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Review Article

Decellularized Scaffolds for Ligament/Tendon Regeneration: Challenges, Opportunities, and Future Directions

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Decellularized scaffolds have gained significant attention in tissue engineering, particularly for tendon and ligament regeneration, due to their ability to retain native extracellular matrix (ECM) architecture and biochemical cues. These decellularized scaffolds offer a biocompatible and bioactive framework that closely mimics the native tissue niche, promoting cellular infiltration, proliferation, and differentiation. Unlike synthetic materials, decellularized tissues can better support functional healing while minimizing immune responses. Tendon and ligament injuries pose a unique challenge due to the dense collagenous structure, limited vascularization, and poor intrinsic healing capacity of these tissues. While autografts and allografts are commonly used, limitations such as donor site morbidity, limited availability, and risk of immune rejection pose treats. Decellularized scaffolds offer a compelling solution by providing mechanical support while promoting natural tissue regeneration. Yet, developing these scaffolds remains challenging, as differences in tissue size, ECM composition, and porosity make it difficult to apply a universal decellularization approach. The major decellularization hurdle is to remove cellular components effectively without compromising the scaffold's structural integrity or biological activity. In addition, achieving immune tolerance and enhancing vascular growth are essential for proper integration and long-lasting performance of the graft. This review highlights the latest advancements in decellularized scaffolds for tendon and ligament repair, discussing the various physical, chemical, and enzymatic methods used for decellularization. It also examines current limitations and strategies being explored to overcome them, such as combining scaffolds with growth factors, stem cells, or pro-angiogenic agents. Beyond musculoskeletal repair, the potential of decellularized scaffolds is expanding into broader applications in tissue and organ engineering. Future research should focus on standardizing decellularization protocols, improving vascular integration, and translating laboratory success into clinically viable products that can enhance patient outcomes in regenerative medicine.

Introduction

Ligaments and tendons are specialized connective tissues essential for joint stability and musculoskeletal function [1]. Ligaments connect bones, restricting excessive joint movement, while tendons link muscles to bones, transmitting contractile forces to enable movement. They comprise primarily of collagen fibers, but they also have substantial amounts of water, proteoglycans, and elastin, which all contribute to their biomechanical capabilities [2,3]

Ligament and tendon injuries are among the most common musculoskeletal disorders, accounting for approximately 50% of all such injuries [4]. In the United States alone, an estimated 17 million ligament injuries require medical intervention annually [5]. Similarly, in India, anterior cruciate ligament (ACL) injuries are particularly prevalent, representing 86.5% of all knee injuries in athletes [6]. National Collegiate Athletic Association (NCAA) Injury system revealed that ACL injuries frequently occur in women's gymnastics and men's football [7]. These injuries often lead to pain, loss of mobility, long-term disability, and a significant economic burden due to prolonged rehabilitation and high re-injury rates.

Ligament injuries, particularly in weight-bearing joints like the knee often result in partial or complete tears, necessitating surgical intervention. Traditional treatment methods include autografts, allografts, and xenografts. Autografts offer advantages such as histocompatibility, non-immunogenicity, and minimal disease transmission risk, but their application is restricted by donor site morbidity and tissue availability. In contrast, allografts and xenografts eliminate donor site concerns but are associated with immune rejection, disease transmission risks, and prolonged healing times [8]. Moreover, the intrinsic healing capacity of tendons and ligaments is poor due to their low vascularity and cellularity, often resulting in incomplete regeneration and scar tissue formation.

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Tendons, in contrast, are inelastic connective tissues that attach muscles to bones, enabling movement by transmitting contractile forces. Tendons consist of 55–70% water, with the extracellular matrix (ECM) predominantly composed of aligned type I collagen fibers (65–80% dry weight) along with minor components like elastin, decorin, biglycan, and fibromodulin. Tendon healing follows a three-phase process: inflammation, proliferation, and remodeling. The inflammatory phase begins immediately post-injury with blood clot formation and fibroblast activation, setting the stage for tissue repair [9]

To overcome the shortcomings of traditional treatments for tendon and ligament repair, Tissue Engineering (TE) has become a promising alternative. TE strategies facilitate functional regeneration while preserving biomechanical integrity [10]. Upon recent advancements, decellularized scaffolds have gained popularity due to their advanced potential in mimicking the native microenvironment of tissues and in supporting regeneration[11]. Decellularization involves the removal of cells from native tissues—such as tendons, ligaments, or other soft tissues—while preserving the biochemical composition, ultrastructure, and mechanical properties of the ECM [12]. These scaffolds retain the inherent bioactive cues necessary for guiding host cell behavior, promoting angiogenesis, and restoring mechanical function. Compared to synthetic scaffolds, decellularized matrices offer superior biocompatibility, tissue-specific architecture, and reduced risk of chronic inflammation or fibrotic response[13].

Despite their promise, several challenges remain, including incomplete decellularization, loss of mechanical strength during processing, variability between tissue sources, and the risk of residual antigenicity[14]. Moreover, translating these materials from laboratory to clinical use requires standardization of protocols, thorough biomechanical validation, and long-term in vivo studies[15,16].

This review aims to provide a comprehensive overview of decellularized scaffolds for ligament and tendon regeneration, discussing their biological basis, decellularization techniques, biomechanical and immunological considerations, preclinical and clinical outcomes, and future directions. Through this synthesis, we aim to highlight current challenges and explore innovative strategies for advancing regenerative therapies in musculoskeletal medicine.

Ligament/Tendon Injury

50% of all musculoskeletal problems in the US are caused by ligament and tendon injuries and damages, which have a substantial negative impact on society and the economy [32]. The delicate balance between joint mobility and stability is upset by these injuries, which frequently impact the knees, hips, shoulders, ankles, elbows, and wrists. This can result in additional degeneration and dysfunction.

Ligament Injuries: Ligaments are among the most frequently injured tissues within a joint. In the United States, approximately 150,000 anterior cruciate ligament (ACL) injuries occur annually, with over 4 million knee arthroscopies performed worldwide each year[33]. ACL tears rank as the second most common injury among college athletes, increasing at a rate of 1.3% per year. Additionally, ACL injuries are a leading cause of knee trauma in children, accounting for 36% of pediatric knee injuries [34]. Ligament injuries are classified as intrinsic - resulting from improper joint motion - or extrinsic, caused by external forces such as sports collisions [35]. Anterior subluxation, pivoting, and severe trauma are some factors that can cause the ligament to overstretch or rip entirely [36].

Tendon Damage: In general, there are two types of tendon injuries - tendinopathy and tendon rupture. The latter presents unique challenges for orthopedic care. These injuries are often caused by microtrauma, high loading, and hyperpronation [3,37]. Flexor tendon injuries typically result from deep cuts, most frequently occurring in individuals aged 20–29, with men being more affected than women. Approximately 25% of these injuries are work-related, predominantly in sectors such as construction and extraction (44%), food preparation and service (14%), and transportation and material moving (12%) (Rhode and Rhode, 2015.). Achilles tendon injuries are among the most common sports-related tendon injuries, with nearly 50% of all sports injuries involving the Achilles tendon. About 75% of Achilles tendon ruptures occur in men aged 30–49, with sports activities being the primary cause [39]. The primary reasons for shoulder pain and Disability are Rotator cuff injuries, especially with age [40]. Research has shown that full-thickness rotator cuff tears are found in about 13% of people in their 50s, 25% in their 60s, and 50% in their 80s [41].

Given the high incidence and impact of ligament and tendon injuries, novel regenerative approaches, such as decellularized scaffolds, offer promising solutions for improving healing outcomes and restoring functional integrity.

Current Treatments in Ligament/Tendon Repair

Ligament / Tendon healing is a slow and often incomplete process. Joint laxity resulting from ligament injuries gradually improves over a period of six weeks to a year [42]. However, a significant proportion of patients continue to experience objective mechanical laxity and subjective joint instability even after this period. Over the years, various strategies have been employed to restore the injured ligament to its pre-injury condition, including rest, mobilization, non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroid injections, and prolotherapy [43]. While these treatments can help relieve pain following a ligament injury, not all of them actively support cellular repair and healing of ligament tissue. In fact, some therapies may negatively impact the healing process by suppressing essential cellular functions required for tissue repair, whereas others promote healing by stimulating key cellular processes involved in ligament regeneration [35].

Table 1: Comparative structural and compositional features of ligaments and tendons

Characteristic	Ligament	Tendon
Cellularity	Composed predominantly of fibroblasts and ligamentocytes [17]	Composed predominantly of tenocytes [18]
Extracellular Matrix	Rich in ground substance; moderate collagen content [19]	Dense collagen matrix; relatively lower ground substance [20]
Collagen Content	High, mainly type I collagen with notable presence of type III	Very high type I collagen with minimal type III [21]
Elastin Content	Contains more elastin, contributing to flexibility[22]	Minimal elastin, emphasizing tensile strength [23]
Water Content	Approximately 60–80%, depending on location and function [24]	Similar water content (60–80%), aiding in viscoelasticity [25]
Fiber Organization	Less organized, with a more interwoven collagen network [26]	Highly aligned parallel collagen fibers along the direction of load [27]
Mechanical Role	Provides joint stability by resisting excessive motion [28]	Transmits mechanical forces from muscle to bone [29]
Orientation	Collagen fibers arranged in a crisscross or mesh-like pattern [30]	Collagen fibers oriented longitudinally along the force axis [31]

Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs have been used for years especially in acute sports-related trauma to manage ligament and tendon injuries. Even though they offer temporary pain relief, several researches show that they provide only mild symptomatic benefits and may impair soft tissue healing[44,45]. Early healing processes are initiated and supported by Prostaglandins by recruiting immune cells to clear debri and initiate tissue repair. NSAIDs work by inhibiting cyclooxygenase (COX) enzymes, thereby blocking prostaglandin production, which is essential for ligament regeneration [46]. This suppression can interfere with the normal healing cascade, potentially delaying recovery. The pain-masking effect of NSAIDs that leads patients to overuse the injured ligament without realizing the extent of tissue damage is the major drawback of NSAIDs [35]. Additionally, both NSAIDs and corticosteroids provide pain relief but do not actively promote tendon or ligament healing. Alternative regenerative strategies, such as tissue engineering approaches and biological scaffolds, are being studied and implemented to enhance functional recovery and promote tendon/ligament regeneration due to the drawbacks and disadvantages of NSAIDs.

Surgical intervention

Severe tendon and ligament injuries require surgical interventions particularly when conservative treatments fail. It is commonly recommended for athletes and individuals experiencing significant functional impairment in daily activities. The definitive goal of surgery is to minimize symptoms, restore stability, and improve joint function. Surgical procedures vary based on factors such as age, injury severity, and activity level and may include: 1. Tendon re-suturing – Directly stitching the torn ends of the tendon together. 2. Tissue removal (debridement) – Eliminating damaged tissue to promote healing. 3. Ligament repair – Suturing torn ligament ends or reattaching using wires or anchors.4. Ligament reconstruction – Using autografts (patient's own tissue) or allografts (donor tissue) to replace damaged ligaments and restore joint stability[47,48].Yet, surgically reconstructed ligaments tend to be less strong, and only 65% of patients resume their pre-injury activity level following ACL reconstruction[49]. Additionally, while knee replacement surgeries are expected to rise significantly by 2030, long-term functional outcomes remain a concern. Studies using standardized functional assessments have shown that post-surgical recovery can vary, highlighting the need for improved surgical techniques and alternative regenerative approaches.

Diet and nutrition

Appropriate diet and nutrition play a crucial role in maintaining ligament health and aiding recovery after injury. According to recent studies, a diet high in saturated fats may contribute to vascular obstruction and reduced oxygen delivery to joints and connective tissues, potentially exacerbating musculoskeletal conditions such as osteoarthritis [50]. While osteoarthritis primarily affects cartilage, similar mechanisms may impact tendon and ligament healing. To counteract this, a nutrient-dense, low-fat diet is advocated that may help, prevent and slow OA progression. Key nutrients essential for ligament and tendon repair include: Vitamin D – Deficiency has been linked with chronic pain and physical function impairment, especially in those taking opioid medications, Chondroitin and Glucosamine Sulfate – These supplements may help reduce OA symptoms and slow disease progression, supporting joint health and cartilage integrity [51,52].

Prolotherapy

Prolotherapy, also referred to as regenerative injection therapy or platelet-rich plasma (PRP) therapy, is a non-surgical procedure applied to musculoskeletal and arthritic pain. This approach involves injecting proliferant solutions such as dextrose or PRP directly into ligaments and tendons to stimulate natural healing and tissue regeneration [53]. Studies have shown that prolotherapy triggers an inflammatory healing response, promoting tissue repair, increases ligament mass and strength, enhancing structural integrity and improves ligament-to-bone attachment, aiding joint stability. Unlike traditional therapies that may inhibit healing, prolotherapy addresses the underlying cause of joint instability and pain. This approach has gained popularity as an effective alternative for treating ligament-related injuries and joint dysfunction [54]

Tissue grafting

Tissue grafting for tendon and ligament repair is classified into three main types: autografts, allografts, and xenografts. Autografts involve harvesting tissue from another part of the patient's body to replace an injured ligament or tendon. A common example is using the hamstring or patellar tendon for ACL reconstruction, which can restore up to 50% of the ligament's pre-injury [55,56]. Despite its effectiveness, autografts come with risks such as donor site pain, instability, and mechanical complications, potentially leading to long-term functional impairment. Allografts and Xenografts serve as alternatives to autografts, eliminating donor site morbidity. Allografts can be artificial or biologically derived [57]. Artificial options like the Ligament Advanced Reinforcement System (LARS) and Kennedy Ligament Augmentation Device (LAD) have shown improvements in knee stability and weight-bearing capacity[58].

Tissue engineered scaffolds

Tissue engineering offers promising therapeutic solutions for tendon and ligament injuries by utilizing advanced composite biomaterials that integrate both natural and synthetic polymers. These materials provide the necessary mechanical strength while supporting cellular attachment, proliferation, and differentiation[59]. Recent innovations such as braided nanofibrous scaffolds and electrospun yarn-like structures have demonstrated superior regenerative outcomes due to their ability to closely mimic the native extracellular matrix architecture and biomechanical properties of tendons and ligaments [60]. For example, Rothrauff et al., 2017 developed braided and stacked electrospun nanofibrous scaffolds that effectively supported the tenogenic differentiation of mesenchymal stem cells, highlighting their potential for tendon and ligament tissue engineering [61]. Among the various scaffolding strategies, decellularized tendon or ligament tissues hold exceptional potential owing to their inherent structural and biochemical similarity to native connective tissues, making them ideal templates for functional regeneration [62].

Decellularized Scaffolds

Decellularized scaffolds have emerged as promising candidates for tendon and ligament repair owing to their preservation of native extracellular matrix architecture, inherent bioactivity, and reduced immunogenic responses. These scaffolds offer a biologically relevant microenvironment that facilitates essential cellular processes such as adhesion, proliferation, and lineage-specific differentiation—features that are often difficult to achieve with entirely synthetic constructs [63,64]. In contrast to conventional grafts, decellularized matrices provide a naturally derived, biocompatible framework that enhances tissue remodeling and functional regeneration with minimal adverse reactions. A variety of decellularization techniques have been optimized to produce tendon-derived ECM scaffolds from both allogeneic and xenogeneic tissues. While decellularized bone has seen widespread clinical use, the translational potential of decellularized matrices from cartilage, skeletal muscle, tendon, and ligament is still under active investigation, particularly in the context of orthopedic and musculoskeletal repair.

Decellularization methods

Decellularization is the process of removing all cellular and nuclear components from tissues or organs while preserving the native architecture, biochemical composition, and biomechanical properties of the extracellular matrix (ECM). The resulting decellularized matrices serve as biological scaffolds, maintaining the functional ECM proteins necessary for tissue regeneration[65].

To achieve effective decellularization while minimizing immunogenicity, various techniques are employed, classified into three major categories – 1. Physical Methods – Utilize mechanical forces or temperature changes to disrupt cellular integrity. 2. Chemical Methods – Involve detergents and solvents to lyse and remove cellular components. 3. En-

zymatic Methods – Use enzymes to selectively degrade cellular material while preserving ECM structure [66]. Each method has its advantages and limitations, often needing a combination of approaches to achieve optimal decellularization while maintaining the functional properties of the scaffold.

Physical Treatments

Physical therapy, including agitation, sonication, high pressure, and freeze-thaw cycles, is used frequently to break cell membranes, leading to the discharge of cellular contents into the extracellular matrix (ECM) surrounding the cells. Such techniques assist in triggering decellularization through the disruption of cellular structures. Despite this, physical treatments are not usually enough for full decellularization of dense tissues. The significant drawback is that wash solutions cannot penetrate deeper tissue layers efficiently, and there remains some residual cellular material behind [67]. Physical treatments are thus complemented with chemical treatments for more efficient decellularization[68].

Freeze Thaw Cycle: The freeze-thaw cycle (Temperature range: "80°C to 37°C) is one of the commonly applied techniques for decellularizing ligaments and tendons, especially for its efficiency in lysing cells in dense tissues. The process destabilizes cell membranes by creating ice crystals, which results in intracellular content release and allows for the removal of cellular components. Freeze-thaw cycles are often combined with chemical or enzymatic treatments to enhance ECM porosity, making it more receptive to subsequent modifications [69]. However, even in the absence of additional treatments, repeated freeze-thaw cycles alone can effectively remove native cells while preserving ECM integrity. For instance, this method has successfully decellularized peripheral nerve sheaths while maintaining the flexibility of the basal lamina [70].

Force And Pressure: High hydrostatic pressure (HHP) is an effective method for tissue decellularization, offering advantages over chemical treatments. HHP disrupts cell membranes and structures, enabling rapid decellularization without harsh chemicals [71]. Pressures exceeding 600 MPa can independently dismantle cell structures, though this approach is often combined with chemical treatments to ensure complete cell removal while preserving extracellular matrix (ECM) integrity [72]. However, careful washing is essential to eliminate cellular debris, and attention must be paid to potential ECM damage due to ice crystal formation under high entropy conditions.

Nonthermal Irreversible Electroporation (NTIRE): It is an emerging decellularization technique that uses electrical pulses to create micropores in cell membranes, leading to cell death while preserving the extracellular matrix (ECM). Unlike chemical or enzymatic treatments, NTIRE does not rely on thermal or harsh chemical exposure, minimizing ECM damage [73]. This approach effectively preserves the key biochemical and structural properties of the ECM, making it a promising option for tissue engineering. However, further research and standardization are necessary to optimize its efficacy and enable its broader application in tendon and ligament decellularization [74].

Supercritical Carbon Dioxide: Supercritical carbon dioxide (CO) exhibits both liquid- and gas-like properties at its critical temperature (31.1°C) and pressure (7.40 MPa), making it highly suitable for biological applications. In tissue decellularization, its primary advantage is its complete removal from the tissue, eliminating the need for extensive washing. However, due to its non-polar nature, a co-solvent such as ethanol is required to facilitate the removal of polar phospholipid membranes. Supercritical CO has been successfully employed to decellularize various tissues, including rat hearts, porcine corneas, bovine and porcine pericardium, and human adipose tissue [75]. This method effectively preserves the structural integrity and mechanical properties of the extracellular matrix, highlighting its potential as an advanced decellularization technique [76,77].

Chemical Treatment

Chemical methods are among the most widely used approaches for decellularization, employing agents such as surfactants, acids, and base. These agents facilitate cell removal while preserving the extracellular matrix (ECM) structure to varying degrees[78].

Acids and Bases: Acids and bases are commonly used in decellularization for their ability to dissolve cytoplasmic components and eliminate nucleic acids. While effective, their aggressive nature may result in the loss of essential growth factors and compromise the mechanical integrity of the extracellular matrix (ECM). Peracetic acid, a highly corrosive and strongly oxidizing agent, is often used in combination with other decellularization agents and is known to increase ECM stiffness[79]. Other acids, such as formic acid, acetic acid, and citric acid, have also been utilized for specific applications, including lymph node decellularization in Lewis rats[80].

Commonly Used Bases: Calcium hydroxide, Sodium sulfide, Sodium hydroxide, Ammonium hydroxide – Primarily used for hair and dermal removal. Alkaline treatment is typically combined with other decellularization methods to enhance efficiency [81,82].

Detergents: Detergents are amphiphilic molecules widely used in decellularization for their ability to disrupt hydrophobic and hydrophilic interactions, leading to cell membrane solubilization and DNA-protein dissociation. Their effectiveness is influenced by exposure time, as prolonged treatment can degrade essential extracellular matrix (ECM) proteins. Ionic detergents such as SDS, SDC, and Triton X-200 are highly disruptive and efficient at removing cellular components; however, SDS, while achieving complete cell removal, significantly reduces glycosami-

Table 2: Summary of the physical methods in decellularization

Method	Description
Freeze thaw cycle	Can have more than one freeze thaw cycle Breaks the cell membrane through the formation of ice crystals. Temperature ranges from -80°C - 37°C
Force and Pressure	Decellularization process is quick Applying high pressure above 600MPa can disrupts cell membrane inside tissue
Nonthermal	Uses electrical pulses to create pores in cell membranes, causing cell death. It preserves ECM if heat is controlled, though further standardization is needed.
Supercritical Carbon dioxide	Temperature is 31.1°C Pressure is 7.40 MPa It requires a polar co-solvent like ethanol to remove polar components like phospholipid membranes. Successfully decellularizes rat heart tissue, porcine corneas, bovine and porcine pericardium, and human adipose.

Table 3: Summary of the chemical treatments in decellularization

Method	Description
Acid and bases	Acids and bases dissolve cytoplasmic components and remove nucleic acids during decellularization. Peracetic acid is highly corrosive, oxidizing, increases ECM stiffness, often used with other methods. Alkaline treatments combined with other agents for effective decellularization.
Detergents	Disrupt hydrophobic-hydrophilic interactions to solubilize cell membranes. Break lipid-protein interactions, aiding in cell and genetic material removal. Zwitterionic detergents are used with SDS or SD for decellularizing lungs (rat, pig, human)
Hypertonic and hypotonic solutions	Used to remove cellular components via osmotic effects, leading to cell lysis, dehydration, and death. Often used as chemical methods for decellularization and for cleaning ECM of residual chemicals and cellular remnants.

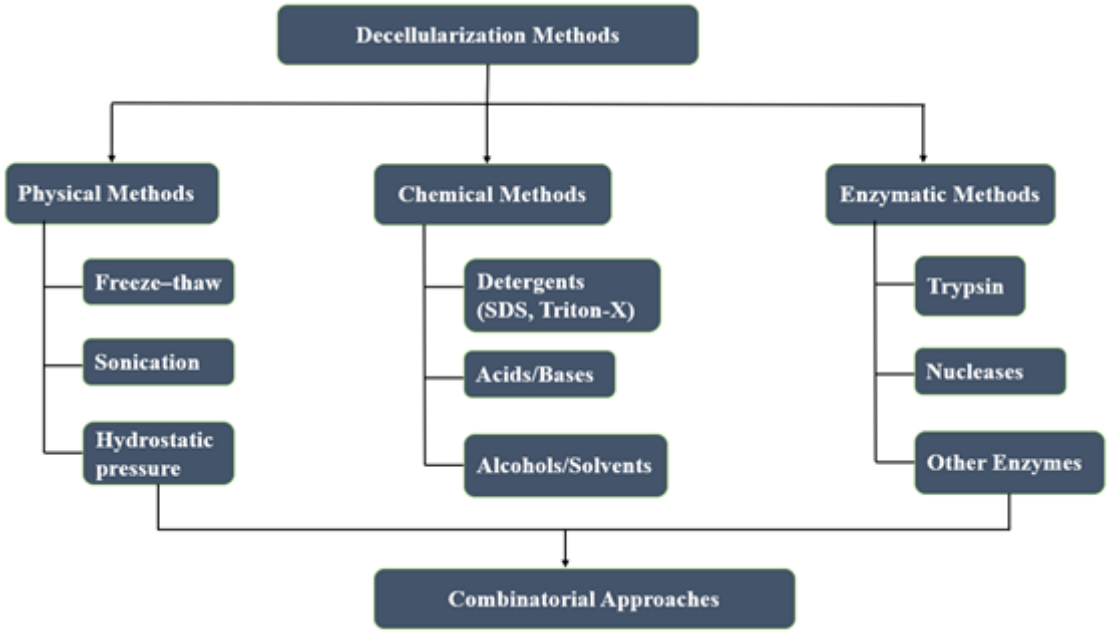

Figure 1: Schematic representation of decellularization methods used in ligament and tendon tissue engineering. Physical methods (e.g., freeze–thaw, sonication, hydrostatic pressure), chemical methods (e.g., detergents such as SDS and Triton-X, acids/bases, and alcohols/solvents), and enzymatic methods (e.g., trypsin, nucleases, and other enzymes) are commonly employed either alone or in combination. Combinatorial approaches are often adopted to balance effective cellular removal with preservation of extracellular matrix structure and functionality

noglycan (GAG) content and disrupts collagen structure [83]. Non-ionic detergents like Triton X-100 are milder, disrupting lipid-based interactions while preserving ECM structure and bioactive molecules such as VEGF and TGF-â, which are crucial for tissue regeneration. Zwitterionic detergents like CHAPS offer intermediate strength and a balance between decellularization efficiency and ECM preservation. Although SDS was found to best preserve ECM components like collagen and laminin, CHAPS left behind more cytoplasmic proteins, indicating incomplete decellularization [84,85].

Hypertonic and Hypotonic Solutions : Osmotic treatments involve exposing cells to solutions with varying solute concentrations, inducing lysis and facilitating decellularization. Hypertonic solutions (e.g., sodium chloride) help remove intracellular proteins. Hypotonic solutions (e.g., Tris-HCl) effectively remove nuclei and DNA but may cause tissue swelling and antigen dispersion. These solutions are often supplemented with enzymatic or chemical treatments to ensure complete cell removal [86].

Table 4: Summary of the enzymatic methods in decellularization

Enzymes	Description
Nucleases	Hydrolytic enzymes that break DNA and RNA through phosphodiester bond cleavage, allowing the removal of cells during decellularization without compromising proteins.
Trypsin	A serine protease that breaks down collagen and elastin in the ECM; EDTA is often used together with trypsin to dislodge cell-matrix adhesions. Concentration and exposure time should be optimal so as not to damage the ECM.
Lipase	Hydrolyzes ester bonds in lipids, helping in delipidation, though complete lipid removal is difficult to achieve.
Dipase	A protease that cleaves fibronectin and collagen IV in basement membranes, useful for specific peptide cleavage but damaging to ECM components.
Collagenase	Breaks peptide bonds in collagen, facilitating tissue breakdown
Benzonase	Genetically engineered endonuclease that degrades DNA and RNA without proteolytic activity, commonly used in combination with other decellularization agents and can be easily removed by washing.
Phospholipase	An enzyme that hydrolyzes phospholipids, which aids in cell solubilization but significantly reduces glycosaminoglycan (GAG) content.

Enzymatic Treatment

Enzymatic treatments are commonly employed to enhance decellularization by assisting in the removal of unwanted cellular and genetic materials. The success of these treatments relies on preserving essential extracellular matrix (ECM) components required for regenerating the functional properties of the target tissue or organ. Enzymes are particularly effective because they selectively target and degrade specific cellular structures or cell-ECM adhesion points. Common enzymatic agents used in decellularization include nucleases, trypsin, collagenase, lipase, dispase, thermolysin, and á-galactosidase [87]. The remaining cellular components are broken down and eliminated with the aid of these enzymes. The lack of ability of enzymatic techniques to accomplish total decellularization, which frequently calls for the employment of additional agents, is a significant disadvantage. Nucleases such as benzonase, for instance, are very good at cleaving nucleotide sequences, which improves cell lysis. Trypsin, a serine protease, is also effective in decellularization, but it may cleave ECM proteins such as collagen and elastin, potentially reducing glycosaminoglycan content. Similarly, nucleases like DNase and RNase are highly targeted in degrading nucleic acids, contributing to efficient cell lysis (Table 4).

Nucleases: Nucleases catalyze the hydrolysis of both deoxyribonucleotide and ribonucleotide chains, facilitating cell removal during decellularization. Deoxyribonuclease (DNase) is a widely used nuclease due to its specificity for removing DNA content while preserving proteins. It works by cleaving nucleic acid sequences, breaking down DNA and RNA to render them inactive, prevent replication, and assist in their

elimination [88].

Typsin: Trypsin, a serine protease, is a potent decellularization enzyme. It works by degrading ECM proteins, including collagen and elastin, though it has the advantage of better preserving glycosaminoglycan content[89]. Trypsin is often paired with ethylenediaminetetraacetic acid (EDTA), a chelating agent that disrupts cell-matrix adhesions. This combination is commonly used in cell culture and decellularization protocols [90]. It is critical to optimize trypsin concentration (typically between <0.03% to 0.5%) and limit exposure to no more than 12 hours to achieve efficient decellularization, as highlighted in studies involving porcine coronary arteries [91].

Lipase: Lipase catalyzes the hydrolysis of ester bonds in lipids, facilitating delipidation during decellularization. However, complete lipid removal can be difficult, and some lipid components may remain after treatment [92].

Dipase: Dispase is a protease that targets and cleaves specific peptides, such as fibronectin and collagen IV, in basement membranes. While effective in breaking down cellular components, it may also damage the basement membranes and ECM, potentially impacting tissue structure and function [93].

Collagenase: Collagenase plays a critical role in decellularization by breaking the peptide bonds in collagen, contributing to the breakdown of tissues during the decellularization process [94].

Benzonase: Benzonase is a genetically engineered endonuclease that specifically degrades DNA and RNA without exhibiting proteolytic activity. It is easy to remove through repeated washing and is commonly used in conjunction with other decellularization methods [95,96].

Phospholipase: Phospholipase hydrolyzes phospholipid components of cell membranes, aiding in cell solubilization during decellularization. However, one significant drawback of using phospholipase is its tendency to significantly reduce glycosaminoglycan (GAG) content, which could impair the functional properties of the ECM [97].

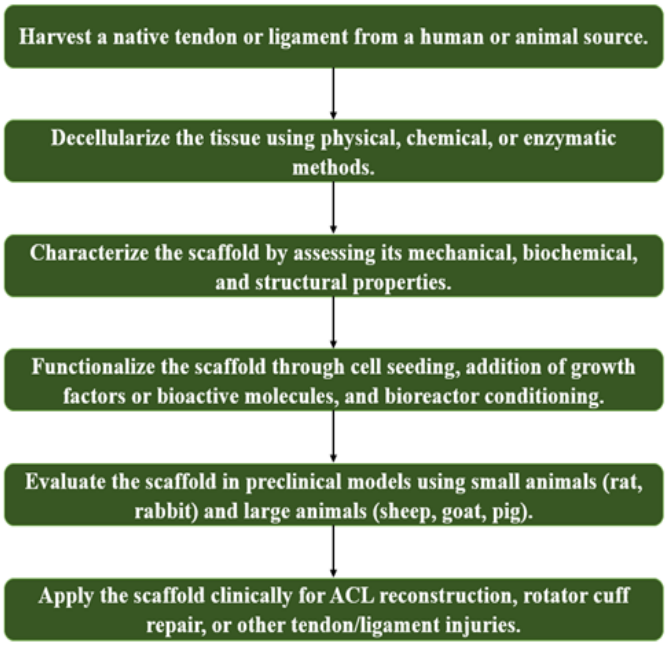

Figure 2: Stepwise workflow for the development and application of decellularized tendon/ligament scaffolds. Native tissues harvested from human or animal sources are decellularized using physical, chemical, or enzymatic methods. The resulting scaffolds are characterized for their structural, biochemical, and mechanical properties, followed by functionalization with cells, growth factors, or bioreactor-based conditioning. Preclinical testing in small and large animal models precedes clinical translation for applications such as ACL reconstruction, rotator cuff repair, and other tendon/ligament injuries

Benefits and limitations of decellularization

Benefits: 1. Decellularization maintains the natural extracellular matrix (ECM) structure, that promotes cell attachment, migration, and tissue regeneration that preserves the ECM. This helps in the formation of functional tissues when used as scaffolds in tissue engineering. 2. Reduced immune rejection: By removing cellular components, decellularization minimizes the chances of immune rejection, allowing for the creation of scaffolds that can be used in allogeneic or xenogeneic transplants. 3. Promotion of Tissue Regeneration: The ECM components, like collagen, elastin, and glycosaminoglycans that are preserved help to provide a supportive microenvironment conducive to cell differentiation and proliferation, which is essential and crucial for successful tissue regeneration.

Limitations: 1. Incomplete Decellularization: Major limitation is the difficulty in achieving complete decellularization. Residual cellular debris or DNA can cause inflammatory responses or affect the scaffold's integrity. 2. Altered Mechanical Properties: Decellularization can sometimes compromise the mechanical properties of the scaffold, including its strength and elasticity, though these can often be restored through additional treatments. 3. Variable Efficacy of Enzymatic Treatments: The use of enzymatic agents for decellularization is not always fully effective in removing cellular and ECM components, and different tissues may require distinct protocols. 4. Loss of Specific ECM Components: Some decellularization methods may cause the loss or degradation of important ECM components, such as glycosaminoglycans, which can limit the functional properties of the scaffold.

Properties of decellularized scaffolds in tissue regeneration

A decellularized scaffold must be biocompatible, ensuring the absence of adverse immune responses while supporting cell attachment, proliferation, and differentiation. The preserved extracellular matrix (ECM) serves as a biological template for tissue regeneration by providing structural and biochemical cues.

For example, Xie et al., 2019 and colleagues developed a book-shaped decellularized ECM scaffold from tendon tissue, which promoted the differentiation of bone marrow-derived stem cells (BMSCs) into tenocytes, thereby enhancing the mechanical strength and functionality of regenerated tendon tissue [98]. Similarly, Suzuki et al., 2022 evaluated the mechanical properties of pericardium decellularized using high-hydrostatic pressurization (HHP) combined with surfactants. Their results demonstrated that the ECM architecture and strength were preserved, making the decellularized pericardium suitable for scaffold fabrication in tissue engineering applications [99].

Whitlock et al., 2012 examined freeze-dried human Achilles allografts for anterior cruciate ligament (ACL) restoration [100]. Their method effectively removed inflammatory material while increasing scaffold porosity. The enhanced porosity facilitated cell infiltration, while the tensile properties were maintained close to those of native tissue. In another study, Seyler et al., 2017 evaluated the tensile properties of native porcine patellar tendons and decellularized, oxidized tendon scaffolds [101]. Results demonstrated no significant differences in ultimate tensile load, stiffness, or elastic modulus between the groups. However, the decellularized scaffolds exhibited greater elongation at failure, indicating improved flexibility. These findings suggest that decellularization combined with oxidation can preserve essential mechanical properties while enhancing flexibility, thus broadening their suitability for tissue engineering.

Zhou et al., 2022, developed acellular tendon fibers (ATFs) by removing cellular debris while maintaining the native structure and mechanical integrity of tendon fibers [102]. When cultured with rat tendon cells, ATFs promoted cell adhesion and differentiation. In vivo studies further demonstrated that ATFs supported fibroblast-like cell activity when introduced into muscle tissue. This study highlighted that decellularization with trypsin and deoxyribonuclease preserved both the

strength and architecture of tendon fibers, making them effective scaffolds for tendon and ligament regeneration.

These studies emphasize that successful decellularization should remove immunogenic cellular components while retaining native ECM composition, ultrastructure, and biomechanical properties. An ideal scaffold should also support angiogenesis, nutrient diffusion, and integration with host tissues. Moreover, the choice of decellularization technique—such as enzymatic, detergent-based, or physical methods (e.g., freeze–thaw cycles, pressure-based approaches)—can significantly influence the scaffold's integrity and regenerative potential.

Other Tissue Engineering Strategies to Enhance Tendon/ ligament Tissue Regeneration

Biological scaffolds

Natural polymers, such as collagen [103], silk [104], and fibrin [105,106], are most widely used in tissue engineering due to their excellent biocompatibility, which ensures that they integrate well with the body and support cellular functions. Synthetic polymers like PLGA (Poly(lactic-co-glycolic acid)) and PCL (Polycaprolactone) offer controlled degradation rates and mechanical strength, which are crucial for the scaffold's longevity and function [106,107]. Hybrid scaffolds combine both natural and synthetic materials to leverage the advantages of each.

One innovative design approach in scaffold creation is zonal mimicry, where multilayered scaffolds are crafted to simulate the different zones of tissues—such as the transition from ligament to cartilage to bone. This design is important because it supports tissue-specific cell adhesion and differentiation, mimicking the natural tissue structure. Advanced and modern manufacturing techniques allow for precise control over the scaffold's architecture, porosity, and anisotropy, which are essential for managing cell alignment and vascularization, both of which are crucial for successful tissue regeneration. Furthermore, scaffolds tend to be functionalized with bioactive molecules or peptides to modify cellular interactions and more closely resemble the features of the extracellular matrix (ECM) [108,109].

Cell based strategies

Mesenchymal stem cells (MSCs) and tendon stem/progenitor cells (TSPCs) are commonly used in tissue regeneration as they possess the ability to differentiate into various cell types such as ligament, cartilage, and bone cells. These cells can be seeded onto scaffolds with zonal architecture, where fibroblasts are seeded into the ligament region, chondroblasts into the cartilage zone, and osteoblasts into the bone zone. The combination of these multiple cell types ensures better tissue integration, especially at the tendon-bone interface, mimicking natural tissue organization [110,111]. Emerging techniques like gene editing and cellular programming are being explored to enhance the differentiation potential and functional integration of stem cells. For instance, genetically engineered stem cells may overexpress factors like BMP2 and bFGF, improving healing and regeneration [112,113].

Another promising strategy involves the use of exosomes, small extracellular vesicles secreted by MSCs. Exosomes carry bioactive molecules such as proteins, lipids, and microRNAs, which help regulate the local microenvironment, promote cell-to-cell interactions, and stimulate tissue repair processes. These exosomes have been shown to enhance tendon-bone healing by promoting angiogenesis and reducing inflammation [114].

Growth factors

Growth factors are crucial signaling proteins that regulate key cellular activities such as migration, proliferation, and differentiation. For example, BMPs (Bone Morphogenetic Proteins) promote osteogenesis, aiding the bone regions of scaffolds[115]. FGFs (Fibroblast Growth Factors) stimulate cell proliferation and angiogenesis, which are vital for ligament/ tendon regeneration [116]. VEGF (Vascular Endothelial Growth Factor) stimulates angiogenesis, enhancing nutrient supply and supporting tissue integration[117]. These growth factors can be embedded in scaffolds for controlled, gradual release, which ensures sustained signaling over time, facilitating effective tissue regeneration. Additionally, Platelet-Rich Plasma (PRP), derived from a patient's blood, is rich in growth factors and can promote inflammation reduction, cell proliferation, and angiogenesis. When combined with biomaterials like hydrogels or scaffolds, PRP can be delivered over time, enhancing tendon-bone healing[118,119].

Biophysical modulation

Biophysical cues play a critical role in tissue regeneration by guiding the structural and functional properties of regenerated tissues. Some of the approaches include: 1. Mechanical loading: Mechanical loading is a technique where both static and dynamic mechanical forces are applied *in vitro* to simulate the natural stress environment of ligaments. This helps promote proper cellular alignment and ECM production, which is essential for the structural integrity of the regenerated tissue[120]. 2. Pulsed Electromagnetic Fields (PEMFs) and Electrical Stimulation: Both methods stimulate cellular metabolism, promote tissue maturation, and enhance collagen synthesis, contributing to improved tissue regeneration[121]. 3. Low-intensity Pulsed Ultrasound (LIPUS) and Extracorporeal Shock Wave Therapy (ESW) have been shown to enhance tendon-bone healing, promoting osteogenesis and fibrocartilage formation [122]. By integrating these strategies, it is possible to enhance tissue regeneration, creating functional and robust tissue-engineered constructs for clinical applications in tendon, bone, and ligament repair.

Preclinical Studies

1. Sheep Model for ACL Reconstruction: A study aimed to evaluate the bio-integration and effectiveness of mineral fiber-reinforced screws used in ACL reconstruction. Nine female sheep underwent ACL reconstruction using autologous tendon grafts secured with 4.75 mm mineral fiber-reinforced screws. Over the course of 28 to 132 weeks, the study observed progressive tissue growth and bone integration. By 104 weeks, the screws were nearly fully bio-integrated, replaced by bone, with minimal inflammatory responses. Graft ossification and cellularization continued to improve, indicating strong tendon-to-bone integration. The study found no significant adverse effects, with a reduction in pro-inflammatory cells and minimal presence of M2-macrophages and giant cells. The study confirmed the efficacy and safety of these screws in achieving stable graft fixation, bone remodeling, and fewer complications than those typically associated with polymer-based implants [123].

2. Rabbit Model for Rotator Cuff Repair: This study investigated the use of decellularized tendon matrix (DTM) putty for enhancing tendon-to-bone healing in a rabbit model. Eight New Zealand White rabbits with chronic supraspinatus tendon tears were treated with DTM putty. The DTM retained its natural bioactivity, stimulating the proliferation of tenocytes and adipose-derived stem cells. Histological evaluations revealed improved repair quality, including enhanced calcification at the bone-tendon interface, closely resembling the natural fibrocartilaginous enthesis. The treatment showed minimal adverse tissue reactions, suggesting that DTM putty is an effective strategy for improving tendon-to-bone healing outcomes. This study highlights DTM putty's potential as a tendon repair biomaterial [124].

3. Rabbit Model for Achilles Tendon Repair (Decellularized Tendon + BMSCs): In this study, decellularized tendon scaffolds seeded with bone marrow–derived mesenchymal stem cells (BMSCs) were assessed in a rat Achilles tendon defect model. The constructs exhibited improved collagen alignment, decreased inflammatory response, and significantly greater tensile strength than unseeded decellularized scaffolds. Histology revealed organized extracellular matrix deposition, indicating that stem cell seeding of decellularized scaffolds greatly improves tendon repair outcomes [125].

These preclinical studies provide evidence of the effectiveness of various biomaterials and therapies in improving tendon repair and orthopedic conditions. Mineral fiber-reinforced screws and decellularized tendon matrix putty demonstrated favorable bio-integration, enhanced healing, and minimal adverse reactions, making them promising candidates for clinical use in tendon and ligament repair. These studies underscore the potential for these advanced materials and therapies to en-

hance orthopedic repair outcomes in preclinical settings.

Challenges and Opportunities

Despite their promise, the use of decellularized tendon (DT) scaffolds in tendon regeneration continues to face several challenges that slow down translation to clinical practice. A central issue is the variability of decellularization protocols, which differ in detergents, antigen removal steps, and assessment criteria. This lack of standardization makes it difficult to compare results across studies, thereby complicating the establishment of regulatory guidelines [126,127]. Furthermore, incomplete antigen removal remains a persistent concern. Even small remnants of DNA fragments, á-gal epitopes, or MHC antigens can elicit host immune responses, and inconsistent reporting of these markers across studies hampers accurate prediction of long-term safety and biocompatibility [128].

Another obstacle is the mechanical performance of DT scaffolds. Native tendons and ligaments are capable of withstanding exceptionally high loads, yet many decellularized constructs exhibit inferior strength and durability. Reinforcement through crosslinking or incorporation of synthetic supports is often necessary to prevent failure under surgical fixation and early rehabilitation stresses [129]. Additionally, much of the encouraging data stems from rodent or rabbit models of acute tendon injury, which do not adequately replicate the chronic degenerative tears and demanding mechanical environment of human tendons. This gap in translational relevance raises questions about the predictive value of preclinical outcomes [130]

Finally, regulatory and clinical validation remains limited. Few randomized controlled trials have evaluated DT scaffolds in human subjects, and standardized criteria for success are largely lacking. This scarcity of high-quality clinical evidence delays regulatory approval and impedes broader adoption [127]. Addressing these challenges will require coordinated efforts to harmonize decellularization protocols, establish robust preclinical models that better simulate human pathology, and generate rigorous clinical evidence. At the same time, these barriers present opportunities: standardization could unlock more reliable comparisons, mechanical reinforcement strategies could enhance scaffold durability, and the accumulation of clinical data could accelerate translation, ultimately positioning DT scaffolds as a valuable platform in tendon tissue engineering.

Future Directions

Moving forward, several strategies could accelerate the clinical translation of decellularized tendon scaffolds. A key priority is the standardization of decellularization protocols and reporting practices. Establishing consensus metrics—such as acceptable thresholds of residual DNA, preservation of critical extracellular matrix components like glycosaminoglycans and collagen, and defined mechanical benchmarks—would enable meaningful cross-study comparisons and facilitate regulatory approval. The functionalization of decellularized extracellular matrix (dECM) with targeted delivery systems represents another promising avenue. Incorporating controlled-release vesicles, growth factors, or small extracellular vesicles (sEVs) into dECM scaffolds, including in innovative platforms such as microneedle–dECM systems, has already shown enhanced tendon-to-bone healing and immunomodulatory effects in preclinical models. These combinatorial approaches may greatly improve local efficacy[131]. Mechanical performance also remains a focus. Hybrid constructs that integrate dECM with synthetic porous fibrous scaffolds or utilize crosslinked coatings are being explored to combine biologic signaling cues with the strength and durability required for clinical repair. Alongside this, advances in tendon-derived stem/progenitor cell (TDSC) biology and mechanobiology hold particular promise. The use of TDSCs in synergy with dECM scaffolds, coupled with mechanical preconditioning in bioreactors, may accelerate the generation of tendon-like tissues and improve graft integration[132].

Finally, translation to the clinic will depend on rigorously designed randomized trials. Controlled studies with standardized endpoints—including tendon healing integrity, biomechanical properties, imaging parameters, and functional outcome scores—are urgently needed to validate the safety and efficacy of optimized dECM-based scaffolds in relevant patient populations. Together, these directions underscore a future where biological fidelity, mechanical competence, and translational rigor converge to realize the full potential of DT scaffolds in tendon regeneration.

Conclusion

Decellularized scaffolds are the new revolution in ligament and tendon tissue engineering, offering unparalleled opportunities for repairing and regenerating damaged tissues. Scaffolds offer a natural extracellular matrix that supports cellular attachment, proliferation, and differentiation, which is important for tissue integration and functional recovery. Although significant, there are still challenges ahead, such as optimizing decellularization methods to preserve the ECM structure and standardizing protocols for consistency in results. While physical, chemical, and enzymatic techniques have advanced scaffold preparation, issues persist regarding immunogenicity, mechanical strength, and long-term functionality. The main thrust of research in tissue repair is being made through advanced strategies, such as the integration of growth factors, stem cell therapies, and biophysical modulation, aimed at improving cellular interactions, vascularization, and biomechanical properties. Further, interdisciplinary efforts combining advanced fabrication techniques with *in vivo* validation are crucial for overcoming current limitations and accelerating clinical translation. Through continuous research and collaboration, decellularized scaffolds will revolutionize ligament and tendon repair with sustainable and effective solutions for patients.

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