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Research review paper

Tissue engineering strategies applied in the regeneration of the human intervertebral disk

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ABSTRACT

Low back pain (LBP) is one of the most common painful conditions that lead to work absenteeism, medical visits, and hospitalization. The majority of cases showing signs of LBP are due to age-related degenerative changes in the intervertebral disk (IVD), which are, in fact, associated with multiple spine pathologies. Traditional and more conservative procedures/clinical approaches only treat the symptoms of disease and not the underlying pathology, thus limiting their long-term efficiency. In the last few years, research and development of new approaches aiming to substitute the nucleus pulposus and annulus fibrosus tissue and stimulate its regeneration has been conducted. Regeneration of the damaged IVD using tissue engineering strategies appears particularly promising in pre-clinical studies. Meanwhile, surgical techniques must be adapted to this new approach in order to be as minimally invasive as possible, reducing recovering time and side effects associated to traditional surgeries. In this review, the current knowledge on IVD, its associated pathologies and current surgical procedures are summarized. Furthermore, it also provides a succinct and up-to-date overview on regenerative medicine research, especially on the newest tissue engineering strategies for IVD regeneration.

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Contents

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1. Introduction

Low back is subjected to most of the load transfer arising from weight bearing, which is further increased during body motion (Van Hoof et al., 2012). Such biomechanical stress may cause disk problems and pain, affecting people of all ages (O'Sullivan, 2005). Low back pain (LBP) is one of the main causes of medical visits, work absenteeism and hospitalization (Luo et al., 2004). It is estimated that the lifetime prevalence of LBP pain is between 50% and 80% in industrialized countries (affecting 15% to 30% of us at any given time), with a peak incidence in the ages between 45 and 64 (Andersson, 1999; Cassidy et al., 2005). This painful condition is responsible for an enormous impact in developed societies, from a social, economic and medical point of view. Estimated costs related to low back disorders exceed $100 billion per year in the USA alone (Katz, 2006). Back pain is usually defined as (i) acute if it lasts less than 6 weeks; (ii) sub-acute if comprised between 6 weeks and 3 months; and (iii) chronic if it lasts more than 3 months. LBP can be classified as "specific" when it is associated to a pathological cause (e.g. congenital spondylolisthesis, ankylosing spondylitis, neoplasms, infections, vertebral fracture and disk disease) or "non-specific" (about 90% of cases) (Kumar et al., 2012; Woolf and Pfleger, 2003). It has also been recognized that it is sometimes difficult to distinguish secondary motives from organic causes in clinical practice (Kumar et al., 2012). Worldwide research on the incidence of LBP is scarce, but the prevalence of this condition varies according to the definition used and the population studied. A large study reported the incidence of 28 cases per 1000 people/year in the nineties, in the Netherlands (Van den Velden et al., 1991). The incidence of LBP with sciatica was 11.6 per 1000 people/year, affecting slightly more men than women (Widanarko et al., 2012), and occurring more

frequently in the working population and among older people. Lifetime prevalence of LBP varies between 58% and 84%. The occurrence of LBP is associated with age (Woolf and Pfleger, 2003), physical fitness, smoking, bone mass index and strength of both back and abdominal muscles (DePalma et al., 2012). Despite the previously exposed, in most of the patients with signs/symptoms of LBP, degenerative changes in the intervertebral disk (IVD) can be found, and this fact should be considered and deserves further reflection. In fact, LBP is usually the first symptom of intervertebral disk degeneration (IDD), which eventually may progress to multiple spinal disorders such as disk herniation (Fig. 1). Spondylolisthesis and spinal stenosis, with the consequent neurological symptoms (e.g. anxiety, depression, and pain behavior) have also been reported (Cheung and Al Ghazi, 2008; Coventry et al., 1945; Raj, 2008; Roberts et al., 1995; Shankar et al., 2009; Urban and Winlove, 2007). Treatment of LBP and associated IDD usually requires bed rest, physiotherapy or medical care targeted to pain relief (e.g. administration of analgesic and anti-inflammatory drugs) (Di Martino et al., 2005). In more severe cases, it often requires surgical intervention since the IVD does not heal spontaneously (Cakir et al., 2009). In order to understand what research directions can be taken to prevent and reverse IDD, the complete knowledge of the anatomy, functioning and biomechanics of the IVD, as well as its associated pathologies is of foremost importance. Low success of disk replacement is the paradigm when treating IDD. Thus, the potential of using new surgical methods based in tissue engineering and regenerative medicine (TERM) strategies has been attracting a great deal of attention. The several stages of IDD often require different treatment strategies. TERM strategies using cellular approaches are suitable for treating IDD (Murrell et al., 2009), namely the use of stem cells which should preferably be isolated from young patients due
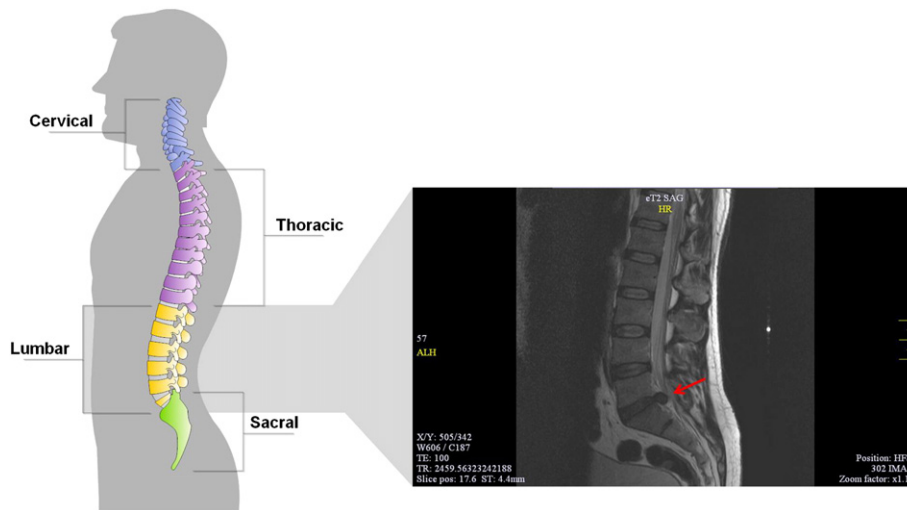
Fig. 1. Graphical representation of spine structure and sagittal MR image of the lumbar spine showing herniation at L5–S1 (lumbar segment 5 and sacral segment 1). The vertebral spine is composed by 24 articulating vertebrae (separated by intervertebral disks) and 9 fused vertebrae (5 form the sacrum and the other 4 the coccyx). The spine is divided into four regions according to their location: cervical (7 vertebrae), thoracic (12 vertebrae), lumbar (5 vertebrae) and sacral. As a result of trauma, wear or disease the outer ring of the disk (annulus fibrosus, AF) can rupture, and the inner core (nucleus pulposus, NP) may protrude (herniate) causing pressure on the nerve root, as shown by MRI.

to higher cellular viability (Civriz Bozdag et al., 2012), but strategies combining the use of hydrogels, stem cells and growth factors hold great promise for treating pathologic processes that occur early in the course of the IDD. Both young and old individuals can be treated by tissue engineered approaches, but the strategy used greatly depends on the stage of degeneration and patient's age. In fact, some authors believe that the replacement of the nucleus only is considered to be a potential alternative approach when it is aimed at restoring the mechanical functionality of the degenerated disk in patients with an almost intact annulus (Reitmaier et al., 2012). In this review, the composition of the IVD, consequences of its degeneration or removal, and procedures and limitations of the currently available treatments for IDD associated pathologies will be overviewed. Additionally, this review will focus on the most recent advances on alternative biomaterials and the promising TERM strategies for the regeneration of IVD.

2. Anatomy and physiology of the intervertebral disk (IVD)

2.1. Location on the human body and structural organization

The IVD is a cartilaginous structure located between two vertebral bodies, which are surrounded by ligaments and muscles. There are a total of 23 disks in the entire length of the spinal cord, each one with approximately 8–10 mm in height and 4 cm in diameter (Shankar et al., 2009). The cervical disks are thinner as compared to the lumbar disks, although the composition is nearly the same. The IVDs provide flexibility to the spine and support compressive loads arising from body weight and muscle tension. The complex fibro-cartilaginous IVD structure (Fig. 2) is divided into different regions: (i) nucleus pulposus (NP), (ii) annulus fibrosus (AF), and (iii) cartilaginous endplate (CEP) (Coventry et al., 1945).

2.1.1. Nucleus pulposus composition
The NP is located in the central region of the IVD and is mainly composed by a gelatinous-like extracellular matrix that is rich in proteoglycans (PGs), collagen fibers and water (Shankar et al., 2009; Urban and

Roberts, 2003). This structure is much less dense than the surrounding AF. It contains collagen (col) type II (mainly) and loosely organized elastin, which is responsible for supporting the PG's framework. The aggrecan is the major PG present in the NP, which covalently links highly sulfated glycosaminoglycan (GAG) chains (e.g. chondroitin and keratin sulfate). These negatively charged molecules create an osmotic drive force that maintains the water in the nucleus, and thus, are responsible for the characteristic ability of the healthy NP to retain water (Urban et al., 2000). As compared to the other regions of the disk, the NP has lower collagen content, but higher concentration of aggrecan and water.

Apart from these major constituents, the NP also contains minor quantities of other types of collagens (e.g. IX, VI and III), small proteoglycans (e.g. decorin, biglycan, lumican and fibromodulin) and other proteins and glycoproteins (Urban et al., 2000).

NP cells have a rounded morphology but are distinct from articular chondrocytes (Zhao et al., 2007). Despite expressing chondrogenic markers such as Sox-9, col-II, and aggrecan (Nerurkar et al., 2010; Vonk et al., 2010), it has been also described that human NP cells possess specific NP-markers such as CD24, CA12, HIF-1, OVOS2, PAX1 and FOXF1 (Fujita et al., 2005; Minogue et al., 2010a, 2010b; Rajpurohit et al., 2002). KRT19 was also considered a candidate for NP cell characterization and could be used to distinguish human NP from AF tissue since it is highly expressed in NP and at significantly lower levels in AF (Rutges et al., 2010a). However, its expression was only detected in the disks of young individuals. NP cells have low mitotic capacity and ability to regenerate, but they remain viable below physiological pH (7.4), under hypoxia, and low glucose levels. Some authors have observed the presence of notochordal cells within the NP during embryonic development, but these cells seem to become increasingly sparse after birth and may then completely disappear (Kim et al., 2009; Zhao et al., 2007). The origin of human NP cells is far from being completely understood (Roberts et al., 2006; Urban et al., 2000). Some works suggest that human NP cells descend from the notochord while others indicate that NP cells result from the migration of cells from the CEP and inner AF into the interior of the disk, after notochord receding (Weiler et al., 2010; Zhao et al., 2007). While notochordal cells with
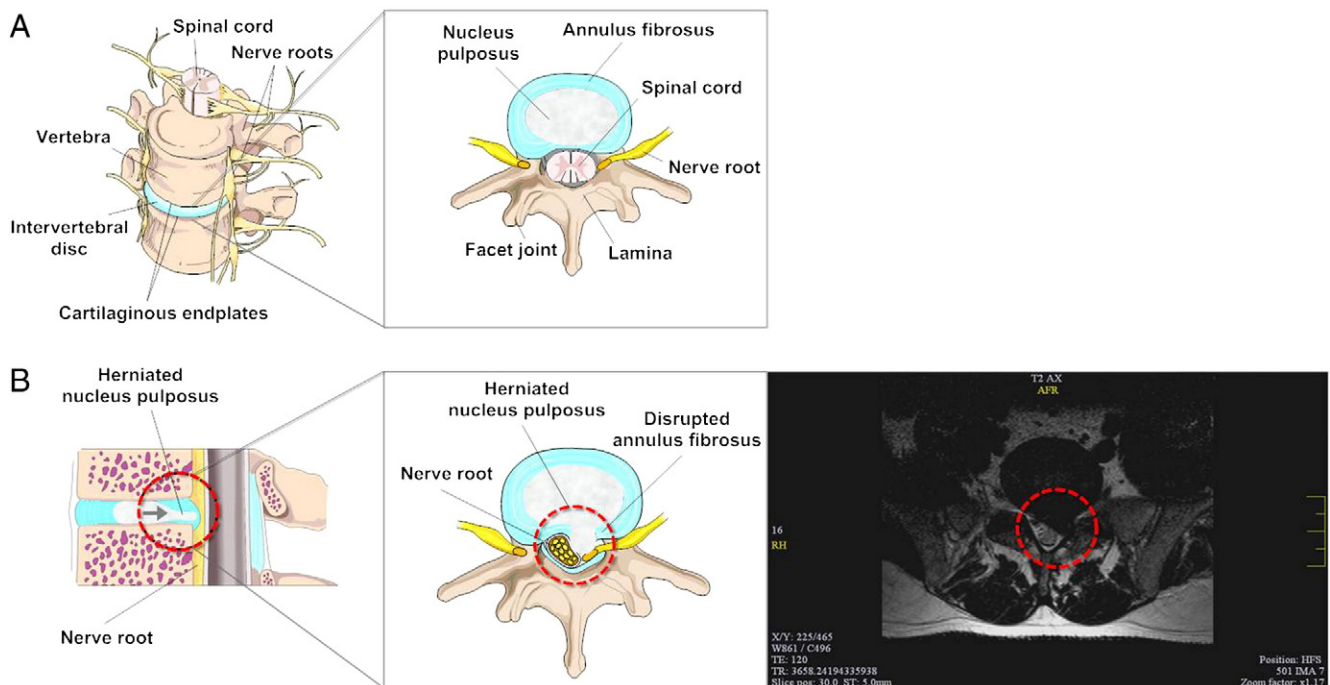

Fig. 2. Normal and herniated intervertebral disk (IVD). (A) Graphical representation of a vertebral segment showing IVD positioning between two adjacent vertebrae and superior view of the structure of a normal IVD; (B) graphical representation of an herniated IVD (sagittal and superior view) and corresponding MR image. The dashed circle indicates the protruded NP.

typical morphologic and phenotypic features persist in some species (e.g. rats, mice, rabbits) throughout the entire life, in humans some studies have reported their presence only in the first few years of life, suggesting that cell population is gradually replaced by another cell population (i.e. mature NP cells) with characteristics more similar to cartilage chondrocytes (Chan et al., 2011; Smith et al., 2011). However, the suggested absence of notochordal cells on adult NP is only based on morphologic and histologic studies. Moreover, recent studies have reported the presence of cells with a notochordal-like phenotype in adult humans, determined by the expression of notochordal-related molecular markers, such as cytokeratin types KRT8, KRT18, KRT19 and galectin-3 (Chan et al., 2011; Gantenbein-Ritter and Chan, 2012; Smith et al., 2011; Weiler et al., 2010).

Several recent studies also reported the presence of immature cell types expressing mesenchymal stem cell (MSC) markers in normal and degenerated IVD (Blanco et al., 2010; Brisby et al., 2013; Henriksson et al., 2009b; Risbud et al., 2007). Blanco et al. (2010) have demonstrated that the human degenerated NP contains MSC, which are quite similar to MSC obtained from the bone marrow of the same patients, although they lack the adipogenic differentiation ability. In similar works (Brisby et al., 2013; Henriksson et al., 2009b; Risbud et al., 2007), cells isolated both from AF and NP tissues obtained from normal and degenerated disks were shown to express classic MSC markers. It was also demonstrated that AF- and NP-derived cells evidenced ability to differentiate into osteogenic, chondrogenic and adipogenic lineages.

2.1.2. Annulus fibrosus composition

The AF is a structure which is much more fibrous than the NP, with higher collagen (mainly of type I) content, but low water percentage (Shankar et al., 2009). This fibrous tissue encircles the NP and is highly organized and oriented in concentric rings, i.e. collagen fibers forming lamellar layers. Elastin fibers are present within the collagen fibers, which are responsible for allowing the disk to return to its original position after a load charge. The AF layers are firmly attached to the endplates and inserted into the anterior and posterior longitudinal ligaments. The AF fibers are strongly attached to the anterior longitudinal ligament, whereas the attachment to the posterior ligament is weaker. This fact may explain why posterior protrusions of the disk occur more frequently than anterior bulging (Coventry et al., 1945).

AF cells are described as fibrochondrocytes, due to their expression of markers typical of both fibroblasts (e.g. col-I) and chondrocytes (e.g. col-II, and aggrecan) (Vonk et al., 2010; Zhao et al., 2007). These cells thus share many phenotypic features attributed to the fibrochondrocytes found in meniscus tissue, such as their elongated morphology.

2.1.3. Cartilaginous endplate composition

Formed by hyaline cartilage, the CEP separates the NP and AF from the adjacent vertebral bone (Fig. 2A). The CEP is not attached to the subchondral vertebral bone but is instead strongly connected to the AF, preventing the highly hydrated NP from protruding into the vertebrae. The CEP normally present a thickness inferior to 1 mm (variable across the width of the IVD), but they are thinner in the region adjacent to the NP (Moore, 2006). In early life, the endplates are larger and highly vascularized; at the third decade of life essentially there are almost no blood vessels present (Raj, 2008).

The biochemical composition of the CEP is quite similar to the other regions of the IVD since it comprises a gel of hydrated PG molecules reinforced by a network of collagen fibrils. The diffusion of nutrients between the subchondral bone of the vertebra and the interior of the disk is facilitated by the morphology of the healthy CEP since the collagen fibrils accumulate at the periphery of the CEP while the center is mostly composed by water and PGs.

In a recent study performed by Liu et al. (2011), the presence of MSC in degenerated human CEP was also demonstrated. In that study, the

isolated cells showed similar behavior to MSC derived from bone marrow and were, in fact, superior in terms of osteogenesis and chondrogenesis.

2.2. Metabolism of the intervertebral disk

IVD cells are responsible for the homeostasis of the extracellular matrix (ECM). Cell metabolism occurs mainly by anaerobic glycolysis pathway, with the consumption of glucose and formation of lactic acid taking place at a high rate (Bibby and Urban, 2004). It is, therefore, vital to cells' survival that an appropriate supply of glucose and adequate removal of lactic acid from the cell environment occurs, for maintaining the balance between anabolism and catabolism. Although disk cells use very low quantities of oxygen, through pathways that are not clear yet, its presence is required for the maintenance of cell activity (Grunhagen et al., 2006). The transport of nutrients and metabolites to and from the disk cells is not efficient due to the avascular nature of the IVD, and thus disk cells depend on the existent blood supply at the disk boundaries for obtaining nutrients (Grunhagen et al., 2006). During the process of aging and disk degeneration, several factors, other than avascularity, can decrease the blood supply to the disk, reducing the availability of nutrients to cells and causing loss of cell activity and viability. The metabolism regulation includes the presence of a variety of molecules (e.g. cytokines, enzymes, enzyme inhibitors, and growth factors), a continuous rise in osmolarity and the mechanical loads applied normally in daily activities (Bibby and Urban, 2004; Grunhagen et al., 2006; Iatridis et al., 2006).

Since the main route for obtaining energy is anaerobic glycolysis, the disk environment is normally slightly acidic (6.9–7.1) due to the concentration of lactic acid. With age and degeneration, the capillary blood vessel network decreases, leaving the nucleus with a very small supply of oxygen and high levels of toxic metabolites. Thus, in IDD, the levels of lactic acid increase and consequently pH decreases. his alteration can affect matrix synthesis and maintenance. In addition, it can also compromise the cells metabolism and survival if the pH reaches more acidic levels (<6.0). The lack of oxygen also compromises the ability of cells to make and repair the matrix, although disk cells seem to be able to survive for several days with-out oxygen (Grunhagen et al., 2006; Urban and Winlove, 2007).

In healthy adult IVD, there are only a few (or even none) blood vessels (Roberts et al., 1995). They are originated from branches of the segmental artery and are mostly present in the CEP before the first year, but in adult life they are restricted to longitudinal ligaments and sporadically to the outer portions of the AF (Shankar et al., 2009). A significant part of IVD vascularization comes from the subchondral bone of the vertebral body, and it is responsible for the blood supply of the inner AF and NP.

Another important component of IVD nutrition is the CEP. In infants, the endplates are thicker, with canals that allow substances and oxygen to reach disk cells. With aging the endplates become thinner and the canals disappear. However, nutrition of adult disk cells is still possible since the nutrients are still able to diffuse. In IDD, calcifications have been observed in both the AF and the NP, especially near tears and clefts (Rutges et al., 2010b). Rutges et al. (2010b) clearly showed that hypertrophic differentiation occurs during IDD. However, the authors did not clarify why hypertrophic differentiation and mineralization is most prominent at the interface between the NP and AF (transition zone). Despite, one possible explanation provided by the authors is that it may be related to the existence of local biomechanical loading profiles. The endplates also tend to become thinner and eventually, "calcification" can occur with age and progression of IDD, being this way a significant barrier to diffusion of nutrients and metabolites (Moore, 2006).

2.3. Functions of the intervertebral disk

There are three major functions of the IVD: (i) resist the compression and support body weight, (ii) act to provide some movement on the vertebral trunk, and (iii) join the vertebral bodies of the vertebral trunk (Cheung and Al Ghazi, 2008). Also, due to its fibrous structure, the AF encloses the nucleus, avoiding it to protrude as a result of the high pressure experienced during compressive movements. The compressive loading experienced by the spine, forces the water to move out of the nucleus, resisting this way to compression. Depending on the loading conditions, the hydrostatic and osmotic pressures of NP ranges from 0.1 to 3 MPa (Iatridis et al., 1997b). Wilke et al. (2001) also reported on intradiscal pressures during different exercises. The pressure for unsupported sitting was 0.46 MPa; relaxed standing was 0.5 MPa; standing flexed forward was 1.1 MPa; lifting a 20 kg weight with a round flexed back was 2.3 MPa, while with the flexed knees was 1.7 MPa, among others. However, the NP will not be able to maintain its compressive properties upon load if it has low water content (as it happens in IDD). Thus, it is the AF that needs to act as a fibrous solid in order to resist compression directly. The high pressure could lead eventually to fissuring and fragmentation of the AF, which when fails to function, leads to herniation of the NP (Figs. 1 and 2B), and frequently to the extrusion of the NP (Adams and Roughley, 2006). In this case, tissue engineering approaches applied to regeneration of IVD must focus not only the restoration of NP functionality, but address also the problem of AF closure. More recently, nucleus replacements consist of soft polymeric materials, such as hydrogels or silicone that are able to undergo larger deformations under compression. Ideally, the deformation that a substitute material can withstand should ensure tension to the annular fibers in a similar way to natural nucleus (Ahrens et al., 2009). An advantage of hydrogels over silicone is their potential ability to replicate a physiological fluid flow and to provide a micro-environment for disk cells to boost the regeneration of the intervertebral disk (Reitmaier et al., 2012).

3. IVD degeneration and associated pathologies

3.1. Changes in IVD structure and composition

As previously stated, IDD is a common pathology affecting the IVD and, although a relation between age and IDD seems to exist, there are no clear data that support IDD as a process of aging (Sowa et al., 2008; Urban and Roberts, 2003). It seems more likely that two distinct pathways may exist since there is clinical evidence of different rates of degeneration among individuals of different ages (Battié et al., 2004). IDD may be secondary to injury or inflammation, whereas aging represents a normal process of cellular and molecular changes leading to disk maturation. Anyway, the changes occurring in disk structure are similar in both cases, so it is difficult to differentiate pathologic alterations from those who are age-related (Roberts et al., 2006; Urban and Roberts, 2003). There is clear evidence that significant age-related changes initiate at the end of the first decade of life and become more considerable in the first half of the second decade (Boos et al., 2002). These alterations differently affect all the anatomic regions that compose the IVD, being more pronounced firstly in the CEP, followed by the NP, and finally the annulus (mainly in the outer AF) later in life. However, even earlier than the first decade of life (in the age of 2), several mild degenerative changes were observed such as decay and/or disappearing of nothocordal cells, decrease of NP cells proliferation, mild cleft formation, changes in cell content, extracellular matrix degeneration in the CEP and diminution of blood vessels in the AF and CEP (Boos et al., 2002; Chan et al., 2011; Urban and Roberts, 2003).

Loss of demarcation between the annulus and the nucleus increases with age, from the second decade onward. More structural changes in the cartilage endplates appear with increasing age, namely: (i) cracks

and thinning of the end plate, (ii) altered cell density, (iii) microfracture in the adjacent subchondral bone, and (iv) bone sclerosis.

The changes observed during intervertebral disk aging also include: (i) diminished blood supply, (ii) decrease in PGs (aggrecan) and water contents, (iii) increase in collagen proportion, (iv) reduction of oxygen levels and pH (due to lactic acid), and (v) pressure increase in the NP, associated with changes in the CEP (Boos et al., 2002; Sowa et al., 2008). The most significant biochemical change associated to IDD is, in fact, the loss of PGs, which is responsible for the loss of osmotic pressure of the IVD and dehydration (Raj, 2008), thus affecting the mechanical properties of the NP (decreasing disk ability to resist compressive forces) and disk height (Urban and McMullin, 1988). In this condition, the IVD is also known as black disk. As determined by rheology, the native human NP presents a shear modulus of 7–20 kPa that corresponds to a Young's modulus of 20–60 kPa (Iatridis et al., 1997b; Leahy and Hukins, 2001). These values were, however, obtained post mortem, and they may not translate the real native NP properties (Iatridis et al., 1997a). The biochemical changes related to GAGs, originated by the loss of aggrecan rather than an increase in the production of collagen, are greatly associated with the decrease of hydration on the matrix and disk degeneration/aging process (Urban and Winlove, 2007).

3.2. Influence of oxidative stress

IDD progression has also been associated with the development of oxidative stress (Cheng et al., 2011). Reactive oxygen species (ROS) are overproduced during the IDD process, which may be responsible for inducing the apoptosis of NP cells. However, it has been reported that IDD can result from cell senescence rather than a decrease in cell number (Gruber et al., 2007; Liebscher et al., 2011). One regenerative approach that could potentially assist in controlling ROS production and cell apoptosis is the use of antioxidant molecules (Turgut et al., 2003). Turgut et al. (2003) have demonstrated that the use of exogenous melatonin in rats reduces CEP vascularity of degenerated IVDs. It is suggested by the authors that this effect may be due to melatonin capacity to act as a broad-spectrum antioxidant and a potent endogenous free radical scavenger, protecting the cells of ROS toxicity.

3.3. De-regulation of biochemical balance

As aforementioned, the disk is able to maintain its normal functions while the rates of macromolecular synthesis and breakdown are in balance. When the disk becomes degenerated due to pathological conditions (e.g. puncture or trauma) and abnormal mechanical loading, this balance is disrupted with the rate of macromolecular breakdown being greater than the synthesis rate (Urban et al., 2000). IVD cells express a wide variety of anabolic regulators, e.g. polypeptide growth factors, such as insulin-like growth factor 1 (IGF-1), transforming growth factor beta (TGF-β), and the bone morphogenetic protein (BMP). However, it is the catabolic regulators (e.g. interleukin 1 (IL-1) and tumor necrosis factor (TNF)-α) that influence the synthesis of matrix-degrading enzymes, such as matrix metalloproteinases (MMPs) and aggrecanases (Lee et al., 2011; Roberts et al., 2000). The latter can alter IVD homeostasis and lead to progression of degeneration, but macrophages can also infiltrate into the IVD and modulate the process of IDD in an autocrine fashion (Bae and Masuda, 2011).

3.4. Genetic factors

As aforementioned, IDD is associated with different risk factors and aging, although recent studies suggest that disk herniation and IDD may be explained in a large degree by genetic factors (Battié et al., 2009; Cheung and Al Ghazi, 2008; Kalb et al., 2012). Indeed, several

works indicated a possible interaction between environmental (like repeated traumatic and microtraumatic events) and genetic factors (such as genes that encode for extracellular matrix components and enzymes involved in their turnover) (Colombini et al., 2008).

3.5. Relation to pain

Around 10% of 50 year-old disks and 60% of 70 year-old disks are severely degenerated with more than 50% of cells within adult disks being necrotic (Urban and Roberts, 2003). Either it is pathological or not, it is still unknown. In fact, age-related changes in matrix composition are not always associated with pain and could merely reflect necessary adaptations to increased mechanical loading due to growth, as well as to reduced metabolite transportation (Adams and Roughley, 2006).

Even so, pain is usually related to IDD. The ingrowth of nerves and microvascular blood vessels that are typically present in a degenerated disk are associated with chronic LBP, even when there is no herniation of the disk (Freemont et al., 1997, 2002). It has been described that in the joints of patients with osteoarthritis, the presence of the cytokine interleukin-1 (IL-1) is responsible for chondrocyte stimulation of the expression of MMPs and vascular endothelial growth factor (VEGF) (Scholz et al., 2010). This induction results in an increase of cartilage matrix degradation by MMPs and also enhances the development of ingrowths of blood vessels originating from the subchondral bone into the cartilage. The establishment of pain occurs from the subsequent ingrowth of bony outgrowths and nerve supply by sensory neurons. As occurs in osteoarthritic cartilage, the cytokine IL-1β is also present in IDD and thus it induces angiogenesis and subsequently re-innervation of IVD accompanied by pain (Lee et al., 2011; Scholz et al., 2010). Therefore, angiogenesis also enhances IDD and contributes to the establishment of pain.

Chan et al. (2011) provide a deeper understanding on the structure and biology of normal and abnormal IVD. IVD has few pain receptors except in the periphery of the disk. Thus, in patients with moderate to severe degrees of inflammation/degeneration of IVD, pain may arise as a consequence of re-innervation (Freemont et al., 1997, 2002).

The microstructural clefts and tears that appear with aging seem to have no significant effect, as long as they remain small (Adams and Roughley, 2006). On the other hand, macroscopic changes that occur mostly between L4 and S1 vertebrae are usually related with pathology.

3.6. Pathologies associated to IDD

One of the most common pathology related to IDD is, in fact, disk herniation that usually causes canal stenosis (Cheung and Al Ghazi, 2008). Disk herniation may vary from protrusion, when the outer annulus lamellae remains intact, to extrusion, when the AF is ruptured, or even to sequestration, which occurs when the herniated part is completely detached from the body of the disk (Roberts et al., 2006).

IDD may cause additional problems, other than spinal stenosis. It may also cause alterations on the stability of the spine (or aggravate pre-existing conditions related to it), like kyphosis, scoliosis and spondylolisthesis (Urban and Roberts, 2003). Isthmic/lytic spondylolisthesis is a condition causing neurological problems and spinal deformity that is multifactorial in origin, i.e. mechanical, genetic, and hormonal factors have been related to appearance of this disease (Ganju, 2002). Fig. 3 shows an L5–S1 secondary herniation in a 40-year-old woman with isthmic lysis. The majority of spondylolysis and spondylolisthesis conditions can be managed with conservative and non-operative treatments, but when pain and neurologic impairment are present, surgical intervention may be required.

IDD alters the height of the disk and, therefore, the mechanical behavior of the spine, interfering with bone structure, as well as muscles and ligaments. Also, the apophyseal facet joints adjacent to the affected disks may be subjected to abnormal loads, which results in osteophyte formation and osteoarthritis changes (Urban and Roberts, 2003). Because osteophyte are bone formations on facet joints and CEP, this will not only affect the effective absorption and distribution of compressive loads, but will also decrease intervertebral foraminal dimensions, which can ultimately result in nerve compression (Woods et al., 2010).

IDD also affects the ligamentum flavum, reducing its tensional force which thus may cause remodeling and thickening. This results in loss of elasticity which causes the ligament to bulge into the spinal canal, leading to spinal stenosis (Urban and Roberts, 2003).

4. Procedures and limitations of the actual treatments of IDD associated pathologies

The common feature of the actual treatments for IDD and associated pathologies is that they only target the symptoms and not the underlying pathology (Raj, 2008). Patients with spinal problems that cannot be managed with conservative treatments are indicated for surgery. Currently available surgical treatments focus on the excision of lesion and release of patient's pain, as well as on the stabilization of the spinal column.

Actually, there are four general categories of surgical procedures (Don and Carragee, 2008) currently used for treatment of IDD-associated pathologies: (i) discectomy and spinal fusion; (ii) disk arthroplasty; (iii) dynamic stabilization; and (iv) minimally invasive approaches such as percutaneous intradiscal pulsed radiofrequency (Fukui et al., 2012), thermal annular procedures (Fukui et al., 2012; Helm et al., 2012), ozone therapy (Magalhaes et al., 2012), local steroid injection (Parr et al., 2009), coblation nucleoplasty (Azzazi et al., 2011; Lemcke et al., 2010) and discectomy (Table 1).

4.1. Discectomy and spinal fusion

Spinal fusion is a surgical technique that is commonly used to join or fuse two or more adjacent vertebrae together in a permanent way. This
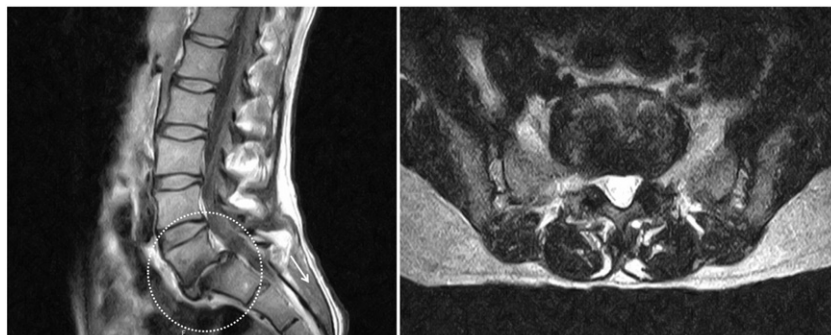

Fig. 3. MRI T1 and T2 images of isthmic lysis with L5–S1 secondary herniation in a 40-year-old female patient.

Table 1
Overview of clinical problems and pathologies associated to intervertebral disk degeneration, its diagnosis methods and current therapies.

Clinical problem/pathology	Diagnosis	Therapy	References
Trauma/fractures	MRI	Discectomy	McAfee (2004)
Spondylolisthesis	X-ray	Spine fusion	Swan et al. (2006)
Spine deformity/instability			Don and Carragee (2008)
Radiculopathy	MRI	Arthroplasty	McAfee (2004)
Myelopathy	X-ray		Ozgur et al. (2005)
			Don and Carragee (2008)
Spinal stenosis	MRI	Dynamic stabilization	Bono and Garfin (2004)
Facet joint arthrosis	X-ray		Barrey et al. (2008)
			Don and Carragee (2008)
Disk herniation	MRI	Percutaneous discectomy	Hijikata (1975)
	X-ray	Chemonucleolysis	Saal and Saal (2000)
		Intradiscal electrothermal therapy	Kim et al. (2002)

type of surgery typically involves the use of bone graft, which is packed between the vertebrae (lumbar or cervical) in order to induce ossification and fuse them together. The bone graft can be obtained by the surgeon from another part of a patient's body, usually pelvic bone (autograft) or from a donor (allograft). The bone graft together with a biomechanical spacer implant will substitute the IVD, which is entirely removed in the process by discectomy in an initial step. In addition, the use of BMP in spinal fusion surgery has been increasing rapidly in the last decade, with successful and promising results (Deyo et al., 2012). Spinal fusion surgery is commonly used for the treatment of spinal disorders such as spondylolisthesis, scoliosis, severe disk degeneration, or spinal fractures. Spinal fusion secondary to fractures, deformity or instability such as spondylolisthesis has been shown to be very effective (Swan et al., 2006). However, due to the complexity of the spine articulations, its benefits in the control of pain related to IDD are inconclusive (Denaro et al., 2009). Spinal fusion compromises spine flexibility and usually leads to pseudoarthrosis, due to the increased strain in adjacent segments which accelerates IDD in the adjacent vertebrae (Don and Carragee, 2008). The standard approach is a traditional open surgery, although new minimally invasive techniques of lumbar fusion have been described with similar results (Lubbers et al., 2012; Ozgur et al., 2005).

Several techniques have been described starting from "simple" instrumented posterior fusion (Zhang et al., 2012). Lumbar interbody fusion (LIF) combines spinal fusion with an interbody device, such as a threaded cage (Schimmel et al., 2012), but other instrumentation may be used, as well. Many types of cage have been developed; two examples of these intervertebral body fusion devices are the polyetheretherketone (PEEK) cage (Dickerman et al., 2008; Liao et al., 2008), which can be filled with cancellous bone as shown in Fig. 4, and polyetherimide (PEI)-based cage reinforced with carbon fibers (Gloria et al., 2008). Gloria et al. (2008) developed a promising threaded composite cage by reinforcing a PEI-based device with carbon fibers in order to tailor the prosthesis stiffness and overcome the high stress shielding and stress concentration effects observed between the prosthesis and bone. The characterization of its biomechanical behavior on a porcine model has shown a compressive stiffness closer to the natural IVD (as compared to commercial titanium devices). However, the movement between the two adjacent vertebrae is still reduced.

Over the last two decades, a tremendous effort has been made to develop an IVD prosthesis that has mechanical properties similar to the spine segment to be replaced (Gloria et al., 2007). Poly(2-hydroxy-ethylmethacrylate) (PHEMA) hydrogels reinforced with poly(caprolactone) (PCL) have been tested for finding application as IVD prosthesis (Ambrosio et al., 1996). It has also been evaluated in the form of a semi-interpenetrating polymer network (s-IPN) composite hydrogel composed of PHEMA and poly(methyl methacrylate) (PMMA) reinforced with poly(ethylene terephthalate) (PET) fibers (Gloria et al., 2007). More recently, the PHEMA/PMMA s-IPN composite hydrogel reinforced with PET fibers has been combined with two hydroxyapatite-reinforced polyethylene (HAPEX™) endplates (which are used to anchor

the device to the vertebral bodies) to manufacture a total customized IVD substitute (Gloria et al., 2011). That works have shown that the PHEMA/PMMA hydrogels reinforced with PET fibers have a mechanical and viscoelastic behavior similar to the natural lumbar IVDs. In addition, the authors proposed and validated a pilot-scale device production process envisioning the manufacture of custom-made implants for human total IVD substitution.

Several approaches in current practice are adapted to anatomical segments, clinical indications and surgeon's experience: anterior lumbar interbody fusion (ALIF) (Schimmel et al., 2012), posterior lumbar interbody fusion (PLIF) (Xu et al., 2013) or transforaminal lumbar interbody fusion (TLIF, Fig. 5) (Xu et al., 2013; Zhang et al., 2012).

Discectomy is a common surgical procedure used to treat lumbar disk herniation. Following removal of the tissue, the space of the excised NP is replaced by fibrocartilaginous granulation tissue. This procedure results in alterations of both the material properties of the intervertebral disk and the biomechanics of the lumbar motion segments, which may influence adjacent upper segmental disk degeneration (Tang and Rebholz, 2013). This effect is increased

Fig. 4. Polyetheretherketone (PEEK) cage filled with cancellous bone used in spinal fusion.
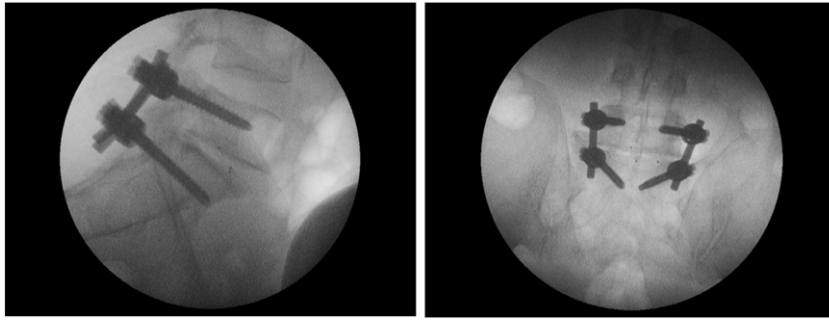
Fig. 5. X-ray images of L5–S1 treated using a transforaminal lumbar interbody fusion (TLIF) technique.

when discectomy is combined to laminectomy procedures with subsequent increased instability (Kawaguchi et al., 2007). These procedures, besides the possibility of complaints' relief on a short-term, might play a role in accelerated spine degeneration (Kawaguchi et al., 2007; Tang and Rebholz, 2013).

4.2. Disk arthroplasty and dynamic stabilization

The arthroplasty consists of total disk replacement with an artificial disk that reproduces the load transmission properties of the natural disk. Theoretically, it maintains spinal movements thus preventing some of the problems related to surgical fusion, namely pseudoarthrosis or limited movements (McAfee, 2004). Arthroplasty is indicated in cases of compression of the spine or raquidian nerves in the intervertebral space. However, clinical results comparing disk arthroplasty with lumbar fusion have been mostly inconclusive (Zigler et al., 2007). Contraindications to disk arthroplasty include spondylolisthesis, facet joint arthritis, central or lateral recess stenosis, fixed deformity, infection, osteoporosis and herniation of NP with radiculopathy that cannot be decompressed by way of anterior approach (Don and Carragee, 2008). These contraindications strictly limit the number of patients that could be selected to arthroplasty. Heterotopic ossification, i.e. abnormal formation of bone within extra-skeletal soft tissues, is one of the complications of disk replacement. The main disadvantage of disk arthroplasty is the reduction of spine movements (Lee et al., 2010). Additionally, it has been demonstrated that the disk implant does not have elastic shock absorption properties and could eventually produce abnormal forces along the facet joints and dimensional changes of the intervertebral foraminal during motion (Bono and Garfin, 2004).

Dynamic stabilization makes use of spacers that will augment the interspinous space and unload the disk and facet joints, thus reducing compression of the nerves (Don and Carragee, 2008). This procedure is indicated in the treatment of LBP caused by spinal stenosis and facet joint arthrosis, although there is no convincing evidence supporting the use of this technique. Dynamic stabilization systems are designed to stabilize the column segments, preserving the original disk and the facet joints (Meyers et al., 2008). The advantage of this approach, as compared to the previous ones, is that it prevents the side effect of adjacent level degeneration (Barrey et al., 2008). Nevertheless, stability of these devices in long-term use is still unknown.

4.3. Minimally invasive approaches

Minimally invasive approaches such as the previously mentioned ALIF, PLIF and TLIF constitute the three most common minimally invasive fusion surgeries used. Microdiscectomy (Maroon, 2002), percutaneous discectomy (Hijikata, 1975), chemonucleolysis (Kim et al., 2002), intradiscal electrothermal therapy (Saal and Saal, 2000), endoscopic lumbar discectomy (Alleyne and Rodts, 1997) and percutaneous laser discectomy (Choy, 1998) are other minimally invasive procedures. PLIF is a fusion technique, where the vertebrae are reached through an

incision in the patient's back (posterior). The PLIF procedure requires several steps: (i) pre-operative planning (MRI and CAT scans) of the disk space, (ii) templating, i.e. laminectomy and access the nerve roots, removal of the affected disk followed by preparation of bone surfaces of adjacent vertebrae for fusion, and (iii) implantation of the bone graft, allograft or BMP with a cage. TLIF is similar to PLIF, but the access is made by the side of the spinal canal through a midline incision in the patient's back. This technique presents several advantages such as a reduction of muscle dissection and nerve manipulation during templating. By its turn, ALIF resembles PLIF; however, it is performed from the front (anterior) of the body.

All these techniques are designed for conventional operations involving large anatomic dissections performed via small incisions. The advantages comprise reduction of the risk of infection and healing problems, obvious esthetic benefits (Jaikumar et al., 2002) and improvement of recovery time, i.e. most patients are up and walking within 24 h and easily return to a healthy life. Thus, these surgical approaches greatly contribute to the relief of the medical–economic burdens, which are mainly related to reduction of hospitalization and work absenteeism costs (Thongtrangan et al., 2004). Percutaneous discectomy includes a wide variety of techniques and equipment. The principal method includes the use of an aspiratory probe, which removes the NP, generally using a posterolateral approach. This way the IVD can be decompressed, reducing the pressure in the herniated disk (Raj, 2008). Another common though controversial technique is chemonucleolysis, which uses enzymes such as chymopapain (Kim et al., 2002). This enzyme depolymerizes the PGEs and glycoproteins present in IVD, causing the bulging disk to shrink while leaving the AF intact (Smith et al., 1963). Recently, another interesting procedure was developed (Saal and Saal, 2000). Intradiscal electrothermal therapy is performed by inserting a catheter with thermal resistive coil at the end, into the affected disk. The coil is then heated, modifying the collagen fibers which then become thicker and contract. These changes decrease the ability of the IVD to re-vascularize and re-innervate as occurs in degenerated disks. The collagen modifications also lead to a reduction in AF dimensions, increasing the stability of the disk itself.

Indications for these techniques include radicular pain, contained herniated disk and failure of conservative measures (Choy, 1998; Kim et al., 2002; Onik et al., 1985; Saal and Saal, 2000). On the other hand, contraindications include extruded or sequestered disk and clinical cases where the spinal canal is compromised by more than 50% as a result of disk herniation, spinal stenosis or spondylolisthesis.

Percutaneous vertebral augmentation methods for cement application into the vertebral body have been developed. Vertebroplasty (VP) and kyphoplasty (KP) allow managing symptomatic compression fractures without neurologic impairment (Denaro et al., 2009). In VP, polymethylmethacrylate (PMMA) cement is injected percutaneously into the vertebral body by means of X-ray imaging guidance (Fig. 6). In KP, an inflatable bone tamp is placed percutaneously into the vertebral body.
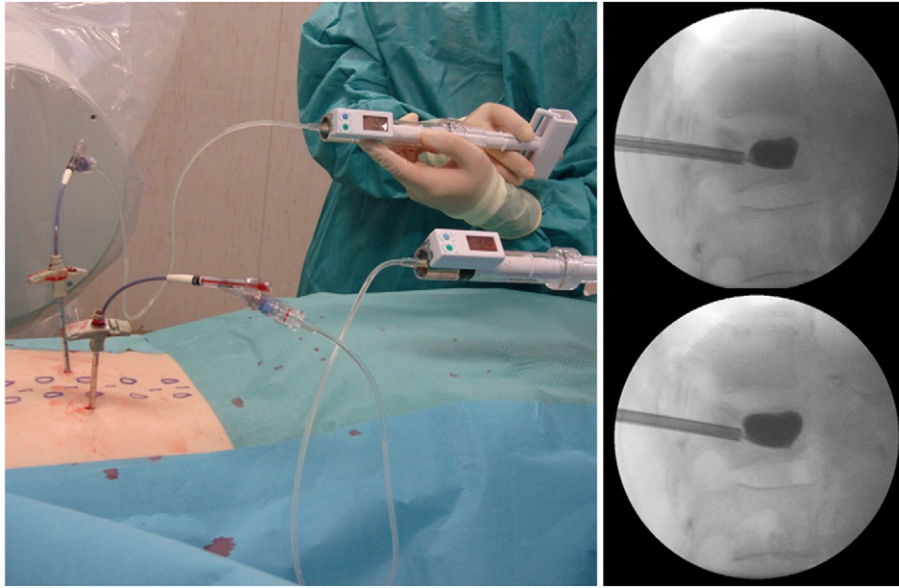
Fig. 6. Photograph of a vertebroplasty (osteoporotic fracture) and respective X-ray images.

5. Tissue engineering strategies for IVD regeneration: pre-clinical studies

Unlike traditional treatments, new therapies are aimed to repair the degenerated disk rather than simply managing the pain (Clouet et al., 2009; Don and Carragee, 2008; Raj, 2008). Because of the previously mentioned alterations associated with IDD in long-term, new treatments based on tissue engineering approaches are focused in restoring disk height and biomechanical factors, as these factors can promote IVD regeneration and re-establish its original function. Cell therapy involving stem cells (Vadalà et al., 2005) and chondrocyte transplantation (Meisel et al., 2007), gene therapy (Cassinelli et al., 2001) and tissue engineering (Richardson et al., 2007) are few strategies that are currently under investigation. Tissue engineering is considered a promising approach for restoring, repairing and regenerating IVD (Fig. 7), but current works are pre-clinical studies. This new approach involves the replacement of the damaged tissue by a biomaterial alone (scaffold) or by the scaffold associated with the appropriated cells and/or biochemical factors (e.g. growth factors, drugs and anti-angiogenic peptides), the so-called constructs (Clouet et al., 2009). Despite the great advances in the use of cell-free materials and growth factors, future research directions can comprise the combination of acellular strategies and cells obtained from different sources. Different biomaterials have been proposed for tissue engineering and regenerative medicine applications, namely polymers of natural origin that can be used as 3D porous scaffolds or hydrogel matrices. A deeper description on biomaterial chemical structure, molecular properties, and further biomedical applications can be found elsewhere (Mano et al., 2007).

5.1. Nucleus pulposus

5.1.1. Acellular strategies

Cell-free scaffolds and growth factors (Masuda and An, 2004), alone or in combination, have been investigated for restoring IVD functions. In the clinical scenario, injectable systems are most appealing as they are possibly administered by means of minimally invasive techniques, allow irregular surgical defects to be filled, facilitate a good surgical practice and decrease the operation time. The hydrogel injectable systems present many advantages as they prevent extrusion and possess a minor risk of migration, and thus allow controlling the delivery of drugs at the damaged site. Hydrogels, prepared from physical crosslinking systems (e.g. based on ionic-crosslinked, and obtained by stereo complexation or hydrophobic interactions) to chemical crosslinking systems (e.g. photo- and enzyme-crosslinked) may be suitable for IVD repair. Although physically crosslinked hydrogels have distinct advantages, such as hydrogel formation under mild conditions, they possess limited stability and mechanical performance. The chemically crosslinked hydrogels have several advantages including improved stability and mechanical properties.

The role of hyaluronan (HA) in tissue engineering for IVD repair has been investigated by different groups. A gelatin/chondroitin-6-sulfate/ HA tri-copolymer was developed and studied by Yang et al. (2005) for its feasibility to act as a bioactive scaffold in human NP regeneration. The HA-based scaffold was able to maintain the viability and proliferation of human NP cells during in vitro culturing while enhancing the production of important ECM molecules (i.e. PGs and col II). Su et al. (2010) reported on an injectable oxidized hyaluronic acid/adipic acid dihydrazide (oxi-HA/ADH) hydrogel which has been tested to repair nucleus pulposus. That work has shown that the chemically crosslinked oxi-HA/ADH hydrogel is biocompatible and maintains its gel matrix in a PBS-rich environment for at least 35 days, in vitro. In addition, the role of HA as anti-inflammatory compound was described by Schimizzi et al. (2006) in a rat model. In that work, the effect of injecting HA in the intradiscal space of animals with disk injury model was studied. HA hydrogel was shown to decrease the number of monocytes/macrophages and the release of certain pro-inflammatory cytokines in the initial inflammatory phase of healing. The data showed that HA has the potential to diminish inflammation, fibrosis and scar formation in the post-laminectomy rat model.

A recent study performed in an ex vivo organ culture (i.e. bovine IVDs) demonstrated the positive effect of the factors released under degenerative conditions (e.g. cytokines/chemokines, adhesion molecules or proteolytic enzymes) in promoting human bone marrow-derived MSC recruitment (Illien-Jünger et al., 2012). In that work, it was found that the number of MSC migrating into the disks is higher in degenerative IVDs than that in simulated physiological IVDs. Moreover, the results showed that the recruitment of IGF-1-transduced MSC promoted a significantly higher production of PGs than non-transduced MSC. These findings may have direct implications for IVD regenerative treatment, which can be directed to recruitment of endogenous progenitor cells or delivery of cells carrying biological factors/ inhibitor molecules that can either activate the resident cells to initiate tissue repair or slow down the degenerative process.
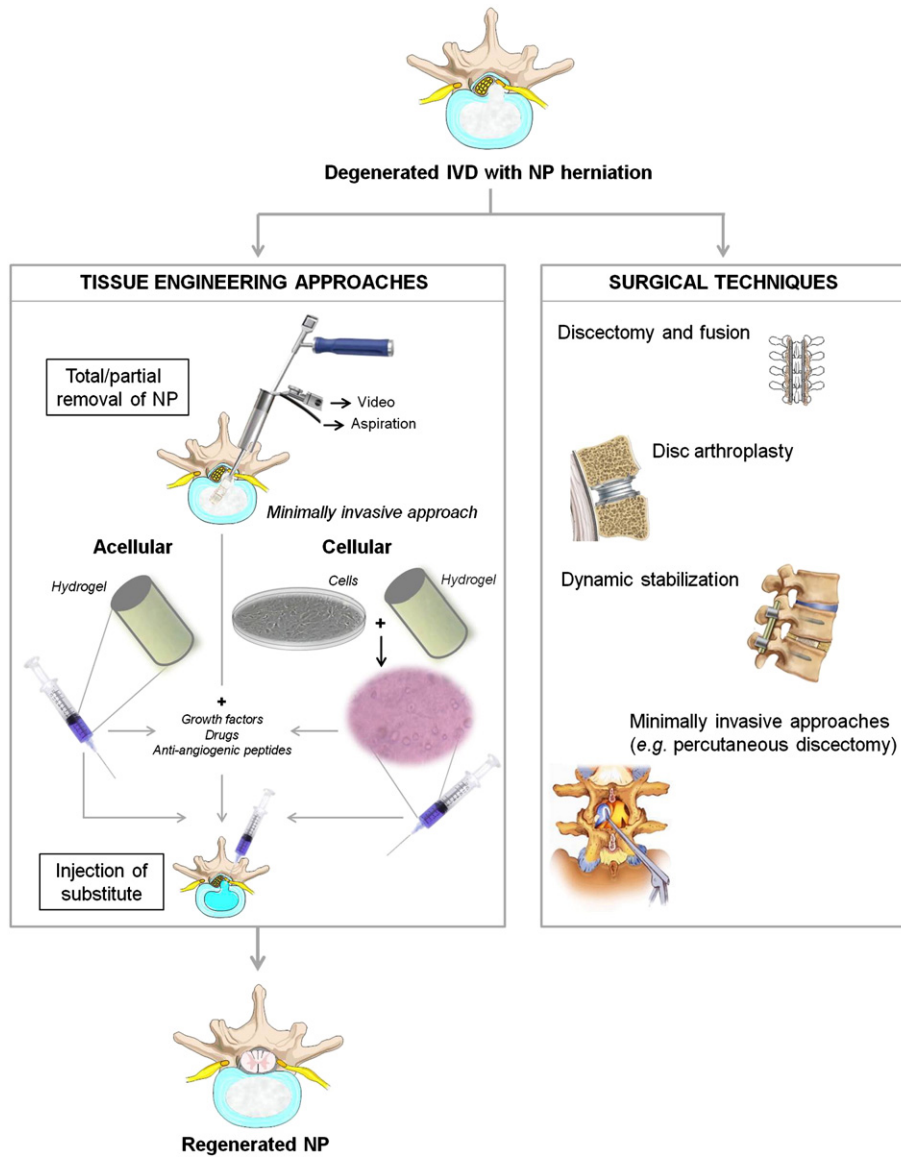
Fig. 7. Tissue engineering acellular and cellular strategies versus current surgical techniques applied in the treatment of intervertebral disk degeneration (IDD).

Another interesting study reported by Miyamoto et al. (2006) investigated the effects of intradiscal injection of osteogenic protein-1 (OP-1) on the biomechanical properties of IVDs, in a rabbit annular-puncture IDD model. Results have demonstrated that OP-1 significantly restored disk height as compared to disks treated with lactose, thus proving to be a valuable molecular tool to improve the anabolic activity in the IVD.

Akeda et al. (2006) investigated the effect of platelet-rich plasma (PRP) on porcine IVD cells, in vitro. Results have demonstrated that PRP stimulated cell proliferation and ECM production, showing that local administration of PRP can be useful for IVD repair.

Bertolo et al. (2011) investigated the effect of different molecules (e.g. dexamethasone (Dex), triiodothyronine (T3) and insulin) on human IVD cells that were cultured in alginate beads. The in vitro studies revealed that TGF-beta I increased the expression of col II and also that aggrecan synthesis was stimulated in cultures containing Dex. That work demonstrated that Dex is an effective drug for enhancing viability and chondrogenesis while insulin and T3 do not have a relevant therapeutic effect. More recently, Liang et al. (2012) reported a novel nanostructured 3D poly(lactide-co-glycolide) (PLGA) scaffold for finding application in tissue engineering of NP. The scaffolds were loaded with Dex and basic fibroblast growth factor (FGF) embedded heparin/poly(L-lysine) nanoparticles via a layer-by-layer system. That study has shown that the system is biocompatible, and it enables the release of both FGF and Dex. This promising study has demonstrated in vitro that the novel scaffolds loaded with Dex and FGF promoted stem cell proliferation and differentiation into NP-like phenotype while reducing the inflammatory response.

Other acellular-based experimental strategies for treating IDD, which are in the initial phase of pre-clinical development, focus on intradiscal injection of genes (Lotz et al., 2012). This technique involves the transfer of exogenous genes, which encode for therapeutic protein synthesis, to target cells (e.g. by using viral vectors). The introduced gene is integrated in the genome of the target cell, and the cell expresses the protein of interest. The use of gene therapy is indicated in patients with no substantial arthritic changes and thus is dependent on early recognition of patients' symptoms and level of disk degeneration. Gene therapy enables to correct homeostasis imbalance and affects the cell in an autocrine manner or in a paracrine manner, e.g. in order to upregulate matrix synthesis such as PGs, GAGs and col II (Cassinelli et al., 2001).

5.1.2. Cell-based strategies

In cellular-based TE strategies, the biomaterial is expected to act as a temporary 3-D matrix for supporting cellular functions. The ultimate purpose is to provide a support with the appropriate biological and mechanical stimuli for live cells to regenerate, restore disk height and, therefore, mimic the anatomical functions of the IVD (Woods et al., 2010). The appropriate scaffold should improve and direct new tissue growth and enable a continuous replacement by the new ECM formed and thus, its degradation, which can occur hydrolytically or enzymatically, should be in balance with tissue regeneration (Chung and Burdick, 2008). It was demonstrated by Bryant and Anseth (2003) that scaffolds comprising degradable and non-degradable units enhanced ECM production and distribution as compared to total non-degradable scaffolds. Slow degradation may hinder new ECM formation, whereas fast degradation may compromise structural support and form maintenance (Chung and Burdick, 2008). Nevertheless, some issues need to be regarded, like disk cell nutrition and compressive loading on the developing disk. These factors are easily modifiable in vitro; however, it is a much more challenging task when considered in vivo. It is desirable that the scaffold permits integration with the CEP and allows inflow of nutrients and outflow of metabolic products, as well as gathers mechanical properties that may symmetrically distribute the IVD forces (Woods et al., 2010). Other characteristics that a scaffold suitable for IVD regeneration should fulfill are biocompatibility, suitable porous architecture, permeability, tissue adhesion, injectability and biodegradability (Clouet et al., 2009). Another important characteristic of the materials is its ability to maintain cells phenotype. Gan and coworkers demonstrated the importance of using an adequate substrate for achieving NP cells proliferation and phenotype maintenance (Gan et al., 2000). In that work, a surface-modified bioactive glass 45S5 (containing a calcium phosphate-rich layer) was shown to be an efficient substrate for rabbit NP cells attachment, proliferation and matrix deposition, as well as for the expression of the typical NP phenotype. In another work, Séguin et al. (2004) used a porous bone substitute material (calcium polyphosphate) to seed and cultivate bovine NP cells in vitro for 6 weeks. The authors concluded that the NP-like tissue obtained in vitro was similar to the native tissue regarding PG content and compressive mechanical properties, although the collagen content was limited to 26% of the native NP. In addition, a recent study in a xenogeneic porcine model (Henriksson et al., 2009a) has shown that transplanted human MSC survived at least for 6 months and were able to produce extracellular matrix typical of a chondrocytic lineage, which was detected on gene and protein levels. In that study, the cells were transplanted into injured porcine IVD by using a 3D-hydrogel carrier (Puramatrix hydrogel) that self assembles in physiological conditions, thus facilitating differentiation and survival of MSC in the disk. In a more recent work, thermoreversible hyaluronan grafted poly(N-isopropylacrylamide) (HA-pNIPAM) hydrogels have been studied as injectable carriers for NP cells due to its easy injectability and mild gelling mechanism (Peroglio et al., 2012). The authors found that a specific cytocompatible HA-pNIPAM hydrogel composition was able to maintain NP phenotype and promote extracellular matrix production after 1 week of in vitro culturing. In addition, this carrier was able to maintain cell viability after injection through a 22 gauge needle and after ex vivo culturing for 1 week in a bovine IVD.

Several materials have been studied (Pereira et al., 2013) for their potential use in tissue engineering approaches for IVD regeneration. One of the most promising approaches for disk regeneration makes use of hydrogels such as methacrylated gellan gum, due to its particularly remarkable similarities with NP, in respect to water content and rheological properties (Reitmaier et al., 2012; Silva-Correia et al., 2011, 2013a, 2013b). Hydrogels are hydrophilic crosslinked polymers which absorb large volumes of water and swell without dissolution (Reza and Nicoll, 2010). In that way, the proper disk height ensures the correct functioning of the IVD and spinal column, avoiding problems like inefficient

fluid transfer, increased loading on the remaining IVD and instability of the spine (Urban and McMullin, 1988). One of the features that need to be evaluated before using hydrogels in tissue engineering applications is its performance in terms of mechanical properties (Kock et al., 2012). Hydrogels' mechanics can be tailored by altering the crosslinking density or the method of crosslinking (either physically or chemically), but a balance should be obtained between the needed mechanical properties and cellular biocompatibility (Chung and Burdick, 2008; Reitmaier et al., 2012; Silva-Correia et al., 2013a).

A wide variety of polymers has been investigated in the last years and used to produce hydrogels, some of them with interesting results (Baer et al., 2001; Bron et al., 2011; Jeon et al., 2009; Pereira et al., 2013; Reza and Nicoll, 2010; Roughley et al., 2006; Silva-Correia et al., 2010, 2011; Su et al., 2010; Thomas et al., 2010).

One example is chitosan, which is a polymer composed by glucosamine and N-acetyl glucosamine derived from chitin extracted from shells of crustaceans (Suh and Matthew, 2000) that can be processed in the form of hydrogel and has been shown to allow an efficient entrapment of IVD cells and/or extracellular matrix proteins (Roughley et al., 2006). This could be an essential property to conjugate the hydrogel with NP cells or extracellular matrix successfully. Chitosan can be induced to form hydrogels, by covalent ionic-crosslinking or by aggregation (Berger et al., 2004a, 2004b). Several attempts have been made to promote specific functionality to chitosan polymer, and to modify their biological, physical and mechanical properties, through the introduction of new side groups that react with the primary amino groups on the molecule (Suh and Matthew, 2000). In one example, chitosan was modified through conjugation with hydroxybutyl groups in order to convert it into a water soluble polymer at low temperature, which then collapses into a solid structure as the temperature rises up to 37 °C (Dang et al., 2006). In the work of Dang et al. (2006), this temperature-responsive polymer was shown to be effective in the maintenance of viability of mesenchymal stem cells and human IVD cells (obtained from degenerated NP and AF) and expression of matrix proteins, being an attractive polymer to be investigated for tissue engineering strategies of the IVD.

Alginate has also been used for multiple tissue engineering applications and in particular for IVD tissue engineering (Baer et al., 2001; Bron et al., 2011; Chou and Nicoll, 2009). It is a polysaccharide extracted from brown algae, and it forms a three-dimensional structure through crosslink polymerization caused predominantly by divalent cations (e.g. calcium) (Chou and Nicoll, 2009). Unfortunately, the stability of the hydrogel decreases over time, possibly due to loss of ions through diffusion or depletion by encapsulated cells (Baer et al., 2001; Bron et al., 2011). Therefore, there is still limited control over the mechanical properties, swelling ratios and degradation profiles of ionic-crosslinked alginate hydrogels. Chemical modification of alginate hydrogels has been reported to induce better stability and control the mechanical properties and degradation rates (Jeon et al., 2009). Alginate is so far the most widely used biomaterial for NP tissue engineering applications (Bron et al., 2011; Huang et al., 2012; Kuo and Ma, 2001). Nevertheless, it has been shown to stimulate in vivo the immune response in mice, even after additional processing to remove impurities (Dusseault et al., 2006; Ménard et al., 2010; Orive et al., 2006). Although several procedures of alginate purification were developed, total removal of the immunogenic contaminants is still not optimized and standardized, thus originating high variability between purified alginates in different research groups (Dusseault et al., 2006; Tam et al., 2006). Despite some improvements that have been achieved concerning the purification of alginate, the biomaterial still presents poor biocompatibility and develops low-grade inflammation process when implanted in the host. The purification processes also induce a number of changes in the polymer's properties, such as increased hydrophilicity and solution viscosity (Tam et al., 2006). Other biomaterials that have been reported for potential applications as NP substitutes are hyaluronic acid (Su et al., 2010), carboxymethylcellulose (Reza and Nicoll, 2010) and

poly(N-isopropyl acrylamide) (Thomas et al., 2010). These polymers also form hydrogels with suitable characteristics to be used as NP substitutes, allowing tissue growth, cellular communication and diffusion of nutrients and metabolic wastes (Kalson et al., 2008). In relation to hyaluronic acid, different chemical modifications of native hyaluronan (HA) have been performed to obtain chemically and mechanically robust materials such as a dodecylamide, HYADD3®, and a photo-linkable ester, HYAFF120®, suitably derived from HA. These hydrogels have shown to possess promising mechanical performance and rheological behavior similar to the native NP tissue and this behavior was not changed upon injection, showing to be suitable for being used in minimally invasive approaches (Gloria et al., 2012).

Gellan gum hydrogels have been initially proposed for cartilage tissue engineering applications (Oliveira et al., 2010b). Gellan gum is a biomaterial of natural origin that is commonly used in the food industry as a thickening agent or stabilizer (Oliveira et al., 2010c). It is a polysaccharide produced by bacterial fermentation and its basic structural unit is composed of glucose, rhamnose and glucuronic acid. Gellan gum is able to form gels with interesting mechanical properties while being heat and acid resistant (Oliveira et al., 2009). In initial studies for use in cartilage regeneration, gellan gum gelation has been established by an ionotropic process (as in other polysaccharides) and, therefore, the presence of cations was necessary for the formation of a stable hydrogel structure (Oliveira et al., 2010c). Gelation occurred as a consequence of temperature decrease, as gellan gum exhibits low viscosity in the range of 42–41 °C and a solid gel configuration at about 39 °C. This process is extremely fast since it takes only about 20 s. The use of gellan gum for IVD regeneration has already started to be explored (Silva-Correia et al., 2011, 2012, 2013a, 2013b).

The new approach in this study is the use of photo-crosslinked methacrylated gellan gum hydrogels (Fig. 8) (Silva-Correia et al., 2010, 2011, 2012). This procedure uses ultraviolet light (UV) to initiate a free radical polymerization reaction that forms covalent crosslinks between functional groups. The functionalization of gellan gum occurred by incorporation of methacrylate units (Silva-Correia et al., 2011). Following exposure to UV, specific chemicals designated as photo-initiators generate free radicals that initiate the polymerization process. The created free radicals convert the aqueous macromer solution into hydrogels. Short exposure to low intensity light and the appropriate choice of a photo-initiator allows for photo-polymerization to occur, with limited effects on cells and bioactive molecules.

Unfortunately, in gellan gum hydrogel as for other ionic-crosslinked polymerized hydrogels (e.g. alginate), an important loss of stability may occur in vivo, with possible misplace of structural integrity (Oliveira et al., 2009, 2010c). Therefore, the chemical modification and photo-polymerization of gellan gum is aimed at obtaining a more stable

Fig. 8. Photo-crosslinked methacrylated gellan gum hydrogel.

structure, without the disadvantages of ionic polymerization. In fact, methacrylated gellan gum hydrogel has been demonstrated to possess improved mechanical properties as compared to gellan gum hydrogel. Pereira et al. (2011) proposed a new strategy to reinforce gellan gum hydrogels. In that work, formulations of low acyl and high acyl gellan gum have been processed as microparticle/matrix systems for application as NP substitutes. Results have shown an improved system with better control over the mechanical properties and degradation rate of the hydrogel. A hydrogel matrix composed of 25% high acyl:75% low acyl (v/v) gellan gum reinforced with 50 mg/mL of microparticles of the same formulation has been shown to possess the closest mechanical properties to the NP. This system also facilitates cell encapsulation and allows obtaining a 3-D cell distribution that structurally mimics the NP, and thus this strategy may find a great potential for applications in NP tissue engineering field.

Also, as already demonstrated by Oliveira et al. (2009), gellan gum hydrogel has been shown to support the growth and extracellular matrix deposition of human articular chondrocytes adequately. In this particular study, chondrocytes encapsulated in gellan gum hydrogels have been shown to remain viable and form extracellular matrix mainly composed of col type II and aggrecan. These findings could be relevant for IVD regeneration. In the meantime, important studies have been performed exploring the use of adipose stem cells combined with gellan gum hydrogels (Oliveira et al., 2010a). When considering IVD regeneration, the use of a different source of cells can avoid the problem of cell scarcity (since the adult NP is naturally acellular) and immunological rejections. The non-cytotoxicity of methacrylated gellan gum hydrogels has already been demonstrated in vitro and in vivo (Silva-Correia et al., 2010, 2011, 2013b), but the main advantage of these hydrogels is that they can be applied by injection using a minimally invasive system, decreasing the complexity and invasiveness of a spine surgery (Oliveira et al., 2009; Silva-Correia et al., 2013a). In a recent study, the rheological/mechanical properties of both ionic- and photo-crosslinked methacrylated gellan gum hydrogels were evaluated in vitro, by performing steady shear analysis, injectability and confined compression stress-relaxation tests (Silva-Correia et al., 2013a). In that study, it was demonstrated that the reactive chemical solutions can be easily injected into and through a 16 gauge needle, and allowed to produce stable and reproducible methacrylated gellan gum hydrogels. Moreover, it was demonstrated by dynamic mechanical analysis that both types of hydrogels present display mechanical properties similar to the native human NP, and maintain their mechanical performance when loaded with cells and during culturing (Silva-Correia et al., 2011, 2013a).

In order to address angiogenesis and consequent symptoms associated to IDD progression, the use of anti-angiogenic scaffolds or drugs, peptides or growth factors with the ability to control of innervation and blood vessels infiltration during tissue repair have been proposed. Regarding the use of anti-angiogenic scaffolds, the NP substitute must prevent vascular invasion while allowing the maintenance of the typical phenotype of NP cells, without a blood supply. Silva-Correia et al. (2012) reported on the angiogenic response of gellan gum and the modified ionic- and photo-crosslinked methacrylated gellan gum hydrogels. Fig. 9 shows the photo-crosslinked methacrylated gellan gum hydrogels implanted in the chorioallantoic membrane (CAM), which is a model used to study angiogenesis in vivo (Ribatti et al., 2006). By analyzing the convergence of the macroscopic blood vessels following 4 days of implantation, it was demonstrated that the gellan gum-based hydrogels are non-angiogenic as compared to positive controls. It was also observed by immunohistochemistry that only the methacrylated hydrogels completely prevented the ingrowth of chick endothelial cells and blood vessel infiltration. Moreover, the histological analysis indicated that no acute inflammatory response was triggered by the gellan gum-based hydrogels. That study clearly evidenced that the methacrylated hydrogels prevented cellular and blood vessel invasion, thus controlling the angiogenic process, which was not observed in
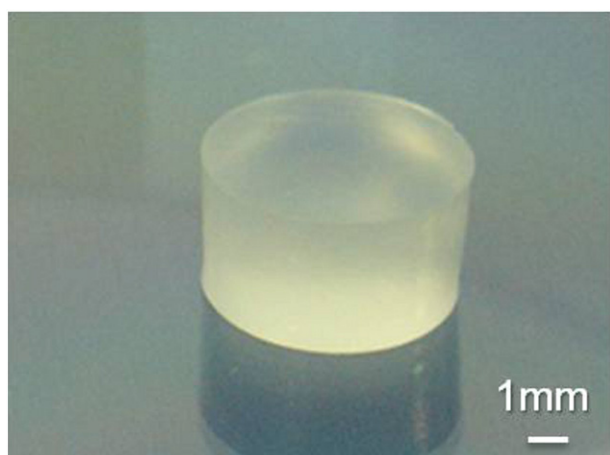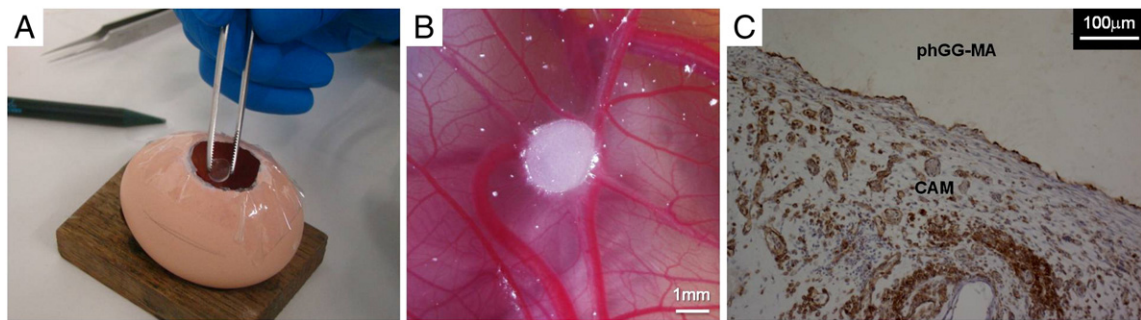
Fig. 9. Angiogenic evaluation of the photo-crosslinked methacrylated gellan gum hydrogel (phGG-MA) in the chick chorioallantoic membrane (CAM). The hydrogel disks are placed on the CAM at day 10 of embryonic development (A) and the assay proceeds until day 14 of embryonic development. The images of the CAM implants are acquired in ovo (B) by the end of the assay using a stereomicroscope. The implants and the underlying and immediately adjacent CAM portions are then excised and treated for immunohistochemical analysis. The angiogenic response is evaluated by analysis of blood vessels convergence and possible blood micro-vessels formation, inflammation and cells ingrowth. Staining with SNA-lectin (C) that specifically binds to chick endothelial cells (brown staining) shows that a monolayer of endothelial cells is contacting with the phGG-MA hydrogel at the interface CAM/hydrogel, but do not allow cells ingrowth and blood vessels infiltration.

gellan gum hydrogels. Other studies have been reported with the aim of developing non-angiogenic hydrogels for tissue engineering applications (Ahmadi et al., 2010), and in particular for articular cartilage and IVD regeneration (Scholz et al., 2010). In both works, the hydrogels tested (i.e. chitosan–glycerol phosphate–hydroxyethyl cellulose hydrogels and polyethylene glycol-crosslinked albumin gel) were shown to possess no angiogenic potential and no endothelial invasion ability. It is expected that injectable hydrogels with such an anti-angiogenic capability will be extremely advantageous and promising for regeneration of NP tissue, by their capacity to target angiogenesis which will ultimately have utility in the treatment of pain associated with structural damage in diseased/damaged tissues.

Interesting strategies to control angiogenesis makes use of anti-angiogenic peptide sequence at the uppermost branching generation of a poly(ε-lysine) dendron, which would enhance the stability in vivo (Meikle et al., 2011). The synthesized anti-angiogenic sequence, when grafted to a dendron scaffold would allow not only to inhibit angiogenesis, but also to control the retention of the peptide in the target tissues due to the tailored-design of the dendron scaffold. Another approach is based on the use of transduced cells expressing the desired levels of a therapeutic gene such as binders of the vascular endothelial growth factor (VEGF) or antagonists (VEGF-inactive analogs) (Matsumoto et al., 2009; Misteli et al., 2010).

The injection of growth factors (e.g. BMP, TGF) or inhibitors of inflammatory cytokines and proteases such as MMPs may be valuable for treating or controlling IDD symptoms since they can enhance the repair process or retard matrix degradation and control the effect of cytokines on nerve ingrowths, respectively (Boyd and Carter, 2006). Fig. 10 shows the MRI images of a 40-year-old male patient with previous L5–S1 discectomy and left-sided laminectomy, where scar tissue and small Schmorl's nodules can be observed. This clinical case is a good example, where TE strategies would be beneficial for pain management and on reducing the progression of further spine-related problems.

5.2. Annulus fibrosus

The closure of the AF is the big challenge that still remains to achieve when considering only NP replacement or when the AF has been compromised. The use of commercially available implants, which are described as modified sutures with anchors (e.g. Xclose©, INclose©), allow suturing and containing the NP in its compartment but present the disadvantage of losing AF tissue and biomechanical functionality (Bron et al., 2009). Another commercial available implant, which is used in adjunction to discectomies, is Barricaid©. This implant completely bridges the defect and reinforces the AF preventing further herniation. Although these implants bring some benefits, the balance between the
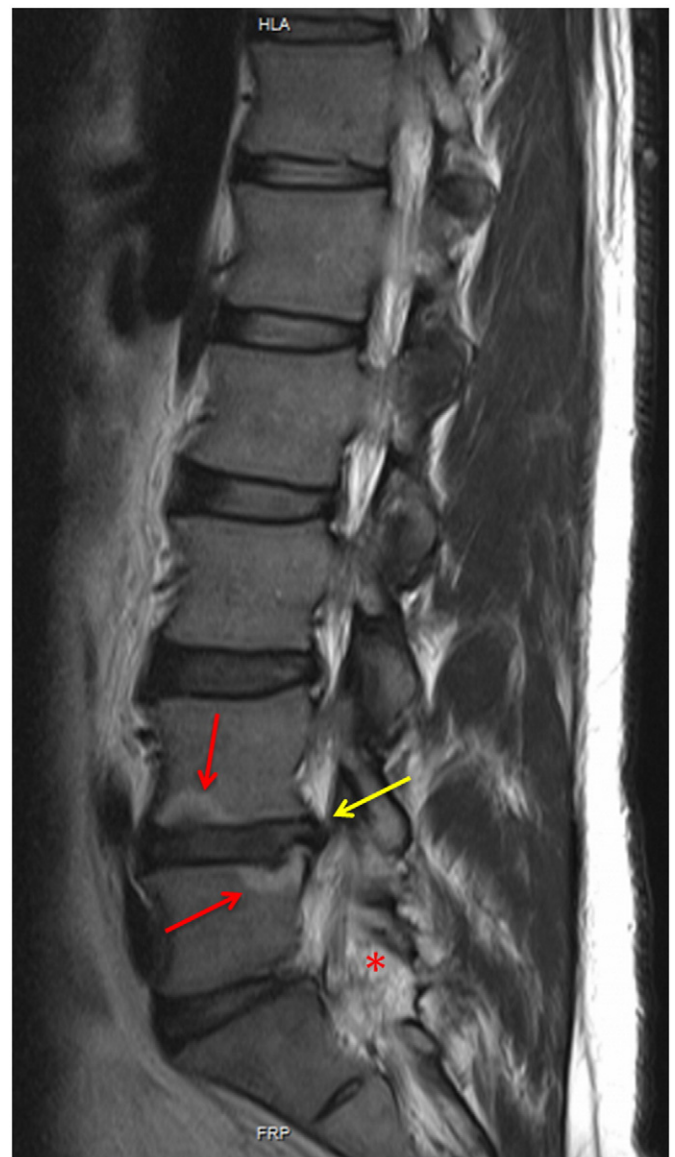

Fig. 10. Male 40-year-old patient with previous L5–S1 discectomy and left-sided laminectomy 4 years ago, notice scar tissue (*); see dehydration and herniation of L4–L5 disk (yellow arrow) and small Schmorl's nodules: protrusions of the cartilage of the intervertebral disk through the vertebral body endplate and into the adjacent vertebra (red arrows).

associated risks and the long-term success of their implantation are not established yet, and this will determine their future use. A more recent approach is investigating the use of biocompatible glues based in fibrin and/or cyanoacrylate as an alternative for the conventional surgical methods for annulus closure (Heuer et al., 2008). Although the tested glues provide some improvements, it appears that longer stability was obtained only when combined with suture; therefore, further optimization is still needed to find the optimal procedure to avoid NP extrusion.

Although there is a major research effort directed towards regenerating NP since it is in this compartment that IDD initiates, other strategies address the regeneration of the AF compartment, or even both NP and AF in an integrated biphasic composite (Bron et al., 2009; Pereira et al., 2013). The restoration of AF is a concept complex to achieve, due to its highly organized structure. The ideal material for AF restoration must: (i) have mechanical properties similar to the native AF tissue, (ii) support the growth of disk cells, and (iii) adhere to the adjacent tissues under physiological levels of strain (Schek et al., 2011). Several materials such as porous silk fibroin (Chang et al., 2007) or atelocollagen honeycomb (Sato et al., 2003) have been investigated for tissue engineering of AF. While some of the candidate materials have been processed in the form of hydrogels and porous scaffolds, others are being tested in the form of electrospun nanofibers (Nerurkar et al., 2009; Vadalà et al., 2012). It is believed that electrospinning technique allows the formation of arrays of aligned polymeric nanofibers, where resident cells can deposit an oriented and organized ECM, thus being able to create a structure that replicates the structural hierarchy of AF. Although some of the tested materials have been shown to fulfill the requirements for being used as AF substitute (e.g. biocompatibility, immunogenicity, fill the AF gap and contain the NP, and allow AF cells, or other delivered cells, to survive/differentiate and maintain the phenotype), the major problem is still the lack of a suitable strategy for delivery and fixation in vivo (Bron et al., 2009). In a recent work, Schek and co-workers developed a genipin crosslinked fibrin hydrogel with mechanical properties in the range of native AF tissue (Schek et al., 2011). Although the results showed that the hydrogel is biocompatible and has excellent adhesive properties, a low cell proliferation and rounded morphology were also observed, which are not adequate for AF regeneration. So, the authors concluded that this biomaterial may be best suited as a sealant for small AF defects or as an adhesive to augment large annulus repairs.

A search in PubMed using the words "intervertebral disk" and "tissue engineering" and "clinical trials" displayed four different papers (Hegewald et al., 2011; Masuda and Lotz, 2010; Meyerrose et al., 2010; Sah and Ratcliffe, 2010), in the last 10 years. From this search it can be concluded that no clinical trials were reported, i.e. three reviews and one basic study were identified. The latter (Hegewald et al., 2011) aimed at characterizing the regenerative potential of cells isolated from herniated disk tissue obtained during microdiscectomy. The authors concluded that there is limited regenerative potential for cells harvested from herniated disk tissue. Thus, that work highlighted the need to perform clinical trials using TE strategies and corroborated the potential of using stem cells as alternative to differentiated cells for treatment of IVD-related problems.

6. Surgical techniques applied to IVD tissue regeneration

Due to the modern technologies, surgeons must develop appropriate surgical procedures, preferably using minimally invasive surgeries or percutaneous injections.

Augmentation of the NP with biomaterials such as the photo-crosslinked methacrylated gellan gum hydrogel, could indeed be used in partial nucleotomies or decompressions (like indicated in disk herniation), as well as in early stages of IDD, where complete nucleus removal and replacement may be required (Boyd and Carter, 2006). One procedure that has been referred and could be theoretically useful in association with new surgical techniques for IVD regeneration using

hydrogels is chemonucleolysis, which consists of the injection of an enzyme into a bulging spinal disk, with the aim of destroying the NP and reducing the disk's size (Kim et al., 2002).

Another procedure such as NP aspiration can also be a valuable option (Revell et al., 2007). In fact, the use of a 16 gauge trochar and cannula in pigs was described, which allow us to reach the NP by insertion into the side of an IVD and apply suction to remove it. However, it seems that disruption of the AF by surgical incision probably propagates degeneration (Roughley et al., 2006).

The needle used, e.g. for delivering enzymes or aspirating the NP, punctures through the AF and makes an incision. It has been shown that even small needles produce annular holes of varying size and shape that remain open, create localized strain concentrations, and induce cell death around the injury site (Iatridis and Hecht, 2012). Although it has been reported that the use of a 27 gauge needle did not cause any significant decrease in disk height (contrarily to 16 or 18 gauge) in a rabbit model (Masuda et al., 2005), other approaches are being investigated to avoid the use of needles in IVD regeneration. Recently, a transpedicular approach emerged as an alternative route to deliver therapeutic agents into the IVD (Vadalà et al., 2013). With this new technique, the NP could be approached through the inferior endplate via the pedicle without affecting the AF, as well as the spinal canal and the neural foramina.

In another study (Meisel et al., 2007), in a dog model, the animals were injected with autologous disk chondrocyte cell cultures using a 25 gauge needle, which was guided into the center of the IVD, using fluoroscopic imaging. Results have shown that autologous disk chondrocyte transplantation using a minimally invasive procedure is a suitable therapeutic option for repairing and inhibiting IDD process. The clinical trial revealed that such strategy reduces pain at 2 years as compared to control population. Concerning the use of hydrogels for IVD regeneration, it is also important to consider the gelation time (Freimark and Czermak, 2009), which should be fast enough to prevent the biomaterial leakage. The setting time should be long enough to allow for accurate positioning during the procedure, yet short enough to prevent its outflow. Therefore, a compromise between material viscosity and surgeon's ability to manipulate its correct placement must be achieved, in order to assure that the substance remains at the site of introduction and completely fills the intradiscal space (Boyd and Carter, 2006). Possible strategies for achieving such goals include optimizing of the intradiscal pressure (Meisel et al., 2007) or annular suturing.

Photo-polymerization has one additional problem. The UV light must be close enough to allow the biomaterial to polymerize.

As aforementioned, extrusion has been shown to be a significant problem that could eventually occur associated with disk hydrogel injection (Carl et al., 2004). Thus, technologies and adequate techniques for repairing the AF must be also developed.

7. Final remarks and future perspectives

The perfect material and regenerative strategy for treating IDD have not been found yet, but there are some promising candidates namely for NP regeneration, such as the ionic- and photo-crosslinked methacrylated gellan gum and hyaluronan-derivative hydrogels. Nevertheless, further studies must be conducted to understand how these hydrogels will be degraded or replaced at the implantation site by the newly formed tissue and how it behaves in terms of mechanical performance. On the other hand, it would be important to evaluate in vivo, the best tissue engineering approach, i.e. the use of acellular (using growth factors) or cellular (autologous approach) strategies. The viability of NP cells in the retained native tissue and implanted cells (e.g. stem cells) is also dependent on the chemical events taking place during the gelation process, such as the degree of crosslinking, temperature and pH. So, further studies, especially in vivo and ex vivo proof-of-concept works are required to obtain more reliable

considerations. In the field of biomaterials for IVD regeneration, namely the development of adequate materials for closure of the AF (e.g. stable and biocompatible glues) and that avoids extrusion of NP, this still remains a huge challenge. Future research directions addressing the total IVD substitution/regeneration should also comprise personalized approaches by means of using reverse engineering, i.e. combining imaging techniques (e.g., MRI and micro-CT) and 3D bioprinting technology. The implantation of custom-made implants mimicking native IVD and possessing an appropriate size, shape, mechanical performance, and biodegradability can improve recovery time after surgery and help to restore the biofunctionality of the spine. The development of finite element analysis models can also help elucidating if the developed materials/prosthesis will be suitable for treating either partial or total IVD defects. The models can comprise descriptions on fibers or hydrogel reinforcement and osmotic tissue swelling/shrinkage, and contain descriptions on the effect of cellularity. Such studies can provide deeper insights on the influence of changes in scaffold acellular/cellularity, permeability, fiber dimensions and orientation to choose the best prosthesis that can withstand mechanical stress while maintaining the weight-bearing capability. The numerical simulations will possibly to develop optimized scaffolds for treating patients with lesions simultaneously affecting NP, AF and adjacent endplates of a disk. Personalized strategies can present several advantages, but it becomes clear that they create new challenges from the surgical point-of-view. Thus, there will be the need of defining and establishing new surgical procedures, which will imply conducting further experiments using animal models or in human cadaver.

Anti-angiogenic materials are also most appealing in IVD regeneration as they prevent vascularization, and may help restore the normal IVD functioning. These novel biomaterials should also prevent re-innervation, which can be an advantage for pain management. Another important factor is the already referred disk nutrition as it is well known that inadequate disk nutrition in adults predisposes to IDD. Therefore, supplementation of the NP with additional cells without improving disk nutrition seems to be destined to failure. Further fundamental studies are needed to investigate hypertrophy mechanism in IDD since the knowledge that can be gain may help develop new cellular therapies to fight IVD degeneration. In respect to the administration of bioactive agents/growth factors, pre-clinical studies suggest that injection therapy can lead to modification of pain behaviors as well as changes in cytokine expression, suggesting that injection of GFs or gene therapy can be promising therapeutic approaches for treatment of chronic discogenic LBP. Despite these, both fundamental studies and clinical trials are further required in order to develop efficient molecular therapies. We are now at the stage where several of the challenges of IVD tissue engineering have been reached. Tissue engineered disks remain viable and non-cytotoxic in small animal models. Human NP cells can be grown outside the body, and administration of stem cells isolated from younger patients may have a higher therapeutic potential as compared to that isolated from older individuals. Regeneration of extracellular matrix is also possible in vivo.

Thus, the next step is to find ways of making both acellular and cellular-based technologies and hopefully personalized approaches to work in the different human spine conditions/diseases, and transfer these from bench to clinic.

Acknowledgments

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