it was clear that, while the organic phase of the differently concentrated SDS solutions turned blue (due to the formation of methylene blue-SDS complexes), the bottom phase obtained with the dNP samples remained transparent, similarly to the negative control. Indeed, the concentration of SDS within the decellularized tissue was significantly lower ($p < 0.0001$) than that in the positive control group ($0.1 \pm 0.0009\%$ vs $0.00008 \pm 0.00003\%$, respectively) (see **Figure 9B**) – 0,1% SDS solution used for decellularization. This suggests that the washes steps after SDS treatment were able to successfully remove detergent residues from the tissue.

Concerning the use of SDS, Ghorbani *et al* examined its potential adverse effects on cell viability and established a threshold of toxicity for a concentration of 0.0001% SDS [208]. As MBAS assay detected only 0.00008% SDS in dNP samples ($< 0.0001\%$), it is highly expected that this decellularized tissue will not be toxic for cells.

HYDROGEL FORMATION

Since the NP is surrounded by AF and other tissues, such as muscles, it is extremely difficult to reach its anatomical location without causing any damage to the adjacent structures (e.g, in microdissectomies, arthroplasty or arthrodesis). Therefore, engineering a hydrogel that can be easily injected either through the AF or transpedicularly with a very thin needle, may be a solution for this problem.

In this work, a hydrogel-based on dNP tissue was developed, following previously optimized conditions. Briefly, after decellularization, the dNP was lyophilized and milled. For hydrogel formation, 40 mg of a dNP powder was solubilized in a solution constituted by 5mg of pepsin dissolved in 1 mL of a 3% acetic acid. The ECM digestion took 72h, followed by a neutralization step with HCL and NaOH. Finally, the solution was buffered with PBS 10X and stored at 4°C, to prevent premature crosslinking (see **Figure 10A**). The gelation of this biomaterial was based on the self-assembly of collagen monomers into fibrils when the temperature was increased up to body temperature, 37°C [204].

First, gelation process was assessed through a turbidity assay (see **Figure 10**). This indirect method evaluates the ability of collagen molecules to self-assembly. This process is mainly constituted by three different phases: lag, growth, and plateau phase. The first one is characterized by an unaltered absorbance since it is correlated with the organization of collagen monomers into intermediate structures – the formation of nucleating centers. The second phase shows a rapid increase in absorbance and coincides with the formation of mature collagen fibrils – nucleating center growth. The last one presents constant absorbance and is reached when all fibrils have formed.

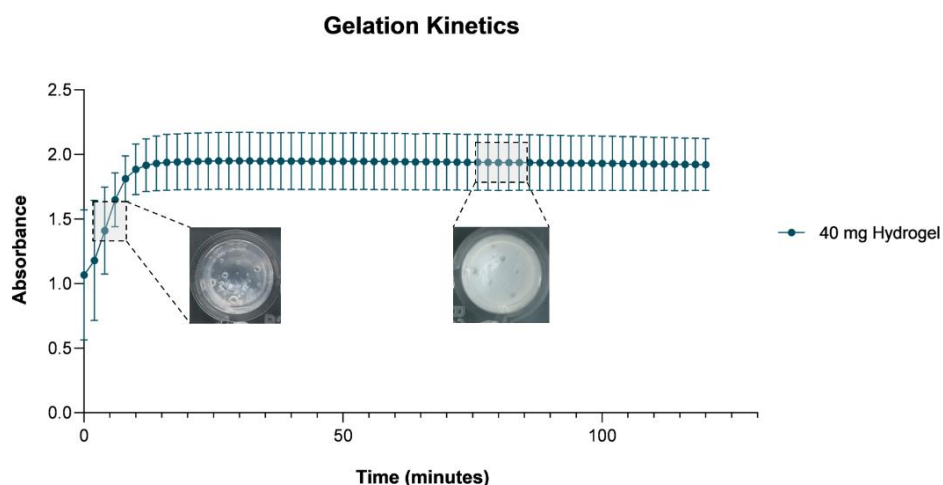


Figure 10: Gelation kinetics via turbidimetric assay. This experiment was performed at 37°C, for two hours. The absorbance was read every two minutes, at 405 nm. Images show macroscopic differences between a pre-gel solution and a polymerized hydrogel at the end of the test.

Observing **Figure 10**, the hydrogel displayed a sigmoidal profile and both growth and plateau phase were easily identified. Indeed, during the first 16 minutes, a dramatic increase in the absorbance was observed (growth phase). After that, a plateau was reached (plateau phase) and the absorbance remained stable until the end of the test. Nevertheless, the lag phase was not detected. Since data was recorded every two minutes, this phase may not have been detected. Another important consideration relies on the presence of nucleating centers within the solution. In fact, although the pre-gel solution was transported on ice, this hypothesis can't be excluded.

To potentiate a favorable biologic response from the host not only at the biochemical but also at the biomechanical level, it is crucial to ensure that the material will resemble the native tissue microenvironment, particularly at the biomechanical level. In this sense, the mechanical properties of the hydrogel were determined with a rheometer (see **Figure 11**).

Firstly, two experiments were performed, a strain sweep (0.01-100%) and a frequency sweep (0.1-100Hz), to determine the linear viscoelastic region (LVR) of the hydrogel. The LVR can be defined as the region in which there is a linear correlation between stress and strain. In this range, no material destruction occurs [209].

Regarding strain sweep, once frequency was fixed at 1 Hz, G' and G'' exhibited a plateau response at low strain amplitudes (from 0.015% to 0.25%) (see **Figure 11B**), with a complex shear modulus (G^*) of 1.063 kPa. This region was defined as the LVR, in which the elastic behavior of the hydrogel was predominant ($G' = 940$ Pa vs $G'' = 311$ Pa). When strain reached higher values, both parameters decreased, thus becoming stress dependent. Moreover, in the non-linear region, a crossover point was identified. In fact, beyond shear strains of 7.95%, G'' assumed higher values, in comparison to G' , and therefore the material started to behave more like a fluid. This change may indicate the destruction of the hydrogel structure, at increased shear rates.

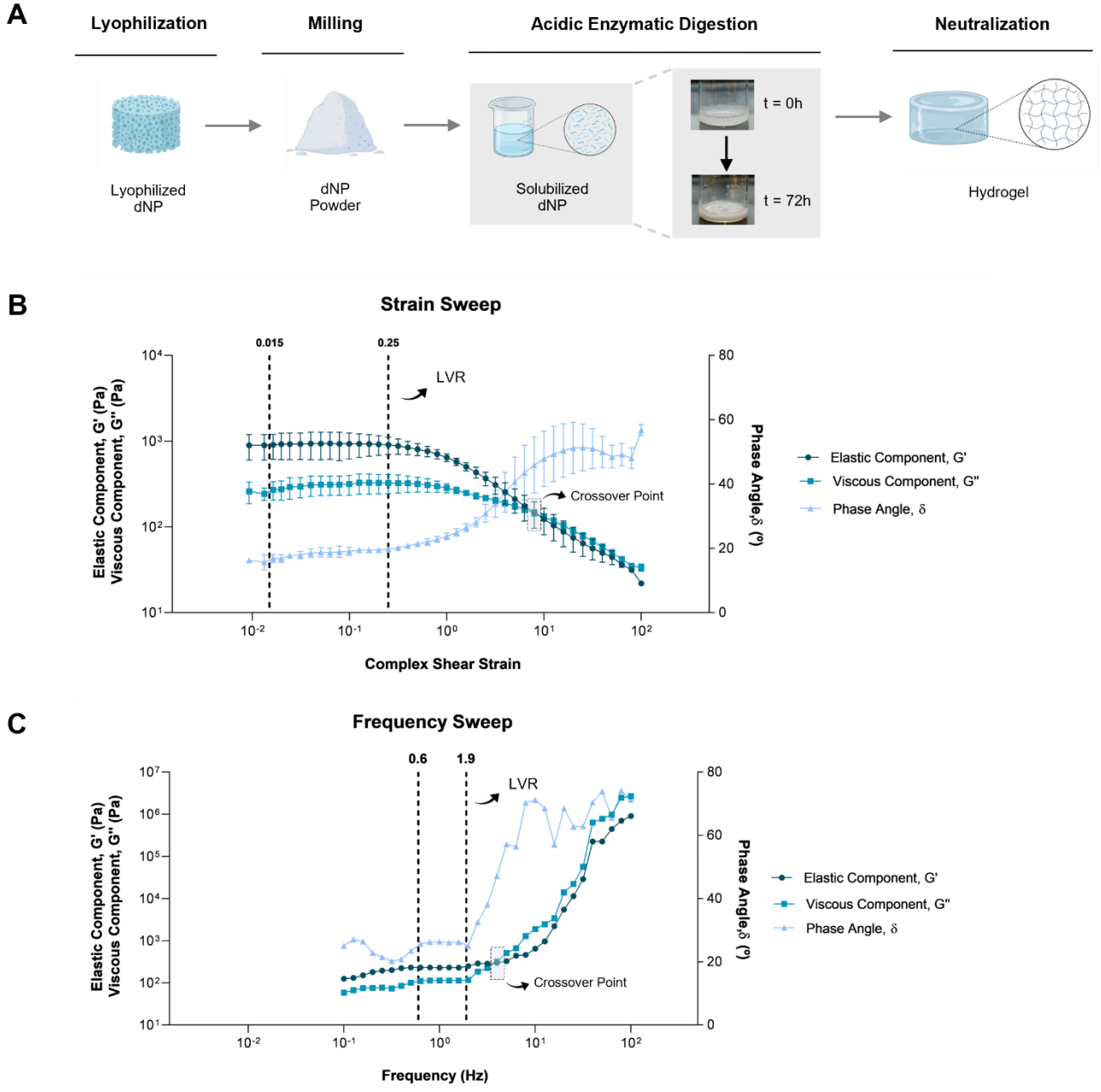


Figure 11: dECM-based hydrogel development and biomechanical characterization. Herein, it is presented a schematical representation of the hydrogel production. Briefly, this process can be divided into four phases: lyophilization, milling, enzymatic digestion and hydrogel neutralization. Previous work showed that the best conditions for hydrogel formation were the following: 40mg/mL of ECM, PBS 10x, digested with 4 mg/mL of pepsin at room temperature (RT) for 72h (A). Moreover, a strain sweep with a fixed frequency of 1Hz (B) and a frequency sweep with a fixed strain of 0.06% (C) were carried out in order to determine the linear viscoelastic region of the present hydrogel. Dashed lines limit the LVR in both experiments. dNP, decellularized nucleus pulposus. LVR, linear viscoelastic region. G' , storage modulus. G'' , loss modulus.

As for the frequency sweep tests, 0.06% was the chosen value for shear strain, as it was included in the LVR region previously determined. Herein, a plateau was reached at low frequencies, particularly in a range of 0.6 Hz to 1.9 Hz (see **Figure 11C**). As 1 Hz (the chosen frequency for the strain sweep experiment) was included in this interval, we selected this frequency for the next tests. Furthermore, it was also observed that the biomaterial reflected a gel-like behavior in the LVR, as G' was higher than G'' (230 Pa vs 114 Pa, respectively). Nevertheless, at higher frequencies (> 4 Hz), the hydrogel performance altered. Indeed, from this point on, both G' and G'' increased dramatically and G'' displayed higher values than G' .

After selecting an appropriate frequency (1 Hz) and strain amplitude (0.06%), a gelation kinetics test, depicted in **Figure 12**, was carried out. The pre-gel solution was placed in the rheometer, at 37°C, and characterized by a higher G'' in the beginning of the test ($G'' = 360.70$ Pa vs $G' = 80.49$ Pa). Nevertheless, approximately 10 seconds later, the crossover occurred ($G' = G'' = 150$ Pa), and the hydrogel started to behave predominantly as a solid. Next, G' and G'' started to increase substantially in the first four minutes and at a slower rate after that. Lastly, a plateau was reached at 21 minutes, for a G' and a G'' value of 2056 Pa and 476 Pa, respectively, as crosslinking took place.

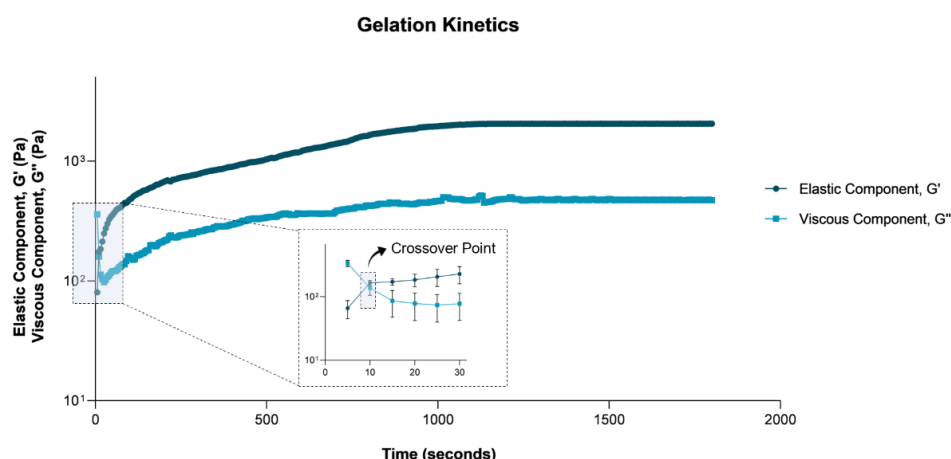


Figure 12: Gelation kinetics via rheological analysis. This experiment was carried out under the following conditions: 1 Hz, 0.06% strain and at 37°C. G' , storage modulus. G'' , loss modulus.

In conclusion, it was validated the formation of a stable hydrogel, through rheological analysis. Indeed, the current material displayed a G^* of 1.063 kPa. Torsion tests found that a healthy human NP had a shear modulus of approximately 10kPa. Thus, it is visible a huge difference between the native biomechanics and that reflected by our hydrogel. To overcome this, some groups have already tested the effect of chemical crosslinking on the mechanical properties of dECM-based hydrogels. Yu *et al* revealed that the use of 0.2% genipin gave rise to a biocompatible material with a similar elastic modulus, in comparison to the NP, thus proving their efficacy [171]. Therefore, the addition of biocompatible crosslinkers, such as genipin, could be a good strategy to create a mechanically competent biomaterial (i. e., a material that can support compressive loads and confer mechanical stability to the IVD).

INJECTABILITY

For injectable materials, there are two key performance parameters of particular importance that should be addressed: the syringeability and the injectability. While the former refers to the ability of transferring a solution from a vial to a syringe, the latter consists of assessing how easily a solution can pass through the needle and be injected into the target site [210]. In this work, we only evaluated the injectability potential of a pre-gel solution. Thus, to test the ability of the solubilized dNPs (*i. e.*, the pre-gel solution) to be injected, the samples were passed through three different needle sizes: 21G, 25G, and 30G. For further comparative purposes, suspension solutions were also examined after their preparation ($t=0$) and 7 days later ($t=7$). A PBS 1X solution was used as a control group. Results can be found in **Figure 13**.

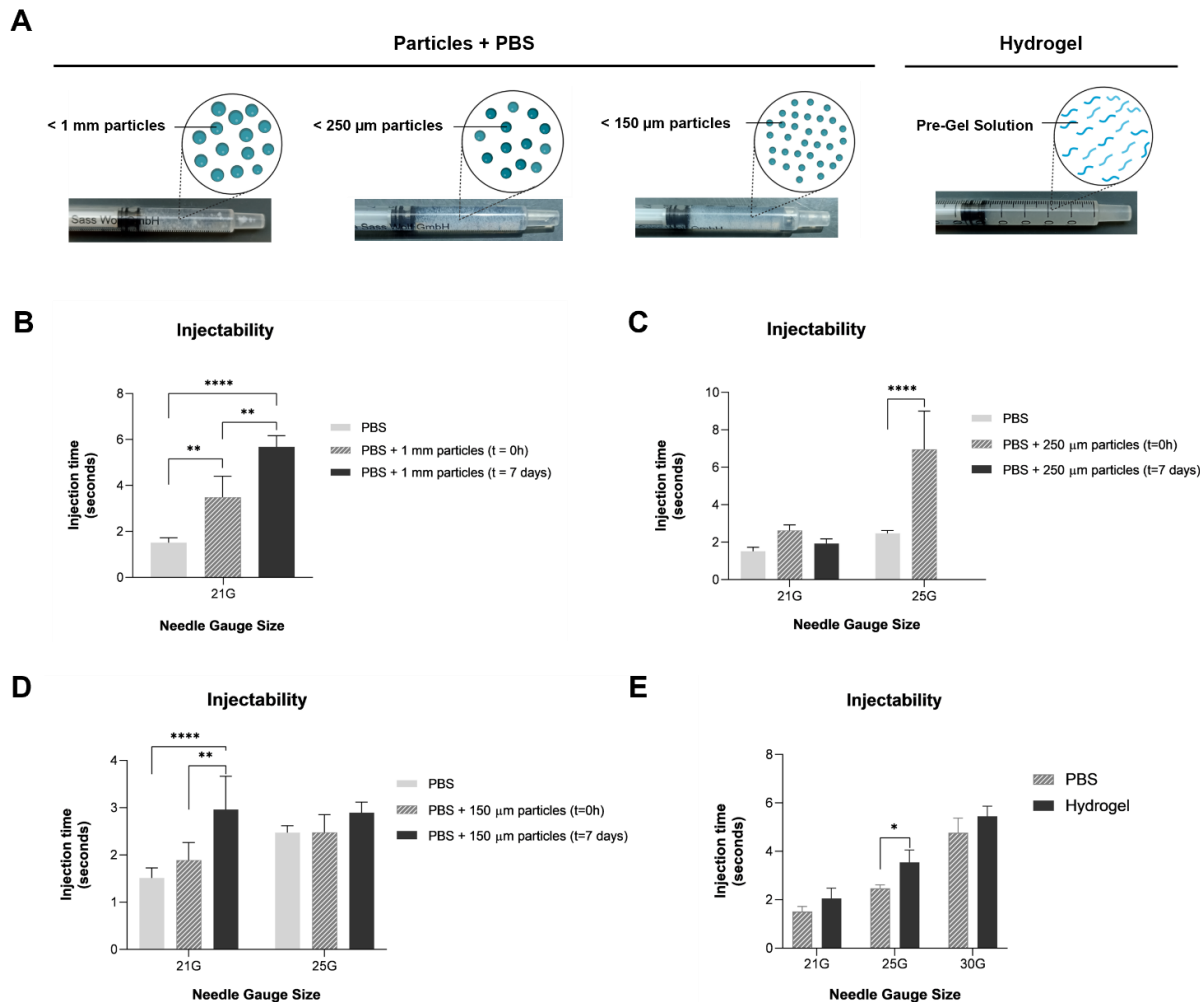


Figure 13: Assessment of the dNPs (solubilized and in particles) injectability. Herein, the multiple experimental groups that were tested were schematically represented (A). In fact, along with the pre-gel solution, the particles themselves were also injected through different needle sizes. Injectability potential was examined by recording the time needed to extrude 500 μ L of the following solutions: <1 mm (B), <250 μ m (C), and <150 μ m (D) particles suspended in a PBS solution, and finally, pre-gel solution (E). A two-way ANOVA was carried out (N=3). * $p<0.05$, ** $p<0.01$, **** $p<0.0001$. PBS, phosphate-buffered saline.

Regarding the prepared suspensions with less than 1 mm particles, it was observed that they were only able to pass through a 21G needle. In addition, the time required for the solution ejection was significantly higher than the one recorded for the PBS control. As for the 25G and 30G needles, since their diameter is lower than 1 mm (particle size), the ejection was not accomplished (see **Figure 13B**).

In turn, when the dNP powder was separated by particle size, using sieves with a mesh size of 250 μm and 150 μm , the observed effect varied. In this case, both suspensions were able to pass through 21G and 25G needles. Nevertheless, none of them was ejected through a 30G needle (see **Figure 13C** and **Figure 13D**), except for the hydrogel.

Additionally, some conclusions could also be drawn, regarding the different timepoints at which injectability was tested. Overall, an increased injection time was detected at day 7, in comparison to $t=0\text{h}$. In particular, although 250 μm particles were extruded through 25G at $t=0\text{h}$, the same did not occur after 7 days. A possible explanation for these differences relies on the potential swelling ability of the dNPs. Indeed, when in contact with PBS, these particles may have absorbed water from the surrounding, which led to an increased swelling size and consequently to ejection blockade.

Concerning the pre-gel solution, a similar tendency was identified in PBS control and experimental group. Indeed, in both, as needle diameter became smaller, the injection time recorded was higher (see **Figure 13E**). Furthermore, in this case, the pre-gel solution passed through all the needles tested, unlike the other solutions, which confers a substantial advantage to hydrogel formulations.

In conclusion, some considerations should be highlighted. In fact, when it comes to injectable applications, the needle size must be carefully selected, in order to minimize pain and damages caused to adjacent tissues. Concerning the first point, it is known that this parameter greatly impacts the pain produced during injections. Thus, to mitigate the discomfort caused to the patient, a needle with a small diameter must be chosen [211]. As for the second, in our particular case, it should be noted that the NP (our target) is surrounded by other tissues, such as the AF. As such, it is important to choose a needle that does not have a further effect on degeneration. In fact, Wang *et al* studied the effect of 25G and 21G needles on bovine AF structure. Results revealed that a more relevant damage was found when this tissue was submitted to a puncture, using the 21G [212]. Curiously, Issy *et al* analyzed the same parameters on Wistar rats, using 21G and 30G needles and the results demonstrated that even the use of the smaller size induced significant alterations in the IVD [213]. In fact, further studies need to be carried out, in order to understand which needle size is better.

SWELLING BEHAVIOR AND HYDROGEL DEGRADATION

After confirming hydrogel formation via rheology and turbidimetry, two important properties were measured: swelling and degradation rate. These properties are very important to assess hydrogel stability. With this aim in mind, a hydrogel was placed in a PBS solution at 37°C. Results are shown in **Figure 14**.

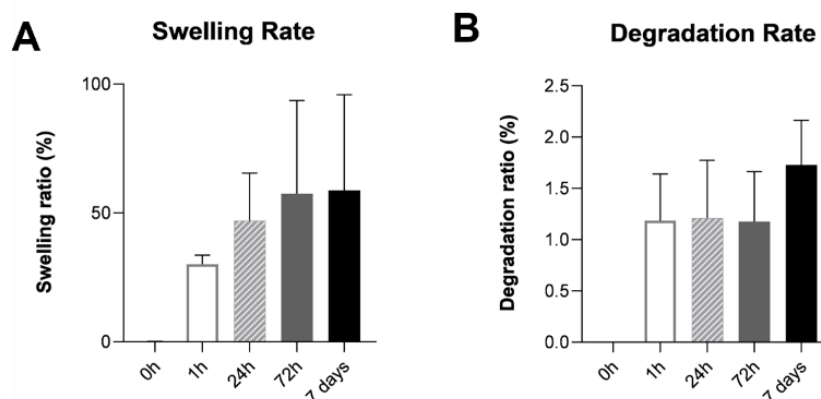


Figure 14: Swelling percentage (A) and degradation rate (B) of NP dECM-based hydrogels. Both parameters were evaluated over 7 days. There were no statistical differences between groups in both graphs. A one-way ANOVA analysis was carried out for swelling and degradation rate (N=3).

Regarding **Figure 14A**, it was observed an increase of the swelling ratio over time, and a dynamic equilibrium was reached at about 72h after immersion in PBS, with a maximum swelling ratio of approximately 58%. This plateau was achieved when swelling and elastic forces of the hydrogel network were balanced.

As for the degradation rate, the percentages remained constant in the first 72h (approximately, $1.2 \pm 0.43\%$) and slightly increased at 7 days ($1.73 \pm 0.43\%$). Nevertheless, differences were minimal and not statistically significant (see **Figure 14B**). This suggests that hydrogels did not dissolve or disintegrate while swelling occurred, remaining stable for 7 days in PBS.

By analyzing these results, several aspects should be highlighted. First, the swelling nature of the hydrogel can be explained by the hydrophilic components, that constitute this biomaterial. In fact, the presence of GAGs embedded in the collagen network may play a crucial role herein, as they are known for attracting water molecules, due to the high negative charge [214]. Another possible justification relies on hydrogel porosity, which enables water entrapment. Second, the NP is a highly hydrated tissue. As such, when developing a therapy for its regeneration, it is important to choose a biomaterial that is capable to provide a swelling pressure, in order to maintain optimal tissue hydration level [215]. In this case, it was demonstrated that our gel has an intrinsic ability to swell, which is

beneficial for the proposed clinical application (NP regeneration). Nevertheless, more tests should be performed in this area, in particular to assess hydrogel's ability to reswell and recover its initial shape after being subjected to cyclic loading [216]. Finally, considering the degradation analysis, it was demonstrated the formation of a robust and stable material. However, although these preliminary data seemed promising, further studies must estimate its degradation profile in a microenvironment similar to that present in the degenerated disc. For that, it is crucial to quantify the enzymes present in the degenerated disc [217]. Alternatively, degenerated IVDs' culture media or that of pro-inflammatory organ culture models could be used.

EVALUATION OF YOUNG BOVINE NP CELLS

In order to study the biological behavior of this biomaterial, young bovine cells were isolated from fresh NPs. After isolation, they were cultured under normoxic conditions to allow their growth. It is important to have in mind that the cells in the disc are under hypoxia, that seems to have an important function in modulating cell expression [218]. Therefore, the conditions in which these experiments were carried out do not fully mimic the *in vivo* microenvironment.

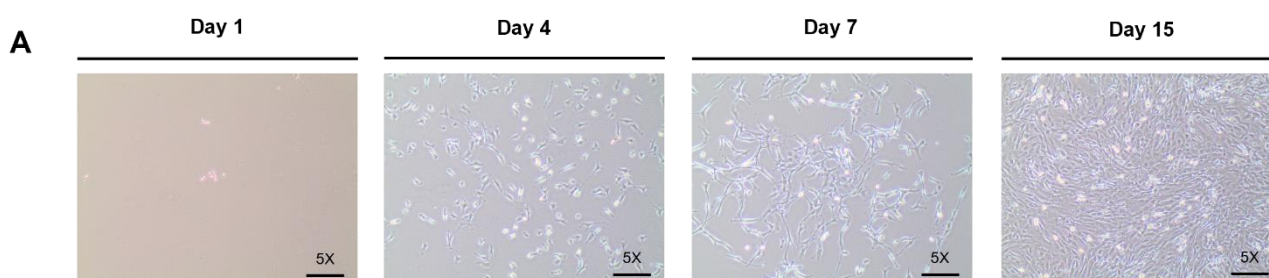


Figure 15: NP cell isolation. Images from were acquired with a 5X magnification.

In **Figure 15**, it is visible a change in morphology and cell density over time. In fact, while at day four, most of the cells still presented a rounded shape, by day 15, they were well spread on the cell culture flask. Moreover, they revealed a colony growth, which indicates their capacity to adhere to the cell culture flask.

CYTOTOXICITY

Previous tests confirmed the efficacy of the adopted decellularization protocol and indicated a hydrogel with promising features. Nevertheless, its biocompatibility also had to be assessed, in order to guarantee the production of a safe biomaterial.

According to the European regulation, hydrogels are classified as medical devices. Thus, their manufacture must follow certain standards to ensure the safety and quality of these materials. Concerning the biological behavior of medical devices, International Organization for Standardization (ISO) 10993 comprises a series of standards to study the biocompatibility.

Considering ISO 10993-5, three different assays were taken into account: extract test, indirect contact, and direct contact. In this case, only the extraction and direct contact assays were performed, as described in **Figure 16**.

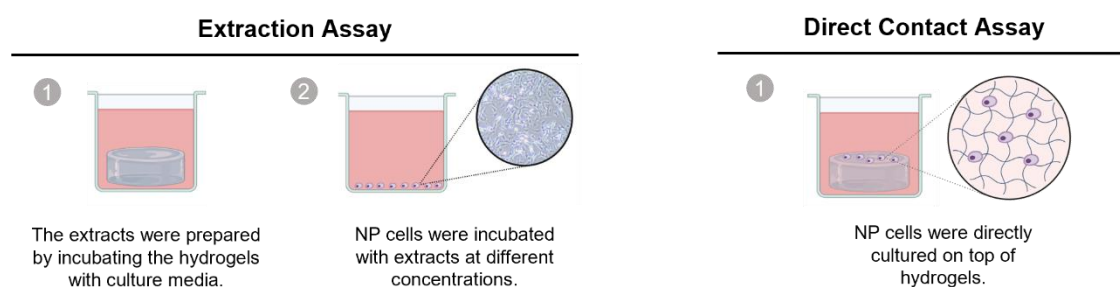


Figure 16: Schematic representation of the two assays (extraction and direct contact) that were conducted for assessing hydrogel toxicity. NP, nucleus pulposus.

Moreover, for quantitative purposes, ISO 10993-5 established that a reduction of cell viability by more than 30 % must be considered a cytotoxic effect. Results from extracts and direct contact assays are presented in **Figure 17** and **Figure 18**, respectively.

For this experiment, cells treated with 70% ethanol were used as a positive control. As expected, over time, a significant decline in cell viability ($p < 0.0001$) was observed ($14 \pm 0.56\%$, by day 7). This change was accompanied by a severe morphological alteration, mainly characterized by a rounded shape and a loss of cell-cell interactions (see **Figure 17A**). None of the tested concentrations exhibited such a drastic decrease, as the positive control (see **Figure 17B**). Among all experimental groups, cells treated with 100% extracts were those that revealed a more significant ($p < 0.05$) reduction in viability (a decrease of 22% after 7 days). In turn, in the presence of 75% extracts, the decrease was much less evident (approximately, 10%). Finally, cells cultured with lower doses (50% and 25%) showed the same tendency. First, the viability percentage increased until day 3. Then, it slightly decreased. Nevertheless, cell viability was higher than 70% in all experimental groups. As for

cell morphology, results were in agreement with the quantitative analysis. Indeed, while it was visible the presence of some rounded cells in the group treated with 100% extracts, in the other three, cells retained their spindle shape, as the negative control.

According to these results, a different trend was detected, regarding extract concentration. While at higher doses (100% and 75%), viability was affected, at lower concentrations, cell behavior did not seem to be significantly altered. This can be explained by the presence of compounds with a certain toxicity that became diluted in the “75%”, “50%” and “25%” groups.

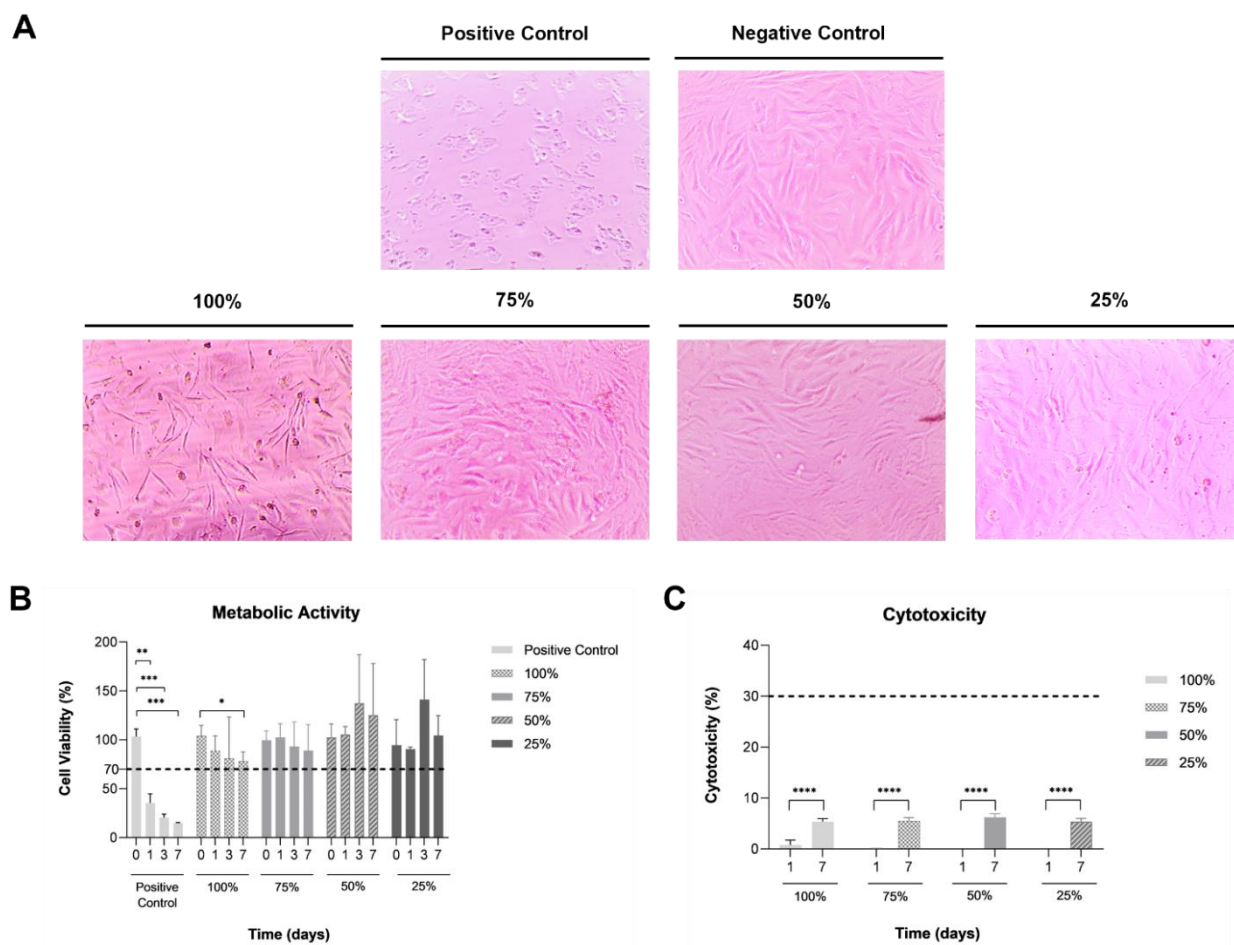


Figure 17: Cytotoxic analysis of the extracts. To evaluate cytotoxicity, cell morphology was assessed (A). Images were taken 7 days after cells were treated with the extracts, with a 10X magnification. A quantitative analysis was performed, regarding the metabolic activity (B) and cytotoxicity (C). For cell viability, a percentage below 70% indicates toxicity. A two-way ANOVA, followed by a Tukey's multiple comparisons test was carried out for metabolic activity and cytotoxicity measurement (N=3). * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. LDH, lactate dehydrogenase.

As for the direct contact assay, after one day in culture, cell viability decreased from $71.21 \pm 20.73\%$ to $49.91 \pm 7.15\%$ (see **Figure 18A**). In turn, from day 1 to day 7, a significant increase ($p < 0.001$) was observed ($49.91 \pm 7.15\%$ to $68.12 \pm 7.15\%$), possibly due to cell proliferation. Nevertheless, viability remained under 70%. In this particular case, the obtained values may not

reflect toxicity. Instead, they can be explained by a low performance of the resazurin assay. As described in **Figure 16**, for this test, cells were cultured on top of the hydrogels, however, it is highly possible that some had migrated into deeper zones. Thus, when resazurin was added to the culture medium, its entrapment and low diffusion may have prevented this dye from reaching the inner parts of the biomaterial, reducing the accuracy of the colorimetric method [219].

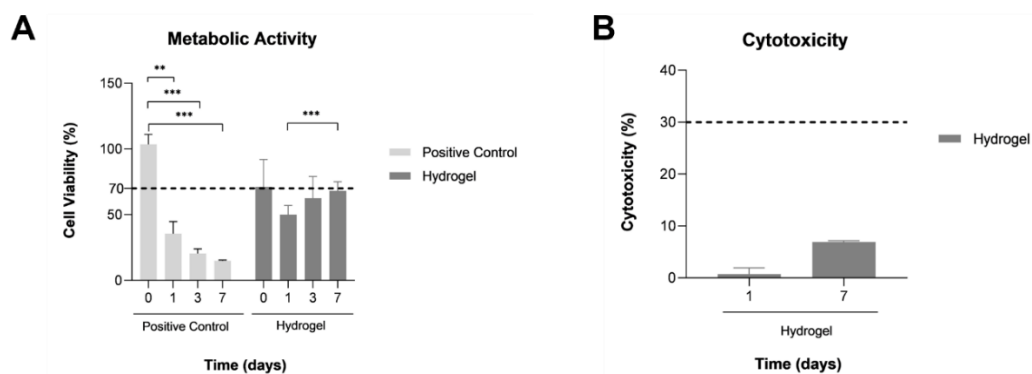


Figure 18: Metabolic activity (A) and cytotoxicity analysis (B) for direct contact assay. For cell viability, a percentage below 70% indicates toxicity. A two-way ANOVA was carried out for metabolic activity and cytotoxicity measurement (N=3). **p<0.01. ***p<0.001. LDH, lactate dehydrogenase.

In addition to the metabolic activity, cytotoxicity was also evaluated, via LDH assay, at day 1 and 7. In fact, this is a useful method, which aims to quantify enzymes (in this case, LDH) that are released from damaged cells [220]. In general, an increase in toxicity was observed, regardless of the extract concentration (see **Figure 17C**). Although the differences were statistically significant ($p<0.0001$), the percentage obtained (approximately, 5%) did not exceed 30%. In turn, when in contact with the biomaterial, cytotoxicity was equal to $6.89 \pm 0.24\%$ (see **Figure 18B**). In this case, no statistical differences were found.

By comparing these results with those from metabolic activity, some inconsistencies were detected. Generally, there is a correlation between cell viability and cytotoxicity (lower metabolic activity can be translated into a higher toxicity level) [221]. However, from day 1 to day 7, cells cultured in the presence of 50% extract, 25% extract, and in direct contact with the hydrogel showed an increased cell viability and a higher cytotoxicity. These can be explained by cell proliferation. In fact, although LDH assay identified a higher percentage of cell death, the remaining active cells were able to proliferate and replace the ones that were lost.

In conclusion, although these two assays are valuable tools for determining biomaterial biocompatibility of injectable materials, it is crucial to have in mind their limitations. Thus, it is mandatory to carry out further tests (e.g., toxicity tests in hypoxic conditions) in order to establish a more accurate toxic profile of the hydrogel.

CELL PROLIFERATION

For IVD regeneration, besides the mechanical properties already discussed, the ideal approach would also provide a scaffold able to promote cell growth, differentiation, survival, and migration, as well as matrix production [6]. In this sense, cells were cultured on top of the hydrogels and their proliferative behavior was assessed after 1, 3, and 7 days in culture, through different methods. Results can be found in **Figure 19** and **Figure 20**.

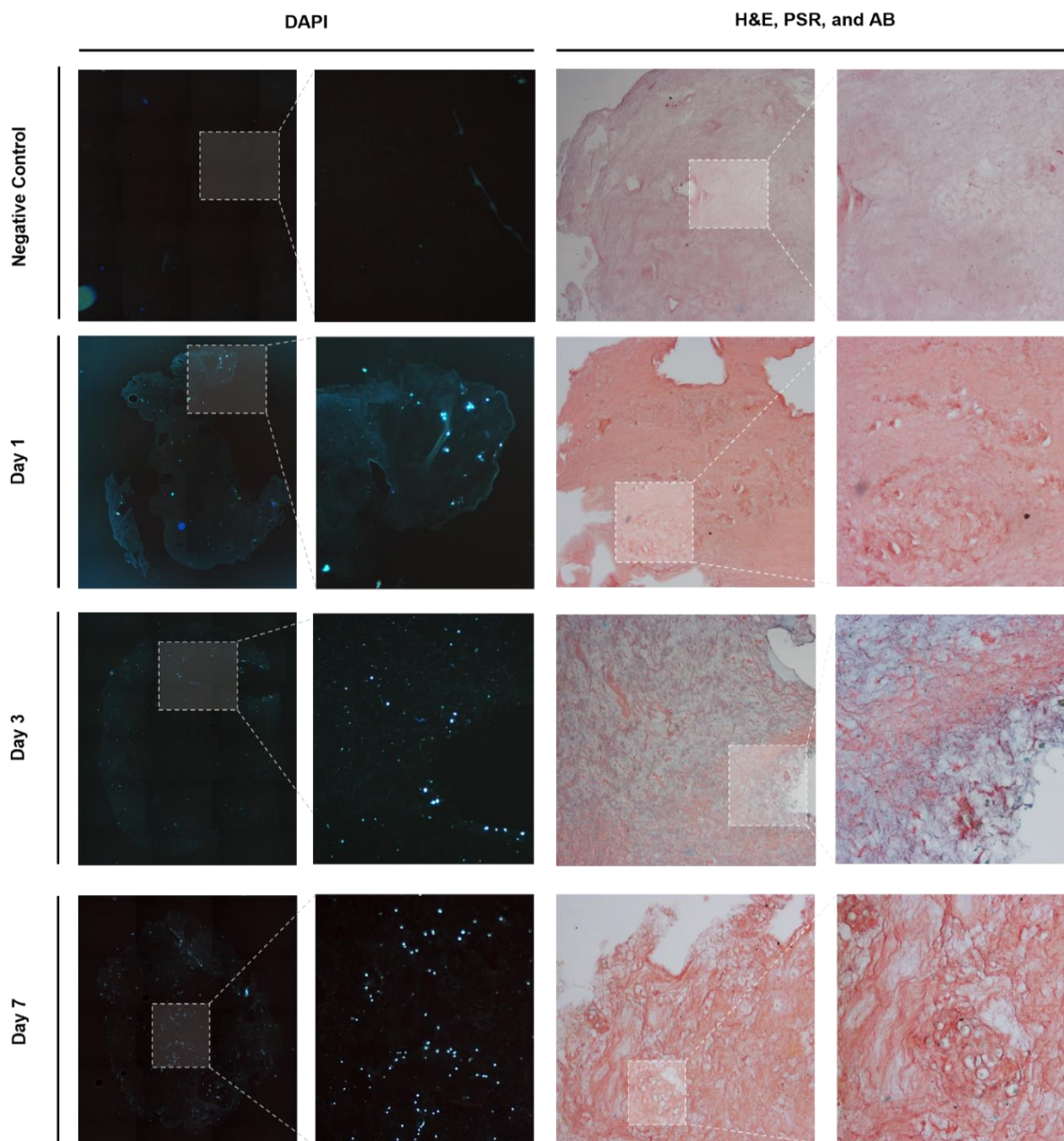


Figure 19: Cell proliferation assessed by DAPI and H&E, PSR and AB staining. Cells were cultured on top of hydrogels and their density and distributions was assessed by H&E, PSR and AB staining. Images from hydrogels were acquired with a 10X and 20X magnification. AB, Alcian Blue. DAPI, 4',6-diamidino-2-phenylindole. H&E, hematoxylin and eosin. PSR, PicroSirius Red.

For a qualitative assessment, gels without cultured cells were used as a negative control (see **Figure 19**). Considering the DAPI staining, no significant fluorescent signal was identified in the control. This confirmed once again that decellularization was well-succeeded and, therefore, that the signal observed in the experimental groups was not associated with the presence of cells that had been inefficiently removed from the dNP and remained in the tissue. As for the hydrogels cultured with NP cells, a significant increase in fluorescence intensity was visible over time, indicative of cell proliferation. In fact, the blue dots were substantially more abundant at day 7 in comparison to day 1 and 3.

Another important conclusion relies on cell distribution. While at day 1, cells were mainly concentrated at the border of the gel, at day 7, they were evenly distributed throughout the entire piece. These results might indicate cell ability to migrate towards the center of the gel.

Regarding H&E, PSR and AB staining, cells were detectable at day 3 and 7. Although DAPI had demonstrated the presence of nuclei within the gel at day 1, no nuclear components were found with H&E staining in the histological slides. This may be explained by a deeper section that might not have captured these cells in these histological cuts. Observing images from day 7, it is possible to identify the presence of cell clusters, possibly as a result of cell proliferation.

In addition, other aspect must be highlighted. Indeed, besides cell proliferation, a change in ECM organization was also detected. In fact, while the matrix was uniformly stained in the negative control (collagen fibers formed a robust network), the ECM components appeared to be sparser in hydrogels at day 3 and 7. It is known that, when ECM is remodeled, there might be changes in pore size [222]. Thus, the presence of a more porous matrix may be a sign of cell activity and, consequently, ECM remodeling. As for quantitative methods, DNA present in hydrogels was also quantified (see **Figure 20**).

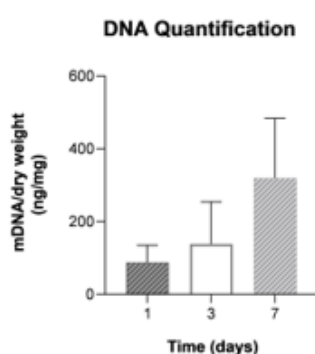


Figure 20: DNA Quantification. Herein, it is presented a quantitative analysis regarding DNA in hydrogels cultured with NP cells. There were no statistically significant differences. A one-way ANOVA, followed by a Tukey's multiple comparisons test was carried out for metabolic activity and cytotoxicity measurement (N=3).

Although no statistical differences were found among the three timepoints, an increase was observed over time (87.67 ± 47.00 ng/mg at day 1 vs 319.68 ± 164.25 ng/mg at day 7). So, it can be inferred that cells proliferated for 7 days. These results are in agreement with the histological images.

OPTIMIZATION OF YOUNG BOVINE IVD DECELLULARIZATION SYSTEM

With the biological confirmation of the dECM-based hydrogel, it is crucial to optimize the decellularization system, in order to reduce the number of time-consuming steps and bring its production closer to an industrial scale.

Preliminary tests assessed the efficiency of three different systems, one with agitation (AS), another with vacuum-assisted perfusion (VAP), and the last one combining both agitation and vacuum (VAS). Decellularization setups are present in **Figure 21**.

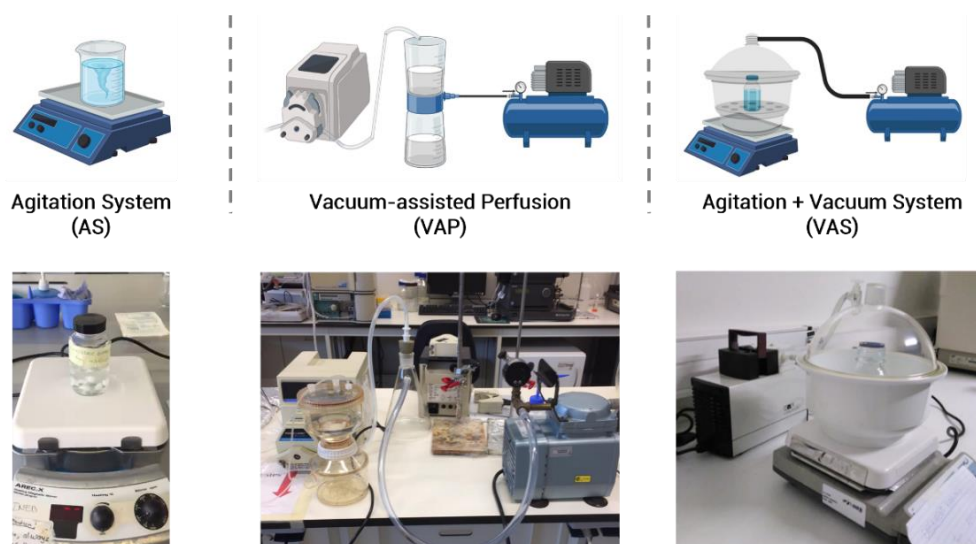


Figure 21: Decellularization Systems for a Scale-Up Approach. Herein, it is represented three systems that were tested, in order to optimize the scale-up conditions for young bovine NP decellularization. Briefly, the AS system was solely constituted by a magnetic stirrer under agitation. The VAP setup was composed of a reusable filter unit with two side-arms, connected to a peristaltic and a vacuum pump. Lastly, the VAS system, in addition to the magnetic stirrer, had a vacuum pump connected to a desiccator.

It was determined that the VAS was the most efficient experimental setup, since it achieved an optimal compromise between cell removal (high %DNA removal) and retention of matrix components (highest %sGAG preservation within the tissue), and therefore, it became the chosen system for further tests. As VAS was the optimized scale-up process, it will be further referred as the scale-up system (SUS).

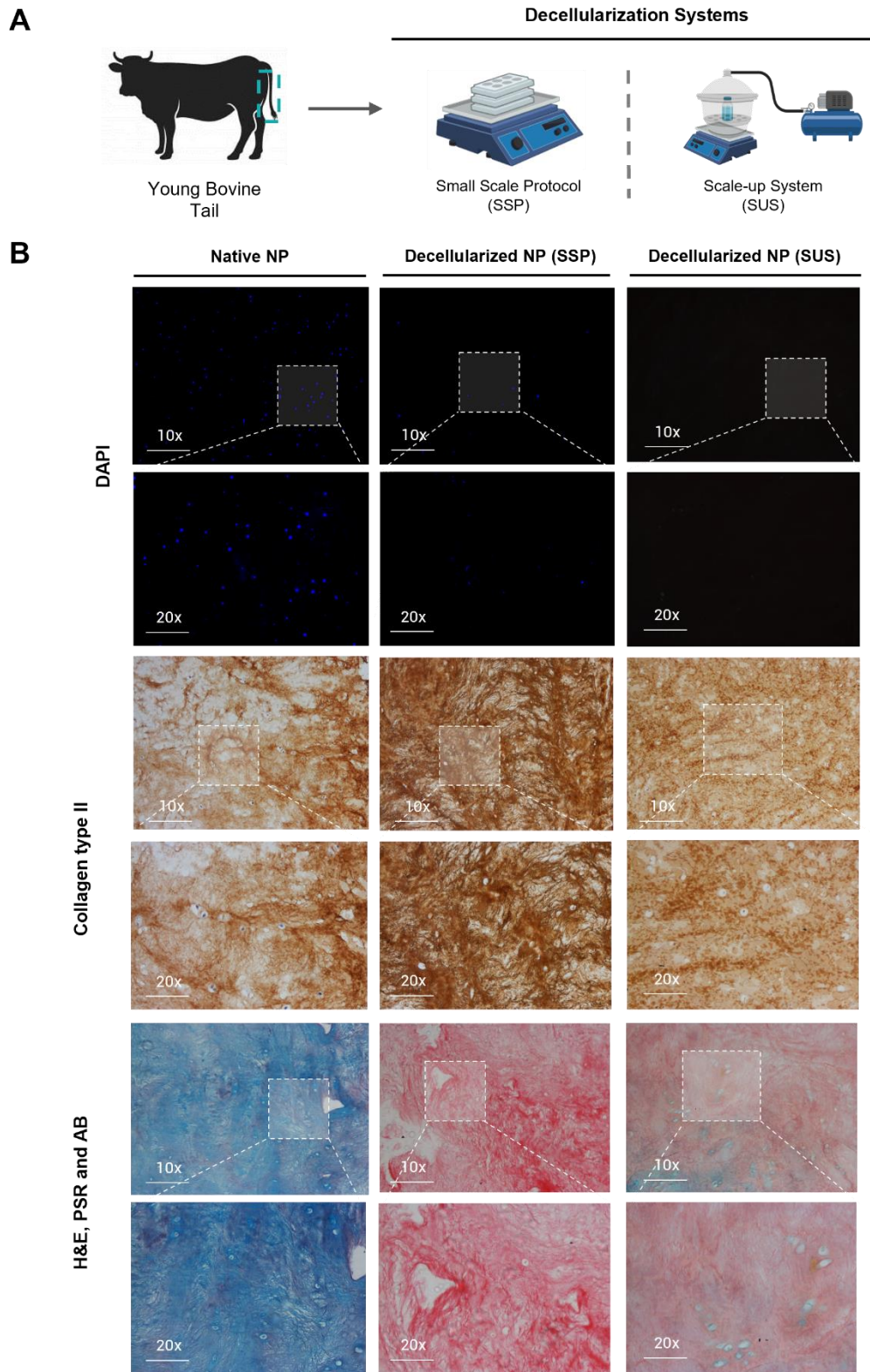


Figure 22: Comparison of two decellularization systems for young IVDs. Herein, two different decellularization systems were tested: SSP with agitation and SUS with both agitation and vacuum (A). Decellularized samples were evaluated qualitatively, by DAPI, PSR, AB, H&E staining and by collagen type II immunohistochemistry (B). All images were acquired with a 10X and 20X magnification. AB, Alcian Blue. DAPI, 4',6-diamidino-2-phenylindole. H&E, hematoxylin and eosin. NP, nucleus pulposus. PSR, PicroSirius Red. sGAG, sulfated glycosaminoglycans. SSP, small-scale protocol. SUS, scale-up system.

DAPI staining showed a significant reduction of cell nuclei (see **Figure 22B**). These results were qualitatively confirmed by H&E staining, that revealed a high degree of cell removal, and quantitatively by DNA quantification. Indeed, while a total mass of 375.54 ± 178.64 ng/mg was found in the native tissue, a DNA content of 11.02 ± 8.16 ng/mg was present in the decellularized tissue, which resulted in a DNA removal efficiency of 95.43%. This percentage was slightly lower in comparison to the previous setup (DNA removal of 98.31%) (see **Figure 23A**).

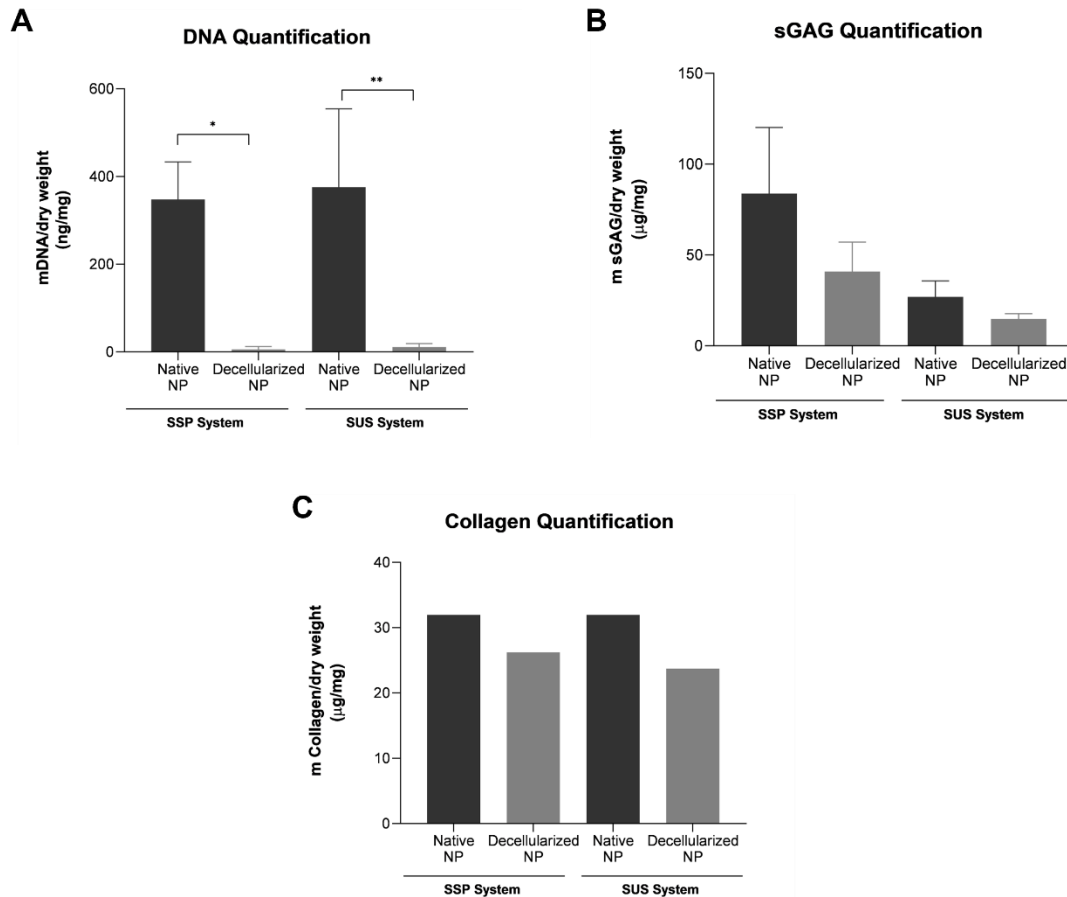


Figure 23: Quantitative analysis of the two decellularization systems tested for young IVDs. Herein, a quantitative DNA (A), sGAGs (B) and collagen (C) analysis is presented. A one-way ANOVA, followed by a Tukey's multiple comparisons test was carried out for DNA and sGAGs quantification (N=3). NP, nucleus pulposus. sGAGs, sulfated glycosaminoglycans. SSP, small-scale protocol. SUS, scale-up system.

As for the GAG analysis, data showed a strong reduction of blue staining in both decellularization procedures. Nevertheless, this setup appears to retain more GAGs than the previous system, as blue staining is still observed within the decellularized tissue (see **Figure 22B**). Furthermore, a quantitative analysis confirmed a high sGAG content decrease (26.92 ± 8.81 µg/mg in native IVD vs 14.74 ± 2.96 µg/mg in dNP) and revealed that SUS was the system that preserved more sGAGs within the tissue,

with a retention rate of approximately 54.76%, in comparison to a retention percentage of 48.75% in the previous decellularization system (see **Figure 23B**).

Finally, regarding collagen type II, none of the systems had a significant impact on the elimination of this molecule (see **Figure 22B**). The quantification revealed a lower preservation of this protein in the young NPs decellularized via SUS, in comparison to the previous system (see **Figure 23C**).

In conclusion, it was shown that the vacuum-assisted decellularization displayed elevated efficiency rates (high %DNA removal and ECM preservation), comparable to that obtained by the previous time-consuming system. Thus, the replacement of the previous setup by this easy and less time-consuming decellularization may be an important step towards the scale-up production of young bovine dNPs.

DECELLULARIZATION OF FETAL BOVINE IVDs

Fetal IVDs have been proposed to have a huge pro-regenerative potential, that may be crucial for IVD therapeutics. In fact, they not only possess unique ECM components (collagen type XII and XIV), that appear to be involved in regeneration and whose expression is suppressed in young disc [26], but also contain collagen fibers with fewer cross-links, responsible for their faster turnover and remodeling [187]. In addition, fetuses express a higher GAG content, important in cell proliferation and migration [188].

Bearing this in mind, the use of decellularized fetal tissue seems to be a promising field that should be further explored. In this sense, the two decellularization systems used for young bovine IVDs were tested for this tissue (see **Figure 24A**) (the small-scale procedure – SUS – and the optimized scale-up process – SUS), as an attempt to scale-up the decellularization process for this material. A qualitative and quantitative analysis were carried out, in order to better understand which system would be more appropriate for fetal decellularization. Results are presented in **Figure 24B** and **Figure 25**.

Concerning DAPI staining, a huge disparity in cell removal efficiency could be observed between the two systems. While in the SSP, a high fluorescence signal was still detected (which was comparable to the native control), in the SUS, almost no staining was found. In fact, some cell nuclei were still present in the tissue, confirming an inefficient cell removal in the former system (see **Figure 24B**). In this case, a higher efficiency in the SUS may be a result of a deeper solution penetration provided by the use of vacuum. DNA quantification revealed a higher decrease in DNA content in SUS decellularization (3168.24 ng/mg in the native vs 43.99 ng/mg in the dNP), when compared to the SSP (4643.78 ng/mg in the native vs 1090.12 ng/mg in the dNP). In addition, while the first system displayed a total DNA mass of less than 50ng/mg, the second did not (see **Figure 25A**). Thus, the material produced with SUS may induce a severe immune response from the host, which turns it into an inappropriate scaffold for IVD regeneration.

Data showed a strong reduction of blue staining in both decellularization procedures. Nevertheless, considering the images obtained from histology, it was visible that NPs obtained by SUS decellularization were those that retained the highest GAG content – presence of blue staining around empty spots in the matrix. Despite the lack of significant differences, a quantitative analysis revealed that the SUS was the system that preserved more sGAGs within the tissue, with a retention rate of approximately 58% (19.04 µg/mg in the decellularized tissue vs 32.53 µg/mg in the native). The SSP, in turn, produced a dNP with a total sGAG mass of 16.08 µg/mg, in comparison to an initial mass of 33.57 µg/mg (conserved approximately 47.91% of these molecules) (see **Figure 25B**).

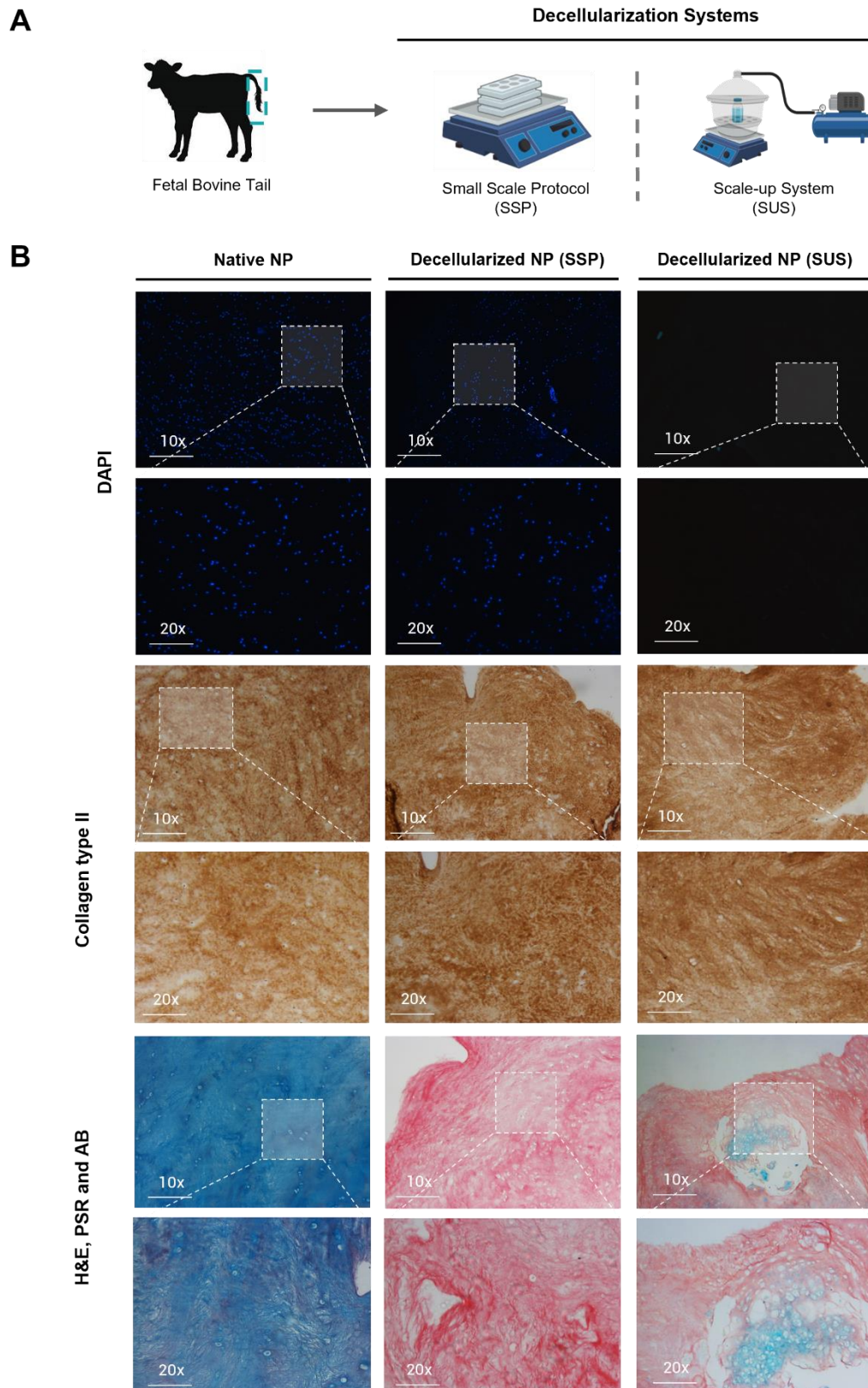


Figure 24: Comparison of two decellularization systems for fetal IVDs. Herein, two different decellularization systems were tested: SSP with agitation and SUS with both agitation and vacuum (A). Their efficiency was tested by DAPI, H&E, PSR and collagen type II staining (B). All images were acquired a 10X and 20X magnification. AB, Alcian Blue. DAPI, 4',6-diamidino-2-phenylindole. H&E, hematoxylin and eosin. NP, nucleus pulposus. PSR, PicroSirius Red. SSP, small-scale protocol. SUS, scale-up system.

As for collagen type II, both experimental groups displayed an identical staining intensity, indicating that none of the systems had a great impact on the retention of this molecule (see **Figure 24B**). Finally, collagen quantification revealed a higher preservation of this protein in the fetal NPs decellularized via SUS, in comparison to the SSP (see **Figure 25C**).

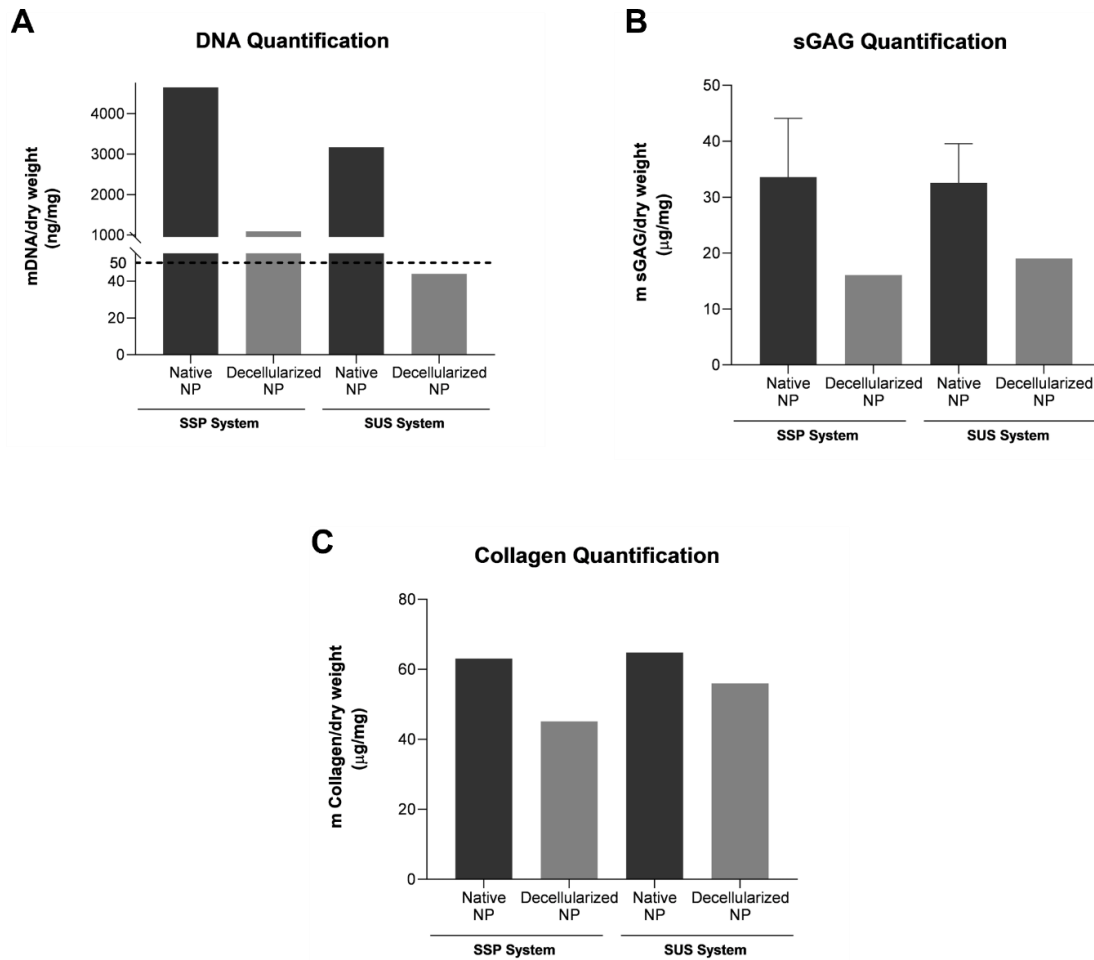


Figure 25: Quantitative analysis of the two decellularization systems used for fetal IVDs. Herein, a quantitative DNA (A), sGAGs (B) and collagen (C) analysis is presented. NP, nucleus pulposus. sGAGs, sulfated glycosaminoglycans. SSP, small-scale protocol. SUS, scale-up system.

In conclusion, these preliminary results showed that a vacuum-assisted decellularization should be preferred for fetal NP, as a more adequate compromise between cell removal and ECM preservation was achieved. In fact, these conclusions are in line with other findings. To date, some groups have developed vacuum-assisted systems for decellularization of different tissues, such as human and rabbit trachea and heart valves. Indeed, these three studies agreed that vacuum was able to reduce the time required for decellularization and produce a dECM-based scaffold with optimal features [223-225]. Nevertheless, more replicates must be performed to confirm that the differences found were due to the use of vacuum and not by chance or by mere variability.

This page was intentionally left in blank.

CHAPTER IV

CONCLUSIONS AND FUTURE PERSPECTIVES

Results revealed that it was possible to obtain hydrogels from young decellularized bovine NPs. Decellularization using a 0.1% SDS-based protocol was effective in removing over 98.31% of DNA while partially preserving GAGs (48.75%) and collagen (82.07%), with no significant traces of SDS being kept in the material after processing.

Hydrogels were produced by an enzymatic digestion, in which 40 mg of lyophilized and milled dNPs were added to a 5 mg/mL pepsin-acetic acid solution. After 72h, the hydrogel was neutralized to physiological pH and buffered with PBS 10X. This material appeared to be mechanically stable for at least one week and, contrarily to decellularized tissue particles <150µm, easily injectable through needles from 21G to 30G. This is important for product storage and preparation under a clinical setting and to be injected without promoting further degeneration in both animals (e.g rat models, dogs) and humans.

Cell culture experiments showed that the developed NP dECM-based hydrogels did not display high levels of cytotoxicity for bovine NP cells which were able to survive and proliferate in the hydrogels, either in direct contact or extraction assays. Further studies should investigate if cells are able to produce healthy endogenous ECM when cultured on these materials.

Finally, envisioning further product development in an industrial context, a scale-up decellularization procedure was successfully validated in both fetal and young bovine IVDs. Indeed, it was concluded that the scale-up system, composed by a magnetic agitator and a vacuum pump, produced promising results, since it achieved an optimal compromise between cell removal (high %DNA removal) and retention of matrix components (highest %sGAG preservation within the tissue), comparable to that obtained using the small-scale more time-consuming bench agitation system.

This NP dECM-based hydrogel could be further tailored, in order to improve both biochemical and biomechanical features. In fact, concerning its biochemical composition, it was observed an extensive GAG loss upon decellularization, possibly due to the use of SDS, thus creating a hydrogel with a low content. In this sense, two strategies could be adopted to overcome this problem: inject the hydrogel together with autologous IVD or MSC cells for ECM production [205], or supplementing the hydrogel with GAG components (e.g., chondroitin sulfate) [206].

As for the biomechanics, it was demonstrated that the produced gel displayed different mechanical properties from the native NP. To improve biomechanical features, the addition of biocompatible crosslinkers, such as genipin, could be a good strategy to create a mechanically competent biomaterial (i. e., a material that can support compressive loads and confer mechanical stability to the IVD). Furthermore, the combination of dECM-based materials with natural or synthetic polymers could also improve these features. Nevertheless, in these cases, cytotoxicity should be reassessed, to guarantee that the biocompatibility is not disturbed.

At the biological level, it is also important to assess the behavior of this hydrogel under hypoxic and pro-inflammatory conditions (which mimic the native nature of the IVD), in order to understand if a similar cell response is obtained. Furthermore, as this material will be injected into a herniated disc, it remains necessary to test its effect on degenerated NP cells, to see if the degenerative characteristics are reverted. Thus, it would be interesting to isolate human NP cells from herniated discs and even cells from dogs (that also suffer from low back pain).

All in all, this work provided essential insights for the establishment of a pioneer treatment for IVD degeneration and Low Back Pain, fostering translation into the clinics and product commercialization. The NP dECM-based hydrogel herein developed also holds potential to improve the already promising MSC-based therapies. In addition, these hydrogels may also be used as potential substitutes of organ-cultures, to further model IVDs under physiological and pathological states.

REFERENCES:

- [1] X. Zhou *et al.*, "Injectable decellularized nucleus pulposus-based cell delivery system for differentiation of adipose-derived stem cells and nucleus pulposus regeneration," *Acta Biomaterialia*, vol. 81, pp. 115-128, 2018/11/01/ 2018, doi: <https://doi.org/10.1016/j.actbio.2018.09.044>.
- [2] W. C. W. Chan, K. L. Sze, D. Samartzis, V. Y. L. Leung, and D. Chan, "Structure and Biology of the Intervertebral Disk in Health and Disease," *Orthopedic Clinics of North America*, vol. 42, no. 4, pp. 447-464, 2011, doi: 10.1016/j.ocl.2011.07.012.
- [3] J. Wang *et al.*, "Therapeutic effect of the injectable thermosensitive hydrogel loaded with SHP099 on intervertebral disc degeneration," *Life Sci*, vol. 266, p. 118891, Feb 1 2021, doi: 10.1016/j.lfs.2020.118891.
- [4] WHO. "Priority diseases and reasons for inclusion: low back pain." http://www.who.int/medicines/areas/priority_medicines/ch6_24LBP.pdf (accessed 22/06/2021).
- [5] R. A. Wachs, E. N. Hoogenboezem, H. I. Huda, S. Xin, S. L. Porvasnik, and C. E. Schmidt, "Creation of an injectable in situ gelling native extracellular matrix for nucleus pulposus tissue engineering," *Spine Journal*, vol. 17, no. 3, pp. 435-444, 2017, doi: 10.1016/j.spinee.2016.10.022.
- [6] M. D'Este, D. Eglin, and M. Alini, "Lessons to be learned and future directions for intervertebral disc biomaterials," *Acta Biomaterialia*, vol. 78, pp. 13-22, 2018, doi: 10.1016/j.actbio.2018.08.004.
- [7] W. JA, R. V, and F. B. "Anatomy, Back, Intervertebral Discs." <https://www.ncbi.nlm.nih.gov/books/NBK470583/> (accessed).
- [8] P. J. Roughley, "Biology of intervertebral disc aging and degeneration: Involvement of the extracellular matrix," *Spine*, vol. 29, no. 23, pp. 2691-2699, 2004, doi: 10.1097/01.brs.0000146101.53784.b1.
- [9] N. Newell, J. P. Little, A. Christou, M. A. Adams, C. J. Adam, and S. D. Masouros, "Biomechanics of the human intervertebral disc: A review of testing techniques and results," *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 69, no. January, pp. 420-434, 2017, doi: 10.1016/j.jmbbm.2017.01.037.
- [10] O. M. Torre, V. Mroz, M. K. Bartelstein, A. H. Huang, and J. C. Iatridis, "Annulus fibrosus cell phenotypes in homeostasis and injury: implications for regenerative strategies," *Ann N Y Acad Sci*, vol. 1442, no. 1, pp. 61-78, Apr 2019, doi: 10.1111/nyas.13964.
- [11] V. Sivakamasundari and T. Lufkin, "Bridging the Gap: Understanding Embryonic Intervertebral Disc Development," *Cell & Developmental Biology*, vol. 1, 05/01 2012, doi: 10.4172/cdb.1000103.
- [12] D. H. Cortes and D. M. Elliott, "The intervertebral disc: Overview of disc mechanics," Springer-Verlag Wien, 2014, pp. 17-31.
- [13] F. Mwale, "Collagen and other proteins of the nucleus pulposus, annulus fibrosus, and cartilage end plates," Springer-Verlag Wien, 2014, pp. 79-92.
- [14] G. Pattappa, Z. Li, M. Peroglio, N. Wismer, M. Alini, and S. Grad, "Diversity of intervertebral disc cells: phenotype and function," *J Anat*, vol. 221, no. 6, pp. 480-96, Dec 2012, doi: 10.1111/j.1469-7580.2012.01521.x.
- [15] F. Galbusera, M. van Rijsbergen, K. Ito, J. M. Huyghe, M. Brayda-Bruno, and H. J. Wilke, "Ageing and degenerative changes of the intervertebral disc and their impact on spinal flexibility," *Eur Spine J*, vol. 23 Suppl 3, pp. S324-32, Jun 2014, doi: 10.1007/s00586-014-3203-4.
- [16] P. P. Raj, "Intervertebral Disc : Anatomy-Physiology-Pathophysiology-Treatment," vol. 8, no. 1, pp. 18-44, 2008.
- [17] P. Y. Hwang, J. Chen, L. Jing, B. D. Hoffman, and L. A. Setton, "The role of extracellular matrix elasticity and composition in regulating the nucleus pulposus cell phenotype in the intervertebral disc: A narrative review," *Journal of Biomechanical Engineering*, vol. 136, no. 2, 2014, doi: 10.1115/1.4026360.

- [18] S. Molladavoodi, J. McMorran, and D. Gregory, "Mechanobiology of annulus fibrosus and nucleus pulposus cells in intervertebral discs," *Cell and Tissue Research*, vol. 379, no. 3, pp. 429-444, 2020/03/01 2020, doi: 10.1007/s00441-019-03136-1.
- [19] S. M. Richardson, F. E. Ludwinski, K. K. Gnanalingham, R. A. Atkinson, A. J. Freemont, and J. A. Hoyland, "Notochordal and nucleus pulposus marker expression is maintained by sub-populations of adult human nucleus pulposus cells through aging and degeneration," *Sci Rep*, vol. 7, no. 1, p. 1501, May 4 2017, doi: 10.1038/s41598-017-01567-w.
- [20] R. J. Moore, "The vertebral endplate: disc degeneration, disc regeneration," (in eng), *European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society*, vol. 15 Suppl 3, no. Suppl 3, pp. S333-S337, 2006, doi: 10.1007/s00586-006-0170-4.
- [21] J. P. G. Urban, S. Smith, and J. C. T. Fairbank, "Nutrition of the Intervertebral Disc," *Spine*, vol. 29, no. 23, pp. 2700-2709, 2004, doi: 10.1097/01.brs.0000146499.97948.52.
- [22] M. Sharabi, K. Wade, and R. Haj-Ali, "The mechanical role of collagen fibers in the intervertebral disc," Elsevier, 2018, pp. 105-123.
- [23] S. Brown, J. Melrose, B. Caterson, P. Roughley, S. M. Eisenstein, and S. Roberts, "A comparative evaluation of the small leucine-rich proteoglycans of pathological human intervertebral discs," *European Spine Journal*, vol. 21, no. 2, pp. 154-159, 2012/05/01 2012, doi: 10.1007/s00586-012-2179-1.
- [24] J. Melrose and P. Roughley, "Proteoglycans of the intervertebral disc," Springer-Verlag Wien, 2014, pp. 53-77.
- [25] J. Melrose, S. M. Smith, R. C. Appleyard, and C. B. Little, "Aggrecan, versican and type VI collagen are components of annular translamellar crossbridges in the intervertebral disc," *European Spine Journal*, vol. 17, no. 2, pp. 314-324, 2008, doi: 10.1007/s00586-007-0538-0.
- [26] J. Caldeira *et al.*, "Matrisome Profiling during Intervertebral Disc Development and Ageing," *Scientific Reports*, vol. 7, no. 1, pp. 1-15, 2017, doi: 10.1038/s41598-017-11960-0.
- [27] C. L. Yang, H. Rui, S. Mosler, H. Notbohm, A. Sawaryn, and P. K. MÜLLER, "Collagen II from articular cartilage and annulus fibrosus: Structural and functional implication of tissue specific posttranslational modifications of collagen molecules," *European Journal of Biochemistry*, vol. 213, no. 3, pp. 1297-1302, 1993, doi: 10.1111/j.1432-1033.1993.tb17881.x.
- [28] W. J. Wang *et al.*, "MMPs and ADAMTSs in intervertebral disc degeneration," (in eng), no. 1873-3492 (Electronic).
- [29] S. S. Sivan, E. Wachtel, and P. Roughley, "Structure, function, aging and turnover of aggrecan in the intervertebral disc," *Biochim Biophys Acta*, vol. 1840, no. 10, pp. 3181-9, Oct 2014, doi: 10.1016/j.bbagen.2014.07.013.
- [30] W. E. B. Johnson, B. Caterson, S. M. Eisenstein, D. L. Hynds, D. M. Snow, and S. Roberts, "Human intervertebral disc aggrecan inhibits nerve growth in vitro," *Arthritis and Rheumatism*, vol. 46, no. 10, pp. 2658-2664, 2002, doi: 10.1002/art.10585.
- [31] W. E. B. Johnson, B. Caterson, S. M. Eisenstein, and S. Roberts, "Human intervertebral disc aggrecan inhibits endothelial cell adhesion and cell migration in vitro," *Spine*, vol. 30, no. 10, pp. 1139-1147, 2005, doi: 10.1097/01.brs.0000162624.95262.73.
- [32] L. M. U. G. B. B. G. V. M. R. J. Š. T. B. R. V. T. T. E. R. i. D. D. D. R. T. I. o. H. C. Gradišnik, mdash, and P. An Optimised, "The Endplate Role in Degenerative Disc Disease Research: The Isolation of Human Chondrocytes from Vertebral Endplate—An Optimised Protocol," *Bioengineering*, vol. 9, no. 4, doi: 10.3390/bioengineering9040137.
- [33] S. Roberts, J. Menage, and J. P. G. Urban, "Biochemical and Structural Properties of the Cartilage End-Plate and its Relation to the Intervertebral Disc," *Spine*, vol. 14, no. 2, 1989. [Online]. Available: https://journals.lww.com/spinejournal/Fulltext/1989/02000/Biochemical_and_Structural_Properties_of_the.5.aspx.
- [34] N. V. Vo *et al.*, "Molecular mechanisms of biological aging in intervertebral discs," *J Orthop Res*, vol. 34, no. 8, pp. 1289-306, Aug 2016, doi: 10.1002/jor.23195.

- [35] D. P. Lama, J. Kulkarni, and B. Tamang, "The Role of Cell Clusters in Intervertebral Disc Degeneration and its Relevance behind Repair," *Spine Research*, vol. 03, 01/01 2017, doi: 10.21767/2471-8173.100035.
- [36] S. Brown *et al.*, "Cell Clusters Are Indicative of Stem Cell Activity in the Degenerate Intervertebral Disc: Can Their Properties Be Manipulated to Improve Intrinsic Repair of the Disc?," *Stem Cells and Development*, vol. 27, no. 3, pp. 147-165, 2018/02/01 2017, doi: 10.1089/scd.2017.0213.
- [37] H. K. Pokharna and F. M. Phillips, "Collagen crosslinks in human lumbar intervertebral disc aging," *Spine*, vol. 23, no. 15, pp. 1645-1648, 1998, doi: 10.1097/00007632-199808010-00005.
- [38] S. S. Sivan *et al.*, "Collagen turnover in normal and degenerate human intervertebral discs as determined by the racemization of aspartic acid," *J Biol Chem*, vol. 283, no. 14, pp. 8796-801, Apr 4 2008, doi: 10.1074/jbc.M709885200.
- [39] P. J. Roughley, L. I. Melching, T. F. Heathfield, R. H. Pearce, and J. S. Mort, "The structure and degradation of aggrecan in human intervertebral disc," (in eng), *Eur Spine J*, vol. 15 Suppl 3, no. Suppl 3, pp. S326-S332, 2006, doi: 10.1007/s00586-006-0127-7.
- [40] Q. Wei, X. Zhang, C. Zhou, Q. Ren, and Y. Zhang, "Roles of large aggregating proteoglycans in human intervertebral disc degeneration," *Connective Tissue Research*, vol. 60, no. 3, pp. 209-218, 2019/05/04 2019, doi: 10.1080/03008207.2018.1499731.
- [41] L. Quero *et al.*, "Hyaluronic acid fragments enhance the inflammatory and catabolic response in human intervertebral disc cells through modulation of toll-like receptor 2 signalling pathways," *Arthritis Research & Therapy*, vol. 15, no. 4, p. R94, 2013/08/22 2013, doi: 10.1186/ar4274.
- [42] P. P. Vergroesen *et al.*, "Mechanics and biology in intervertebral disc degeneration: a vicious circle," *Osteoarthritis Cartilage*, vol. 23, no. 7, pp. 1057-70, Jul 2015, doi: 10.1016/j.joca.2015.03.028.
- [43] S. Umehara, S. Tadano, K. Abumi, K. Katagiri, K. Kaneda, and T. Ukai, "Effects of degeneration on the elastic modulus distribution in the lumbar intervertebral disc," *Spine (Phila Pa 1976)*, vol. 21, no. 7, pp. 811-9; discussion 820, Apr 1 1996, doi: 10.1097/00007632-199604010-00007.
- [44] F.-J. Lyu *et al.*, "Painful intervertebral disc degeneration and inflammation: from laboratory evidence to clinical interventions," *Bone Research*, vol. 9, no. 1, p. 7, 2021/01/29 2021, doi: 10.1038/s41413-020-00125-x.
- [45] P. Patil, L. J. Niedernhofer, P. D. Robbins, J. Lee, G. Sowa, and N. Vo, "Cellular senescence in intervertebral disc aging and degeneration," *Curr Mol Biol Rep*, vol. 4, no. 4, pp. 180-190, Dec 2018, doi: 10.1007/s40610-018-0108-8.
- [46] J. García-Cosamalón *et al.*, "Intervertebral disc, sensory nerves and neurotrophins: who is who in discogenic pain?," (in eng), no. 1469-7580 (Electronic), 2010.
- [47] A. E. Dubin and A. Patapoutian, "Nociceptors: The sensors of the pain pathway," vol. 120, pp. 3760-3772, 2010, doi: 10.1172/JCI42843.
- [48] P. Lama, C. L. Le Maitre, I. J. Harding, P. Dolan, and M. A. Adams, "Nerves and blood vessels in degenerated intervertebral discs are confined to physically disrupted tissue," *Journal of Anatomy*, vol. 233, no. 1, pp. 86-97, 2018, doi: 10.1111/joa.12817.
- [49] R. Buchbinder *et al.*, "Low back pain: a call for action," *Lancet*, vol. 391, no. 10137, pp. 2384-2388, Jun 9 2018, doi: 10.1016/S0140-6736(18)30488-4.
- [50] A. K. Burton *et al.*, "Chapter 2: European guidelines for prevention in low back pain," *European Spine Journal*, vol. 15, no. 2, pp. s136-s168, 2006/03/01 2006, doi: 10.1007/s00586-006-1070-3.
- [51] S. Clark and R. Horton, "Low back pain: a major global challenge," *The Lancet*, vol. 391, no. 10137, p. 2302, 2018, doi: 10.1016/S0140-6736(18)30725-6.
- [52] J. L. Dieleman *et al.*, "US Health Care Spending by Payer and Health Condition, 1996-2016," (in eng), no. 1538-3598 (Electronic), 2020.
- [53] S. Chen *et al.*, "Global, regional and national burden of low back pain 1990–2019: A systematic analysis of the Global Burden of Disease study 2019," *Journal of Orthopaedic Translation*, vol. 32, pp. 49-58, 2022/01/01/ 2022, doi: <https://doi.org/10.1016/j.jot.2021.07.005>.
- [54] K. Fujii *et al.*, "Discogenic Back Pain: Literature Review of Definition, Diagnosis, and Treatment," 2019, doi: 10.1002/jbm4.10180.

- [55] S. Zhang *et al.*, "The role of structure and function changes of sensory nervous system in intervertebral disc-related low back pain," *Osteoarthritis and Cartilage*, vol. 29, no. 1, pp. 17-27, 2021, doi: 10.1016/j.joca.2020.09.002.
- [56] M. Allegri *et al.*, "Mechanisms of low back pain: a guide for diagnosis and therapy," *F1000Res*, vol. 5, 2016, doi: 10.12688/f1000research.8105.2.
- [57] S. Ohtori, G. Inoue, M. Miyagi, and K. Takahashi, "Pathomechanisms of discogenic low back pain in humans and animal models," *Spine Journal*, vol. 15, no. 6, pp. 1347-1355, 2015, doi: 10.1016/j.spinee.2013.07.490.
- [58] J. V. Simmonds and R. J. Keer, "Hypermobility and the hypermobility syndrome," *Manual Therapy*, vol. 12, no. 4, pp. 298-309, 2007/11/01/ 2007, doi: <https://doi.org/10.1016/j.math.2007.05.001>.
- [59] J. C. Lotz and J. A. Ulrich, "Innervation, Inflammation, and Hypermobility May Characterize Pathologic Disc Degeneration: Review of Animal Model Data," *Journal of Bone and Joint Surgery*, vol. 88, no. suppl_2, pp. 76-82, 2006, doi: 10.2106/JBJS.E.01448.
- [60] L. Zhao, L. Manchikanti, A. D. Kaye, and A. Abd-Elsayed, "Treatment of Discogenic Low Back Pain: Current Treatment Strategies and Future Options-a Literature Review," (in eng), no. 1534-3081 (Electronic), 2019.
- [61] E. A. Shipton, "Physical Therapy Approaches in the Treatment of Low Back Pain," *Pain and Therapy*, vol. 7, no. 2, pp. 127-137, 2018/12/01 2018, doi: 10.1007/s40122-018-0105-x.
- [62] R. Chou, "Pharmacological Management of Low Back Pain," *Drugs*, vol. 70, no. 4, pp. 387-402, 2010/03/01 2010, doi: 10.2165/11318690-000000000-00000.
- [63] L. Liu, M. Skinner, S. McDonough, L. Mabire, and G. D. Baxter, "Acupuncture for Low Back Pain: An Overview of Systematic Reviews," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, p. 328196, 2015/03/04 2015, doi: 10.1155/2015/328196.
- [64] NHS. "Acupuncture." <https://www.nhs.uk/conditions/acupuncture/> (accessed).
- [65] I. Urits *et al.*, "Acupuncture for the Management of Low Back Pain," *Current Pain and Headache Reports*, vol. 25, no. 1, p. 2, 2021/01/14 2021, doi: 10.1007/s11916-020-00919-y.
- [66] M. W. Greene and R. J. W. Clemente-Fuentes, "Is transcutaneous electrical nerve stimulation (TENS) more effective than placebo for management of chronic low back pain?," *Evidence-Based Practice*, vol. 19, no. 8, 2016. [Online]. Available: https://journals.lww.com/ebp/Fulltext/2016/08000/Is_transcutaneous_electrical_nerve_stimulation.11.aspx.
- [67] NHS. "TENS (transcutaneous electrical nerve stimulation)." <https://www.nhs.uk/conditions/transcutaneous-electrical-nerve-stimulation-tens/> (accessed).
- [68] N. N. Knezevic, S. Mandalia, J. Raasch, I. Knezevic, and K. D. Candido, "Treatment of chronic low back pain - new approaches on the horizon," (in eng), no. 1178-7090 (Print), 2017.
- [69] K. Patel, P. Chopra, and S. Upadhyayula, "Epidural Steroid Injections. BTI - StatPearls," (in eng), 2022.
- [70] M. Kim, S. Lee, H.-S. Kim, S. Park, S.-Y. Shim, and D.-J. Lim, "A Comparison of Percutaneous Endoscopic Lumbar Discectomy and Open Lumbar Microdiscectomy for Lumbar Disc Herniation in the Korean: A Meta-Analysis," *BioMed Research International*, vol. 2018, p. 9073460, 2018/08/07 2018, doi: 10.1155/2018/9073460.
- [71] E. Elowitz. "Microdiscectomy Spine Surgery: Risks, Complications, and Success Rates." <https://www.spine-health.com/treatment/back-surgery/microdiscectomy-spine-surgery-risks-complications-and-success-rates> (accessed 13-09-2022).
- [72] S. Baliga, K. Treon, and N. J. Craig, "Low Back Pain: Current Surgical Approaches," (in eng), no. 1976-1902 (Print), 2015.
- [73] F. Marsico, S. Paolillo, and P. P. Filardi, "NSAIDs and cardiovascular risk," *Journal of Cardiovascular Medicine*, vol. 18, 2017. [Online]. Available: https://journals.lww.com/jcardiovascularmedicine/Fulltext/2017/01001/NSAIDs_and_cardiovascular_risk.8.aspx.

- [74] S. van Uden, J. Silva-Correia, J. M. Oliveira, and R. L. Reis, "Current strategies for treatment of intervertebral disc degeneration: substitution and regeneration possibilities," (in eng), *Biomater Res*, vol. 21, pp. 22-22, 2017, doi: 10.1186/s40824-017-0106-6.
- [75] R. Mhanna and A. Hasan, "Introduction to Tissue Engineering," *Tissue Engineering for Artificial Organs*, <https://doi.org/10.1002/9783527689934.ch1> pp. 1-34, 2017/05/08 2017, doi: <https://doi.org/10.1002/9783527689934.ch1>.
- [76] B. P. Chan and K. W. Leong, "Scaffolding in tissue engineering: general approaches and tissue-specific considerations," (in eng), no. 1432-0932 (Electronic), 2008.
- [77] F. J. O'Brien, "Biomaterials & scaffolds for tissue engineering," *Materials Today*, vol. 14, no. 3, pp. 88-95, 2011/03/01/ 2011, doi: [https://doi.org/10.1016/S1369-7021\(11\)70058-X](https://doi.org/10.1016/S1369-7021(11)70058-X).
- [78] S. F. Badylak, "Decellularized Allogeneic and Xenogeneic Tissue as a Bioscaffold for Regenerative Medicine: Factors that Influence the Host Response," *Annals of Biomedical Engineering*, vol. 42, no. 7, pp. 1517-1527, 2014/07/01 2014, doi: 10.1007/s10439-013-0963-7.
- [79] A. K. Gaharwar, I. Singh, and A. Khademhosseini, "Engineered biomaterials for in situ tissue regeneration," *Nature Reviews Materials*, vol. 5, no. 9, pp. 686-705, 2020/09/01 2020, doi: 10.1038/s41578-020-0209-x.
- [80] M. J. Jahir-Hussain, N. A. Maaruf, N. E. F. Esa, and N. Jusoh, "The effect of pore geometry on the mechanical properties of 3D-printed bone scaffold due to compressive loading," *IOP Conference Series: Materials Science and Engineering*, vol. 1051, no. 1, p. 012016, 2021/02/01 2021, doi: 10.1088/1757-899x/1051/1/012016.
- [81] K. A.-O. Yamada, N. A.-O. Iwasaki, and H. A.-O. Sudo, "Biomaterials and Cell-Based Regenerative Therapies for Intervertebral Disc Degeneration with a Focus on Biological and Biomechanical Functional Repair: Targeting Treatments for Disc Herniation. LID - 10.3390/cells11040602 [doi] LID - 602," (in eng), no. 2073-4409 (Electronic), 2022.
- [82] D. Ukeba, H. Sudo, T. Tsujimoto, K. Ura, K. Yamada, and N. Iwasaki, "Bone marrow mesenchymal stem cells combined with ultra-purified alginate gel as a regenerative therapeutic strategy after discectomy for degenerated intervertebral discs," (in eng), no. 2352-3964 (Electronic), 2020.
- [83] Y. Zeng *et al.*, "Injectable microcryogels reinforced alginate encapsulation of mesenchymal stromal cells for leak-proof delivery and alleviation of canine disc degeneration," *Biomaterials*, vol. 59, pp. 53-65, 2015/08/01/ 2015, doi: <https://doi.org/10.1016/j.biomaterials.2015.04.029>.
- [84] A. I. Chou, S. B. Akintoye So Fau - Nicoll, and S. B. Nicoll, "Photo-crosslinked alginate hydrogels support enhanced matrix accumulation by nucleus pulposus cells in vivo," (in eng), no. 1522-9653 (Electronic), 2009.
- [85] H. Wang *et al.*, "Utilization of stem cells in alginate for nucleus pulposus tissue engineering," (in eng), no. 1937-335X (Electronic), 2014.
- [86] Y. H. Cheng *et al.*, "Thermosensitive chitosan-gelatin-glycerol phosphate hydrogels as a cell carrier for nucleus pulposus regeneration: an in vitro study," (in eng), no. 1937-335X (Electronic), 2010.
- [87] S. M. Richardson, J. A. Hughes N Fau - Hunt, A. J. Hunt Ja Fau - Freemont, J. A. Freemont Aj Fau - Hoyland, and J. A. Hoyland, "Human mesenchymal stem cell differentiation to NP-like cells in chitosan-glycerophosphate hydrogels," (in eng), no. 0142-9612 (Print), 2008.
- [88] P. Roughley, C. Hoemann, E. DesRosiers, F. Mwale, J. Antoniou, and M. Alini, "The potential of chitosan-based gels containing intervertebral disc cells for nucleus pulposus supplementation," *Biomaterials*, vol. 27, no. 3, pp. 388-396, 2006/01/01/ 2006, doi: <https://doi.org/10.1016/j.biomaterials.2005.06.037>.
- [89] L. J. Smith *et al.*, "In vitro characterization of a stem-cell-seeded triple-interpenetrating-network hydrogel for functional regeneration of the nucleus pulposus," (in eng), no. 1937-335X (Electronic), 2014.
- [90] W. I. Choi *et al.*, "Chitosan functionalized thermosponge nano-carriers for prolonged retention and local delivery of chymopapain at the nucleus pulposus in porcine discs ex vivo," *RSC Advances*, 10.1039/C6RA17848K vol. 6, no. 93, pp. 90967-90972, 2016, doi: 10.1039/C6RA17848K.

- [91] C. Zeng *et al.*, "Silk fibroin porous scaffolds for nucleus pulposus tissue engineering," *Materials Science and Engineering: C*, vol. 37, pp. 232-240, 2014/04/01/ 2014, doi: <https://doi.org/10.1016/j.msec.2014.01.012>.
- [92] S. H. Park, E. S. Cho H Fau - Gil, B. B. Gil Es Fau - Mandal, B.-H. Mandal Bb Fau - Min, D. L. Min Bh Fau - Kaplan, and D. L. Kaplan, "Silk-fibrin/hyaluronic acid composite gels for nucleus pulposus tissue regeneration," (in eng), no. 1937-335X (Electronic), 2011.
- [93] Y. Zhang, J. J. Yoo, and A. Atala, "Chapter 46 - Tissue engineering: bladder and urethra," in *Principles of Tissue Engineering (Fifth Edition)*, R. Lanza, R. Langer, J. P. Vacanti, and A. Atala Eds.: Academic Press, 2020, pp. 845-862.
- [94] R. C. Gupta, R. Lall, A. Srivastava, and A. Sinha, "Hyaluronic Acid: Molecular Mechanisms and Therapeutic Trajectory," *Frontiers in Veterinary Science*, Review vol. 6, 2019. [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fvets.2019.00192>.
- [95] E. C. Collin *et al.*, "An injectable vehicle for nucleus pulposus cell-based therapy," *Biomaterials*, vol. 32, no. 11, pp. 2862-2870, 2011/04/01/ 2011, doi: <https://doi.org/10.1016/j.biomaterials.2011.01.018>.
- [96] W.-Y. Su, Y.-C. Chen, and F.-H. Lin, "Injectable oxidized hyaluronic acid/adipic acid dihydrazide hydrogel for nucleus pulposus regeneration," *Acta Biomaterialia*, vol. 6, no. 8, pp. 3044-3055, 2010/08/01/ 2010, doi: <https://doi.org/10.1016/j.actbio.2010.02.037>.
- [97] C. Woiciechowsky *et al.*, "Regeneration of nucleus pulposus tissue in an ovine intervertebral disc degeneration model by cell-free resorbable polymer scaffolds," (in eng), no. 1932-7005 (Electronic), 2014.
- [98] R. Tsaryk *et al.*, "Collagen-low molecular weight hyaluronic acid semi-interpenetrating network loaded with gelatin microspheres for cell and growth factor delivery for nucleus pulposus regeneration," *Acta Biomaterialia*, vol. 20, pp. 10-21, 2015/07/01/ 2015, doi: <https://doi.org/10.1016/j.actbio.2015.03.041>.
- [99] M. Peroglio *et al.*, "Injectable thermoreversible hyaluronan-based hydrogels for nucleus pulposus cell encapsulation," (in eng), no. 1432-0932 (Electronic), 2012.
- [100] X. Zhou *et al.*, "Genipin cross-linked type II collagen/chondroitin sulfate composite hydrogel-like cell delivery system induces differentiation of adipose-derived stem cells and regenerates degenerated nucleus pulposus," *Acta Biomaterialia*, vol. 71, pp. 496-509, 2018/04/15/ 2018, doi: <https://doi.org/10.1016/j.actbio.2018.03.019>.
- [101] J. L. Bron, V. Koenderink Gh Fau - Everts, T. H. Everts V Fau - Smit, and T. H. Smit, "Rheological characterization of the nucleus pulposus and dense collagen scaffolds intended for functional replacement," (in eng), no. 1554-527X (Electronic), 2009.
- [102] N. R. Malhotra, J. Han Wm Fau - Beckstein, J. Beckstein J Fau - Cloyd, W. Cloyd J Fau - Chen, D. M. Chen W Fau - Elliott, and D. M. Elliott, "An injectable nucleus pulposus implant restores compressive range of motion in the ovine disc," (in eng), no. 1528-1159 (Electronic), 2012.
- [103] H. J. Wilke, C. Heuer F Fau - Neidlinger-Wilke, L. Neidlinger-Wilke C Fau - Claes, and L. Claes, "Is a collagen scaffold for a tissue engineered nucleus replacement capable of restoring disc height and stability in an animal model?," (in eng), no. 0940-6719 (Print), 2006.
- [104] A. Friedmann *et al.*, "Intervertebral Disc Regeneration Injection of a Cell-Loaded Collagen Hydrogel in a Sheep Model," (in eng), no. 1422-0067 (Electronic), 2021.
- [105] S. M. Richardson *et al.*, "The differentiation of bone marrow mesenchymal stem cells into chondrocyte-like cells on poly-L-lactic acid (PLLA) scaffolds," *Biomaterials*, vol. 27, no. 22, pp. 4069-4078, 2006/08/01/ 2006, doi: <https://doi.org/10.1016/j.biomaterials.2006.03.017>.
- [106] M. Santoro, S. R. Shah, J. L. Walker, and A. G. Mikos, "Poly(lactic acid) nanofibrous scaffolds for tissue engineering," (in eng), no. 1872-8294 (Electronic), 2016.
- [107] M. Endres *et al.*, "Intervertebral disc regeneration after implantation of a cell-free bioresorbable implant in a rabbit disc degeneration model," *Biomaterials*, vol. 31, no. 22, pp. 5836-5841, 2010/08/01/ 2010, doi: <https://doi.org/10.1016/j.biomaterials.2010.03.078>.

- [108] J. Zhang *et al.*, "Novel Fabricating Process for Porous Polyglycolic Acid Scaffolds by Melt-Foaming Using Supercritical Carbon Dioxide," *ACS Biomaterials Science & Engineering*, vol. 4, no. 2, pp. 694-706, 2018/02/12 2018, doi: 10.1021/acsbmaterials.7b00692.
- [109] H. Y. Kim *et al.*, "Effect of pore sizes of PLGA scaffolds on mechanical properties and cell behaviour for nucleus pulposus regeneration in vivo," (in eng), no. 1932-7005 (Electronic), 2017.
- [110] L. Xin *et al.*, "Effects of annulus defects and implantation of poly(lactic-co-glycolic acid) (PLGA)/fibrin gel scaffolds on nerves ingrowth in a rabbit model of annular injury disc degeneration," (in eng), no. 1749-799X (Electronic), 2017.
- [111] C.-z. Liang *et al.*, "Dual release of dexamethasone and TGF- β 3 from polymeric microspheres for stem cell matrix accumulation in a rat disc degeneration model," *Acta Biomaterialia*, vol. 9, no. 12, pp. 9423-9433, 2013/12/01/ 2013, doi: <https://doi.org/10.1016/j.actbio.2013.08.019>.
- [112] P. Gentile, V. Chiono, I. Carmagnola, and P. V. Hatton, "An overview of poly(lactic-co-glycolic) acid (PLGA)-based biomaterials for bone tissue engineering," (in eng), no. 1422-0067 (Electronic), 2014.
- [113] A. T. Francisco, P. Y. Hwang, C. G. Jeong, L. Jing, J. Chen, and L. A. Setton, "Photocrosslinkable laminin-functionalized polyethylene glycol hydrogel for intervertebral disc regeneration," (in eng), no. 1878-7568 (Electronic), 2014.
- [114] K. Benz *et al.*, "Rheological and biological properties of a hydrogel support for cells intended for intervertebral disc repair," (in eng), no. 1471-2474 (Electronic), 2012.
- [115] K. M. Salleh, N. A. Zainul Armir, N. S. N. Mazlan, M. Mostapha, C. Wang, and S. Zakaria, "12 - Hydrogel- and aerogel-based composites: Biodegradable hydrogel and aerogel polymer blend-based composites," in *Biodegradable Polymers, Blends and Composites*, S. Mavinkere Rangappa, J. Parameswaranpillai, S. Siengchin, and M. Ramesh Eds.: Woodhead Publishing, 2022, pp. 355-388.
- [116] G. Tang *et al.*, "Advances of Naturally Derived and Synthetic Hydrogels for Intervertebral Disk Regeneration," *Frontiers in Bioengineering and Biotechnology*, vol. 8, no. June, pp. 1-13, 2020, doi: 10.3389/fbioe.2020.00745.
- [117] J. Zhu and R. E. Marchant, "Design properties of hydrogel tissue-engineering scaffolds," *Expert Rev Med Devices*, vol. 8, no. 5, pp. 607-26, Sep 2011, doi: 10.1586/erd.11.27.
- [118] D. R. Pereira, J. Silva-Correia, J. M. Oliveira, and R. L. Reis, "Hydrogels in acellular and cellular strategies for intervertebral disc regeneration," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 7, no. 2, pp. 85-98, 2013, doi: 10.1002/term.500.
- [119] C. A. Heath, "Cells for tissue engineering," *Trends in Biotechnology*, vol. 18, no. 1, pp. 17-19, 2000/01/01/ 2000, doi: [https://doi.org/10.1016/S0167-7799\(99\)01396-7](https://doi.org/10.1016/S0167-7799(99)01396-7).
- [120] G. Vadalà, F. Russo, L. Ambrosio, M. Loppini, and V. Denaro, "Stem cells sources for intervertebral disc regeneration," (in eng), *World J Stem Cells*, vol. 8, no. 5, pp. 185-201, 2016, doi: 10.4252/wjsc.v8.i5.185.
- [121] H. Ishiguro *et al.*, "Intervertebral disc regeneration with an adipose mesenchymal stem cell-derived tissue-engineered construct in a rat nucleotomy model," *Acta Biomaterialia*, vol. 87, pp. 118-129, 2019/03/15/ 2019, doi: <https://doi.org/10.1016/j.actbio.2019.01.050>.
- [122] Y. Ikada, "Challenges in tissue engineering," (in eng), no. 1742-5689 (Print), 2006.
- [123] D. Howard, K. M. Buttery, L. Fau - Shakesheff, S. J. Shakesheff, K. Fau - Roberts, and S. J. Roberts, "Tissue engineering: strategies, stem cells and scaffolds," (in eng), no. 1469-7580 (Electronic), 2008.
- [124] R. Champlin. "Selection of Autologous or Allogeneic Transplantation." <https://www.ncbi.nlm.nih.gov/books/NBK12844/> (accessed).
- [125] C.-P. Huang, C.-Y. Yang, and C.-R. Shyr, "Utilizing Xenogeneic Cells As a Therapeutic Agent for Treating Diseases," *Cell Transplantation*, vol. 30, p. 09636897211011995, 2021/01/01 2021, doi: 10.1177/09636897211011995.
- [126] M. H. Amer, F. Rose, K. M. Shakesheff, M. Mado, and L. A.-O. X. White, "Translational considerations in injectable cell-based therapeutics for neurological applications: concepts, progress and challenges," (in eng), no. 2057-3995 (Electronic), 2017.
- [127] S. M. Willerth and S. E. Sakiyama-Elbert, "Combining Stem Cells and Biomaterial Scaffolds for Constructing Tissues and Cell Delivery," *StemJournal*, vol. 1, pp. 1-25, 2019, doi: 10.3233/STJ-180001.

- [128] X. Zhao, K. Cui, and Z. Li, "The role of biomaterials in stem cell-based regenerative medicine," *Future Medicinal Chemistry*, vol. 11, no. 14, pp. 1777-1790, 2019/07/01 2019, doi: 10.4155/fmc-2018-0347.
- [129] D. M. O'Halloran and A. S. Pandit, "Tissue-Engineering Approach to Regenerating the Intervertebral Disc," *Tissue Engineering*, vol. 13, no. 8, pp. 1927-1954, 2007/08/01 2007, doi: 10.1089/ten.2005.0608.
- [130] J. C. Kennon, M. E. Awad, N. Chutkan, J. DeVine, and S. Fulzele, "Current insights on use of growth factors as therapy for Intervertebral Disc Degeneration," vol. 9, no. 1, pp. 43-52, 2018, doi: doi:10.1515/bmc-2018-0003.
- [131] C.-H. Heldin, "Targeting the PDGF signaling pathway in tumor treatment," *Cell Communication and Signaling*, vol. 11, no. 1, p. 97, 2013/12/20 2013, doi: 10.1186/1478-811X-11-97.
- [132] D. Paglia, H. Singh, T. Karukonda, H. Drissi, and I. Moss, "PDGF-BB Delays Degeneration of the Intervertebral Discs in a Rabbit Preclinical Model," *SPINE*, vol. 41, p. 1, 12/01 2015, doi: 10.1097/BRS.0000000000001336.
- [133] H. E. Gruber, H. J. Norton, and E. N. Hanley, Jr., "Anti-Apoptotic Effects of IGF-1 and PDGF on Human Intervertebral Disc Cells In Vitro," *Spine*, vol. 25, no. 17, 2000. [Online]. Available: https://journals.lww.com/spinejournal/Fulltext/2000/09010/Anti_Apoptotic_Effects_of_IGF_1_and_PDGF_on_Human.2.aspx.
- [134] S. Wrigley, D. Arafa, and D. Tropea, "Insulin-Like Growth Factor 1: At the Crossroads of Brain Development and Aging," (in eng), *Front Cell Neurosci*, vol. 11, pp. 14-14, 2017, doi: 10.3389/fncel.2017.00014.
- [135] P. Buxton, C. Edwards, C. W. Archer, and P. Francis-West, "Growth/Differentiation Factor-5 (GDF-5) and Skeletal Development," *JBJS*, vol. 83, no. 1, 2001. [Online]. Available: https://journals.lww.com/jbjsjournal/Fulltext/2001/00001/Growth_Differentiation_Factor_5_GDF_5_and.4.aspx.
- [136] A. J. L. Walsh, D. S. Bradford, and J. C. Lotz, "In Vivo Growth Factor Treatment of Degenerated Intervertebral Discs," *Spine*, vol. 29, no. 2, 2004. [Online]. Available: https://journals.lww.com/spinejournal/Fulltext/2004/01150/In_Vivo_Growth_Factor_Treatment_of_Degenerated.8.aspx.
- [137] T. Chujo *et al.*, "Effects of Growth Differentiation Factor-5 on the Intervertebral Disc-In Vitro Bovine Study and In Vivo Rabbit Disc Degeneration Model Study," *Spine*, vol. 31, no. 25, 2006. [Online]. Available: https://journals.lww.com/spinejournal/Fulltext/2006/12010/Effects_of_Growth_Differentiation_Factor_5_on_the.6.aspx.
- [138] Q. Chen, C.-E. Lee, B. Denard, and J. Ye, "Sustained induction of collagen synthesis by TGF- β requires regulated intramembrane proteolysis of CREB3L1," (in eng), *PLoS One*, vol. 9, no. 10, pp. e108528-e108528, 2014, doi: 10.1371/journal.pone.0108528.
- [139] D. Yang *et al.*, "Role of Transforming Growth Factor- β 1 in Regulating Fetal-Maternal Immune Tolerance in Normal and Pathological Pregnancy," (in eng), *Front Immunol*, vol. 12, pp. 689181-689181, 2021, doi: 10.3389/fimmu.2021.689181.
- [140] G. Feng, Y. Wan, G. Balian, C. T. Laurencin, and X. Li, "Adenovirus-mediated expression of growth and differentiation factor-5 promotes chondrogenesis of adipose stem cells," (in eng), *Growth Factors*, vol. 26, no. 3, pp. 132-142, 2008, doi: 10.1080/08977190802105917.
- [141] K. Xue *et al.*, "The role and mechanism of transforming growth factor beta 3 in human myocardial infarction-induced myocardial fibrosis," (in eng), *J Cell Mol Med*, vol. 23, no. 6, pp. 4229-4243, 2019, doi: 10.1111/jcmm.14313.
- [142] D. A. Frauchiger, S. R. Heeb, R. D. May, M. Wöltje, L. M. Benneker, and B. Gantenbein, "Differentiation of MSC and annulus fibrosus cells on genetically engineered silk fleece-membrane-composites enriched for GDF-6 or TGF- β 3," *Journal of Orthopaedic Research*, <https://doi.org/10.1002/jor.23778> vol. 36, no. 5, pp. 1324-1333, 2018/05/01 2018, doi: <https://doi.org/10.1002/jor.23778>.

- [143] C. Aluganti Narasimhulu and D. K. Singla, "The Role of Bone Morphogenetic Protein 7 (BMP-7) in Inflammation in Heart Diseases," (in eng), *Cells*, vol. 9, no. 2, p. 280, 2020, doi: 10.3390/cells9020280.
- [144] Y. Imai, K. Miyamoto, H. S. An, E. J. M. A. Thonar, G. B. J. Andersson, and K. Masuda, "Recombinant Human Osteogenic Protein-1 Upregulates Proteoglycan Metabolism of Human Annulus Fibrosus and Nucleus Pulposus Cells," *Spine*, vol. 32, no. 12, 2007. [Online]. Available: https://journals.lww.com/spinejournal/Fulltext/2007/05200/Recombinant_Human_Osteogenic_Protein_1_Upregulates.8.aspx.
- [145] S. Yamakawa and K. Hayashida, "Advances in surgical applications of growth factors for wound healing," *Burns & Trauma*, vol. 7, no. 1, p. 10, 2019/04/05 2019, doi: 10.1186/s41038-019-0148-1.
- [146] W. Hu, Z. Wang, Y. Xiao, S. Zhang, and J. Wang, "Advances in crosslinking strategies of biomedical hydrogels," *Biomater Sci*, vol. 7, no. 3, pp. 843-855, Feb 26 2019, doi: 10.1039/c8bm01246f.
- [147] J. H. Lee and H. W. Kim, "Emerging properties of hydrogels in tissue engineering," *Journal of Tissue Engineering*, vol. 9, pp. 0-3, 2018, doi: 10.1177/2041731418768285.
- [148] K. Y. Lee and D. J. Mooney, "Hydrogels for tissue engineering," *Chemical Reviews*, vol. 101, no. 7, pp. 1869-1879, 2001, doi: 10.1021/cr000108x.
- [149] R. D. Bowles and L. A. Setton, "Biomaterials for intervertebral disc regeneration and repair," *Biomaterials*, vol. 129, pp. 54-67, 2017, doi: 10.1016/j.biomaterials.2017.03.013.
- [150] T. J. DiStefano, J. O. Shmukler, G. Danias, and J. C. Iatridis, "The Functional Role of Interface Tissue Engineering in Annulus Fibrosus Repair: Bridging Mechanisms of Hydrogel Integration with Regenerative Outcomes," *ACS Biomater Sci Eng*, vol. 6, no. 12, pp. 6556-6586, Dec 14 2020, doi: 10.1021/acsbomaterials.0c01320.
- [151] A. A. Thorpe *et al.*, "Thermally triggered hydrogel injection into bovine intervertebral disc tissue explants induces differentiation of mesenchymal stem cells and restores mechanical function," *Acta Biomaterialia*, vol. 54, pp. 212-226, 2017, doi: 10.1016/j.actbio.2017.03.010.
- [152] N. Sadr *et al.*, "Enhancing the biological performance of synthetic polymeric materials by decoration with engineered, decellularized extracellular matrix," (in eng), no. 1878-5905 (Electronic), 2012.
- [153] R.-H. Fu *et al.*, "Decellularization and Recellularization Technologies in Tissue Engineering," *Cell transplantation*, vol. 23, pp. 621-30, 04/09 2014, doi: 10.3727/096368914X678382.
- [154] M. C. Cramer and S. F. Badylak, "Extracellular Matrix-Based Biomaterials and Their Influence Upon Cell Behavior," *Annals of Biomedical Engineering*, vol. 48, no. 7, pp. 2132-2153, 2020/07/01 2020, doi: 10.1007/s10439-019-02408-9.
- [155] M. Fiordalisi, A. J. Silva, M. Barbosa, R. Gonçalves, and J. Caldeira, "Decellularized Scaffolds for Intervertebral Disc Regeneration," *Trends in Biotechnology*, vol. 38, no. 9, pp. 947-951, 2020, doi: 10.1016/j.tibtech.2020.05.002.
- [156] P. M. Crapo, T. W. Gilbert, and S. F. Badylak, "An overview of tissue and whole organ decellularization processes," *Biomaterials*, vol. 32, no. 12, pp. 3233-43, Apr 2011, doi: 10.1016/j.biomaterials.2011.01.057.
- [157] R. H. Fu *et al.*, "Decellularization and recellularization technologies in tissue engineering," *Cell Transplantation*, vol. 23, no. 4-5, pp. 621-630, 2014, doi: 10.3727/096368914X678382.
- [158] G. Agmon and K. L. Christman, "Controlling stem cell behavior with decellularized extracellular matrix scaffolds," *Curr Opin Solid State Mater Sci*, vol. 20, no. 4, pp. 193-201, Aug 2016, doi: 10.1016/j.cossms.2016.02.001.
- [159] L. A. Monaco, S. J. DeWitte-Orr, and D. E. Gregory, "A comparison between porcine, ovine, and bovine intervertebral disc anatomy and single lamella annulus fibrosus tensile properties," *Journal of Morphology*, <https://doi.org/10.1002/jmor.20492> vol. 277, no. 2, pp. 244-251, 2016/02/01 2016, doi: <https://doi.org/10.1002/jmor.20492>.
- [160] T. W. Gilbert, T. L. Sellaro, and S. F. Badylak, "Decellularization of tissues and organs," *Biomaterials*, vol. 27, no. 19, pp. 3675-83, Jul 2006, doi: 10.1016/j.biomaterials.2006.02.014.
- [161] T. Scientific. "Traditional Methods of Cell Lysis for Protein Extraction." <https://www.thermofisher.com/pt/en/home/life-science/protein-biology/protein-biology-learning->

- <center/protein-biology-resource-library/pierce-protein-methods/traditional-methods-cell-lysis.html> (accessed).
- [162] U. Mendibil, R. A.-O. X. Ruiz-Hernandez, S. A.-O. X. Retegi-Carrion, N. Garcia-Urquia, B. A.-O. Olalde-Graells, and A. A.-O. Abarategi, "Tissue-Specific Decellularization Methods: Rationale and Strategies to Achieve Regenerative Compounds. LID - 10.3390/ijms21155447 [doi] LID - 5447," (in eng), no. 1422-0067 (Electronic), 2020.
 - [163] D. Moffat, K. Ye, and S. Jin, "Decellularization for the retention of tissue niches," *Journal of Tissue Engineering*, vol. 13, p. 20417314221101151, 2022/01/01 2022, doi: 10.1177/20417314221101151.
 - [164] P. Srinivasan and D.-J. Hsieh, "Supercritical Carbon Dioxide Facilitated Collagen Scaffold Production for Tissue Engineering," 2022, doi: 10.5772/intechopen.102438.
 - [165] R.-H. Fu *et al.*, "Decellularization and Recellularization Technologies in Tissue Engineering," *Cell Transplantation*, vol. 23, no. 4-5, pp. 621-630, 2014/05/01 2014, doi: 10.3727/096368914X678382.
 - [166] P. M. Crapo, S. F. Gilbert Tw Fau - Badylak, and S. F. Badylak, "An overview of tissue and whole organ decellularization processes," (in eng), no. 1878-5905 (Electronic), 2011.
 - [167] P. Das, Y. P. Singh, B. B. Mandal, and S. K. Nandi, "Chapter 10 - Tissue-derived decellularized extracellular matrices toward cartilage repair and regeneration," in *Methods in Cell Biology*, vol. 157, D. Caballero, S. C. Kundu, and R. L. Reis Eds.: Academic Press, 2020, pp. 185-221.
 - [168] D. M. Giraldo-Gomez *et al.*, "Trypsin as enhancement in cyclical tracheal decellularization: Morphological and biophysical characterization," *Materials Science and Engineering: C*, vol. 59, pp. 930-937, 2016/02/01/ 2016, doi: <https://doi.org/10.1016/j.msec.2015.10.094>.
 - [169] W. Kuang, C. Liu, and H. Xu, "The application of decellularized nucleus pulposus matrix/chitosan with transforming growth factor β 3 for nucleus pulposus tissue engineering," *Cytotechnology*, vol. 73, no. 3, pp. 447-456, 2021/06/01 2021, doi: 10.1007/s10616-021-00469-0.
 - [170] Y. Zhang *et al.*, "Characterization and cytocompatibility of 3D porous biomimetic scaffold derived from rabbit nucleus pulposus tissue in vitro," *Journal of Materials Science: Materials in Medicine*, vol. 32, no. 1, p. 8, 2021/01/20 2021, doi: 10.1007/s10856-020-06480-9.
 - [171] L. Yu *et al.*, "Genipin Cross-Linked Decellularized Nucleus Pulposus Hydrogel-Like Cell Delivery System Induces Differentiation of ADSCs and Retards Intervertebral Disc Degeneration," *Frontiers in Bioengineering and Biotechnology*, Original Research vol. 9, 2021. [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fbioe.2021.807883>.
 - [172] L. Yu *et al.*, "Thermosensitive injectable decellularized nucleus pulposus hydrogel as an ideal biomaterial for nucleus pulposus regeneration," *Journal of Biomaterials Applications*, vol. 35, no. 2, pp. 182-192, 2020/08/01 2020, doi: 10.1177/0885328220921328.
 - [173] S. Illien-Jünger, D. D. Sedaghatpour, D. M. Laudier, A. C. Hecht, S. A. Qureshi, and J. C. Iatridis, "Development of a bovine decellularized extracellular matrix-biomaterial for nucleus pulposus regeneration," (in eng), no. 1554-527X (Electronic), 2016.
 - [174] Y. Peng *et al.*, "Decellularized Disc Hydrogels for hBMSCs tissue-specific differentiation and tissue regeneration," (in eng), no. 2452-199X (Electronic), 2021.
 - [175] C. Fernandez, A. Marionneaux, S. Gill, and J. Mercuri, "Biomimetic nucleus pulposus scaffold created from bovine caudal intervertebral disc tissue utilizing an optimal decellularization procedure," (in eng), *J Biomed Mater Res A*, vol. 104, no. 12, pp. 3093-3106, 2016, doi: 10.1002/jbm.a.35858.
 - [176] J. Xu *et al.*, "Decellularised nucleus pulposus as a potential biologic scaffold for disc tissue engineering," *Materials Science and Engineering: C*, vol. 99, pp. 1213-1225, 2019/06/01/ 2019, doi: <https://doi.org/10.1016/j.msec.2019.02.045>.
 - [177] J. J. Mercuri, S. Patnaik, G. Dion, S. S. Gill, J. Liao, and D. T. Simionescu, "Regenerative Potential of Decellularized Porcine Nucleus Pulposus Hydrogel Scaffolds: Stem Cell Differentiation, Matrix Remodeling, and Biocompatibility Studies," *Tissue Engineering Part A*, vol. 19, no. 7-8, pp. 952-966, 2013/04/01 2012, doi: 10.1089/ten.tea.2012.0088.
 - [178] J. J. Mercuri, S. S. Gill, and D. T. Simionescu, "Novel tissue-derived biomimetic scaffold for regenerating the human nucleus pulposus," *Journal of Biomedical Materials Research Part A*,

- <https://doi.org/10.1002/jbm.a.33001> vol. 96A, no. 2, pp. 422-435, 2011/02/01 2011, doi: <https://doi.org/10.1002/jbm.a.33001>.
- [179] L. Yu *et al.*, "Thermosensitive injectable decellularized nucleus pulposus hydrogel as an ideal biomaterial for nucleus pulposus regeneration," *Journal of Biomaterials Applications*, vol. 35, no. 2, pp. 182-192, 2020, doi: 10.1177/0885328220921328.
- [180] C. Ligorio, J. A. Hoyland, and A. Saiani, "Self-Assembling Peptide Hydrogels as Functional Tools to Tackle Intervertebral Disc Degeneration," *Gels*, vol. 8, no. 4, 2022, doi: 10.3390/gels8040211.
- [181] X. Zhang, X. Chen, H. Hong, R. Hu, J. Liu, and C. Liu, "Decellularized extracellular matrix scaffolds: Recent trends and emerging strategies in tissue engineering," *Bioactive Materials*, vol. 10, pp. 15-31, 2022/04/01/ 2022, doi: <https://doi.org/10.1016/j.bioactmat.2021.09.014>.
- [182] S. Sasikumar, S. Chameettachal, B. Cromer, F. Pati, and P. Kingshott, "Decellularized extracellular matrix hydrogels—cell behavior as a function of matrix stiffness," *Current Opinion in Biomedical Engineering*, vol. 10, pp. 123-133, 2019/06/01/ 2019, doi: <https://doi.org/10.1016/j.cobme.2019.05.002>.
- [183] J. Xu *et al.*, "Decellularised nucleus pulposus as a potential biologic scaffold for disc tissue engineering," (in eng), no. 1873-0191 (Electronic), 2019.
- [184] M. Parmaksiz, S. Dogan A Fau - Odabas, A. E. Odabas S Fau - Elçin, Y. M. Elçin Ae Fau - Elçin, and Y. M. Elçin, "Clinical applications of decellularized extracellular matrices for tissue engineering and regenerative medicine," (in eng), no. 1748-605X (Electronic), 2016.
- [185] A. Gupta, S. F. El-Amin, 3rd, H. J. Levy, R. Sze-Tu, S. E. Ibim, and N. Maffulli, "Umbilical cord-derived Wharton's jelly for regenerative medicine applications," *J Orthop Surg Res*, vol. 15, no. 1, p. 49, Feb 13 2020, doi: 10.1186/s13018-020-1553-7.
- [186] L. Penolazzi *et al.*, "Extracellular Matrix From Decellularized Wharton's Jelly Improves the Behavior of Cells From Degenerated Intervertebral Disc," *Front Bioeng Biotechnol*, vol. 8, p. 262, 2020, doi: 10.3389/fbioe.2020.00262.
- [187] H. N. Lovvorn, 3rd, M. E. Cheung Dt Fau - Nimni, N. Nimni Me Fau - Perelman, J. M. Perelman N Fau - Estes, N. S. Estes Jm Fau - Adzick, and N. S. Adzick, "Relative distribution and crosslinking of collagen distinguish fetal from adult sheep wound repair," (in eng), no. 0022-3468 (Print), 1999.
- [188] B. M. Sicari *et al.*, "The effect of source animal age upon the in vivo remodeling characteristics of an extracellular matrix scaffold," *Biomaterials*, vol. 33, no. 22, pp. 5524-5533, 2012, doi: 10.1016/j.biomaterials.2012.04.017.
- [189] S. Rajasekaran *et al.*, "Part 1: profiling extra cellular matrix core proteome of human fetal nucleus pulposus in search for regenerative targets," *Scientific Reports*, vol. 10, p. 15684, 09/24 2020, doi: 10.1038/s41598-020-72859-x.
- [190] M. Kudelko *et al.*, "PRIMUS: Comprehensive proteomics of mouse intervertebral discs that inform novel biology and relevance to human disease modelling," (in eng), no. 2590-0285 (Electronic), 2021.
- [191] L. J. White *et al.*, "The impact of sterilization upon extracellular matrix hydrogel structure and function," *Journal of Immunology and Regenerative Medicine*, vol. 2, pp. 11-20, 2018/09/01/ 2018, doi: <https://doi.org/10.1016/j.regen.2018.04.001>.
- [192] G. G. Giobbe *et al.*, "Extracellular matrix hydrogel derived from decellularized tissues enables endodermal organoid culture," *Nature Communications*, vol. 10, no. 1, p. 5658, 2019/12/11 2019, doi: 10.1038/s41467-019-13605-4.
- [193] T. E. o. E. Britannica. "Pepsin." Encyclopedia Britannica. <https://www.britannica.com/science/pepsin> (accessed).
- [194] L. A.-O. Piening, D. J. Lillyman, F. S. Lee, A. M. Lozano, J. R. Miles, and R. A.-O. Wachs, "Injectable decellularized nucleus pulposus tissue exhibits neuroinhibitory properties," (in eng), no. 2572-1143 (Electronic), 2022.
- [195] L. T. Saldin, M. C. Cramer, S. S. Velankar, L. J. White, and S. F. Badylak, "Extracellular matrix hydrogels from decellularized tissues: Structure and function," *Acta Biomater*, vol. 49, pp. 1-15, Feb 2017, doi: 10.1016/j.actbio.2016.11.068.

- [196] U. K. Blaschke, E. F. Eikenberry, D. J. S. Hulmes, H.-J. Galla, and P. Bruckner, "Collagen XI Nucleates Self-assembly and Limits Lateral Growth of Cartilage Fibrils," *Journal of Biological Chemistry*, vol. 275, no. 14, pp. 10370-10378, 2000, doi: 10.1074/jbc.275.14.10370.
- [197] P. Duran, M. Alperin, and K. L. Christman, "CHAPTER 7 Decellularized Extracellular Matrix Hydrogels: Fabrication, Properties, Characterization, and Current Applications," in *Decellularized Extracellular Matrix: Characterization, Fabrication and Applications: The Royal Society of Chemistry*, 2020, pp. 116-138.
- [198] S. Uriel *et al.*, "Extraction and Assembly of Tissue-Derived Gels for Cell Culture and Tissue Engineering," *Tissue engineering. Part C, Methods*, vol. 15, pp. 309-21, 09/01 2009, doi: 10.1089/ten.tec.2008.0309.
- [199] T. D. Johnson, S. Y. Lin, and K. L. Christman, "Tailoring material properties of a nanofibrous extracellular matrix derived hydrogel," *Nanotechnology*, vol. 22, no. 49, p. 494015, Dec 9 2011, doi: 10.1088/0957-4484/22/49/494015.
- [200] P. M. V. Raja and A. R. Barron, "Dynamic Mechanical Analysis," ed, 2021.
- [201] T. J. Keane, I. T. Swinehart, and S. F. Badylak, "Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance," (in eng), no. 1095-9130 (Electronic), 2015.
- [202] D. E. Terry, J. Chopra Rk Fau - Ovenden, T. P. Ovenden J Fau - Anastassiades, and T. P. Anastassiades, "Differential use of Alcian blue and toluidine blue dyes for the quantification and isolation of anionic glycoconjugates from cell cultures: application to proteoglycans and a high-molecular-weight glycoprotein synthesized by articular chondrocytes," (in eng), no. 0003-2697 (Print), 2000.
- [203] R. Lattouf *et al.*, "Picrosirius red staining: a useful tool to appraise collagen networks in normal and pathological tissues," (in eng), no. 1551-5044 (Electronic), 2014.
- [204] J. Fernandez-Perez and M. Ahearne, "The impact of decellularization methods on extracellular matrix derived hydrogels," *Sci Rep*, vol. 9, no. 1, p. 14933, Oct 17 2019, doi: 10.1038/s41598-019-49575-2.
- [205] S. Illien-Jünger, D. D. Sedaghatpour, D. M. Laudier, A. C. Hecht, S. A. Qureshi, and J. C. Iatridis, "Development of a bovine decellularized extracellular matrix-biomaterial for nucleus pulposus regeneration," (in eng), *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*, vol. 34, no. 5, pp. 876-888, 2016, doi: 10.1002/jor.23088.
- [206] C. Borrelli and C. T. Buckley, "Injectable Disc-Derived ECM Hydrogel Functionalised with Chondroitin Sulfate for Intervertebral Disc Regeneration," *Acta Biomater*, vol. 117, pp. 142-155, Nov 2020, doi: 10.1016/j.actbio.2020.10.002.
- [207] B. Zvarova *et al.*, "Residual Detergent Detection Method for Nondestructive Cytocompatibility Evaluation of Decellularized Whole Lung Scaffolds," *Tissue Engineering Part C: Methods*, vol. 22, no. 5, pp. 418-428, 2016/05/01 2016, doi: 10.1089/ten.tec.2015.0439.
- [208] F. Ghorbani, M. Ekhtiari, B. Moeini Chaghervand, L. Moradi, B. Mohammadi, and A.-M. Kajbafzadeh, "Detection of the residual concentration of sodium dodecyl sulfate in the decellularized whole rabbit kidney extracellular matrix," *Cell and Tissue Banking*, vol. 23, no. 1, pp. 119-128, 2022/03/01 2022, doi: 10.1007/s10561-021-09921-z.
- [209] I. A. Ansari, G. A. Gupta, J. Ramkumar, and K. K. Kar, "22 - Fly ash-mixed polymeric media for abrasive flow machining process," in *Handbook of Fly Ash*, K. K. Kar Ed.: Butterworth-Heinemann, 2022, pp. 681-713.
- [210] F. Cilurzo *et al.*, "Injectability Evaluation: An Open Issue," *AAPS PharmSciTech*, vol. 12, no. 2, pp. 604-609, 2011/06/01 2011, doi: 10.1208/s12249-011-9625-y.
- [211] H. S. Gill and M. R. Prausnitz, "Does needle size matter?," (in eng), no. 1932-2968 (Electronic), 2007.
- [212] J. Y. Wang, J. C. Mansfield, S. Brasselet, C. Vergari, J. R. Meakin, and C. P. Winlove, "Micro-mechanical damage of needle puncture on bovine annulus fibrosus fibrils studied using polarization-resolved Second Harmonic Generation(P-SHG) microscopy," *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 118, p. 104458, 2021/06/01/ 2021, doi: <https://doi.org/10.1016/j.jmbbm.2021.104458>.

- [213] A. Issy *et al.*, "Does a small size needle puncture cause intervertebral disc changes?," *Acta cirurgica brasileira / Sociedade Brasileira para Desenvolvimento Pesquisa em Cirurgia*, vol. 30, pp. 574-579, 09/09 2015, doi: 10.1590/S0102-865020150080000009.
- [214] J. Casale and J. S. Crane. "Biochemistry, Glycosaminoglycans." <https://www.ncbi.nlm.nih.gov/books/NBK544295/> (accessed 09-09-2022).
- [215] S. S. Sivan *et al.*, "Injectable hydrogels with high fixed charge density and swelling pressure for nucleus pulposus repair: Biomimetic glycosaminoglycan analogues," *Acta Biomaterialia*, vol. 10, no. 3, pp. 1124-1133, 2014/03/01/ 2014, doi: <https://doi.org/10.1016/j.actbio.2013.11.010>.
- [216] B. L. Showalter, N. R. Malhotra, E. J. Vresilovic, and D. M. Elliott, "Nucleotomy reduces the effects of cyclic compressive loading with unloaded recovery on human intervertebral discs," (in eng), no. 1873-2380 (Electronic), 2014.
- [217] T. C. Schmitz *et al.*, "Characterization of biomaterials intended for use in the nucleus pulposus of degenerated intervertebral discs," *Acta Biomaterialia*, vol. 114, pp. 1-15, 2020, doi: 10.1016/j.actbio.2020.08.001.
- [218] J. W. Chen, B. Li, Y. H. Yang, S. D. Jiang, and L. S. Jiang, "Significance of hypoxia in the physiological function of intervertebral disc cells," (in eng), no. 1045-4403 (Print), 2014.
- [219] A. J. Dominijanni, M. Devarasetty, S. D. Forsythe, K. I. Votanopoulos, and S. Soker, "Cell Viability Assays in Three-Dimensional Hydrogels: A Comparative Study of Accuracy," (in eng), no. 1937-3392 (Electronic), 2021.
- [220] A. Kumar P Fau - Nagarajan, P. D. Nagarajan A Fau - Uchil, and P. D. Uchil, "Analysis of Cell Viability by the Lactate Dehydrogenase Assay. LID - 10.1101/pdb.prot095497 [doi]," (in eng), no. 1559-6095 (Electronic), 2018.
- [221] I. Cortés-Ciriano and A. Bender, "How Consistent are Publicly Reported Cytotoxicity Data? Large-Scale Statistical Analysis of the Concordance of Public Independent Cytotoxicity Measurements," *ChemMedChem*, <https://doi.org/10.1002/cmdc.201500424> vol. 11, no. 1, pp. 57-71, 2016/01/01 2016, doi: <https://doi.org/10.1002/cmdc.201500424>.
- [222] K. M. Yamada, A. D. Doyle, and J. Lu, "Cell–3D matrix interactions: recent advances and opportunities," *Trends in Cell Biology*, vol. 32, no. 10, pp. 883-895, 2022, doi: 10.1016/j.tcb.2022.03.002.
- [223] C. R. Butler *et al.*, "Vacuum-assisted decellularization: an accelerated protocol to generate tissue-engineered human tracheal scaffolds," *Biomaterials*, vol. 124, pp. 95-105, 2017/04/01/ 2017, doi: <https://doi.org/10.1016/j.biomaterials.2017.02.001>.
- [224] Y. Luo and L. Ma, "Bioprosthetic heart valves with reduced immunogenic residuals using vacuum-assisted decellularization treatment," *Biomedical Materials*, vol. 15, no. 6, p. 065012, 2020/09/23 2020, doi: 10.1088/1748-605x/abaabf.
- [225] Z. Wang, F. Sun, Y. Lu, B. Zhang, G. Zhang, and H. Shi, "Rapid Preparation Method for Preparing Tracheal Decellularized Scaffolds: Vacuum Assistance and Optimization of DNase I," *ACS Omega*, vol. 6, no. 16, pp. 10637-10644, 2021/04/27 2021, doi: 10.1021/acsomega.0c06247.