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RESEARCH ARTICLE

Three-Dimensional Electron Microscopy of Human Umbilical Cord Tissue Allograft Pre and Post Processing: A Literature Comparison

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ABSTRACT

One in four adults in the US suffer from cartilage degeneration of the Intervertebral Disc (DDD) or load bearing joints (DJD). Since cartilage is avascular, it has a limited regenerative capacity. Conventional non-surgical treatments provide brief symptomatic relief, have sided effects, and do not address the cartilage defect itself. As such, new alternatives are needed. Perinatal birth tissue allografts are a novel frontier for bio-mechanical cartilage engineering research. The tissues of interest include umbilical cord-derived Wharton's Jelly (WJ). This study assessed WJ tissue samples via ZEISS Supra 55VP FieldEmission Scanning Electron Microscope (SEM) at 100 and 300 nm resolution scales. The captured images of pre and post-processed structural tissue matrices in WJ allografts were analyzed against themselves and peer-reviewed SEM images of articular cartilage, intervertebral disc cartilage, and muscle fascia. SEM images of post-processed WJ structural tissue matrices were found to be comparable to structural tissue matrices in human articular cartilage, intervertebral disc cartilage, and muscle fascia on a qualitative and quantitative level. This is the first study that we are aware of, to demonstrate that structural collagen matrices in post-processed WJ allografts are analogous in structure to the cartilage in articular joints, intervertebral discs, and muscle fascia.

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- Wharton's jelly
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Introduction

Advances in regenerative medicine have increased significantly throughout the past decade. Wharton's Jelly (WJ) was initially characterized in 1656 by Thomas Wharton [1]. Since its initial discovery, there has been significant interest in the use of WJ in regenerative medicine applications [2]. Located between the blood vessels of the umbilical cord and the amniotic epithelium, WJ spans the entire length of the umbilical cord, providing protection, cushioning, and structural support [2,3]. Initial research centered on WJ as a cellular product, dependent on the metabolic activity of living cells to exert its primary function [3]. However, current research demonstrates that WJ exerts an effect independent of any cellular activity [3]. Initially classified as a mucoid connective tissue, we now know that WJ functions as an ideal system to transplant chemokine and growth factors, in addition to providing a biomechanical microarchitecture for collagen extracellular matrix formation in collagen-based defects [4].

ECM is a collagen-based, cross-linked network that provides tensile strength and distributes load. Damage, degeneration or diminished function in collagen cross-bridging will lead to loss of mechanical properties of the collagen network

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and result in an impaired ability to resist forces delivered by compression [5]. Collagen-specific structural tissue defects can be identified by physical examination and confirmed with MRI or musculoskeletal ultrasound [6]. A structural tissue defect in numerous skeletal muscles would prove detrimental to both functional outcomes and work productivity. Collagen is the most abundant family of Extracellular Matrix (ECM) proteins and is ubiquitous throughout the body [7].

Collagen fibers are organized into a system of multi-thread ropes, allowing for more flexibility when compared to compact fibers [8]. Human tissue strength is directly dependent upon the sizeable crosslinking of collagen [9]. Collagen types II, III, V, VI, and XII have been isolated from WJ [2,10].

Regeneration of collagen structural tissue defect occurs as ECM is regenerated in the base of the defect, restoring the original relevant characteristics of the damaged tissue [10]. Scar tissue will only achieve a maximum of 80% strength of the native tissue [11]. There is great potential for the WJ matrix derived from the human Umbilical Cord (UC) to act as an ECM scaffold and augment the regenerative process [10]. WJ contains an abundant amount of collagen and hyaluronic acid, both essential components of ECM [2,10]. Since WJ is a comprehensive source of human extracellular matrix proteins and growth factors; there may be numerous clinical applications for collagen-based structural tissue defects [3].

Previously reported uses for the human umbilical cord and its byproducts, including umbilical cord-derived WJ, have focused on the use and proliferation of mesenchymal stem cells [12]. WJ has frequently been studied in terms of cellular activity [13]. However, we have chosen to report on the role of umbilical cord-derived WJ as an extracellular collagen matrix, or micro-architectural framework based on its qualitative and quantitative aspects. Current clinical practice guidelines and research support the homologous use of WJ and its non-cellular role in establishing a microarchitectural framework for ECM in collagen-based structural tissue defects.

Human cell, tissue, and tissue-based products (HCT/Ps) are regulated by the United States Food and Drug Administration (FDA) under title 21, part 1271 of the Code of Federal Regulations (CFR). Under these regulations, the HCT/P must be minimally manipulated, have no systemic effect or be dependent on living cells for its primary function, be intended for homologous use only, and exclude the combination of the product's cells or tissues with another article except for water, crystalloids, or a sterilizing, preserving, or storage agent provided that the storage agent does not raise new clinical safety concerns with respect to the HCT/P [13]. This study aimed to assess WJ tissue samples via a ZEISS Supra 55VP Field-Emission Scanning Electron Microscope (SEM) at 100 and 300 nm resolution scales.

Materials and Methods

All methods were completed in compliance with the FDA and American Association of Tissue Banks (AATB) standards. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Institute of Regenerative and Cellular Medicine (IRCM-2022-311) on 12 January 2022.

Donation and collection

Human umbilical cords were obtained from consenting donors following full-term Caesarian section deliveries. Prior to delivery, donors underwent comprehensive medical, social, and blood testing. Qualtex Laboratories in San Antonio, TX tested all donations for infectious disease in accordance with Clinical Laboratory Improvement Amendments (CLIA) of 1988, 42 CFR part 493, and FDA regulations. Each donor was tested for Hepatitis B Core Antibody (HBcAb), Hepatitis B, Surface Antigen (HBsAg), Hepatitis C Antibody (HCV), Human Immunodeficiency Virus Antibody, (HIV-1/HIV-2 Plus O), Human T-Lymphotropic Virus Antibody (HLTV-I/11), Syphilis (RPR), Cytomegalovirus (CMV), HIV-1/HCV/HBV, NAT, and West Nile Virus (WNV). Each test was performed with an FDA-Approved testing kit [**Appendix A**]. All test results were negative or non-reactive.

Preparation of the pre-processed umbilical cord tissue samples

All procedures were performed in accordance with strict aseptic techniques. In an ISO class 5 biologic safety cabinet, the umbilical cord was rinsed with saline to remove excess blood residue and clots. Various sized cross-sections of the umbilical cord were cut and placed on a sterile drying tray. The cross-sections were then desiccated in a high nitrogen concentration drying chamber.

Preparation of processed umbilical cord tissue samples product

Wharton's jelly was aseptically dissociated from the rinsed umbilical cord. After dissociation, 150 mg of Wharton's Jelly was suspended in approximately 2mL of sterile Sodium Chloride 0.9% solution (normal saline). The sample was not combined with cells, tissues, or articles other than the exceptions outlined in 21 CFR Part 1271.10(a)(3) (Human Cells, Tissues, and Cellular and Tissue-Based Product Regulation).

The manufacture of the HCT/P does not involve the combination of the cells or tissues with another article, except for water, crystalloids, or a sterilizing, preserving, or storage agent, provided that the addition of water, crystalloids, or the sterilizing, preserving, or storage agent does not raise new clinical safety concerns with the respect to the HCT/P [15].

Scanning electron microscope imaging

Pre-processed and post-processed desiccated tissue samples were received by the University of Montana laboratory for analysis and electron microscope imaging. The tissue samples were transferred to a sticky carbon surface (PELCO Tab, Ted Pella, Inc.) and coated with a thin layer of iridium (60 seconds at 25 mA, Emite K575X Sputter Coater) to mitigate charging in the microscope. The tissue samples were then examined via a ZEISS Supra 55VP Field-Emission Scanning Electron Microscope (SEM) at a 100 and 300 nm scale of resolution.

Results

The present study reports the anatomic structural compatibility of a human Umbilical Cord Tissue (UCT) allograft before and after processing to provide objective evidence of minimal manipulation. The resultant comparison establishes examples of homologous use with objective evidence.

Discussion

The expanding applications of regenerative medicine have led to increased patient demand, clinical utilization, and substantial marketing of new advancements in regenerative medicine [1]. Despite the well-recognized secondary and tertiary functions of WJ, there has been minimal research on the anatomic structural compatibility of WJ allografts and the sites of application within collagen-based structural tissue defects. In the current study, WJ allografts were produced through minimal manipulation of human umbilical cord tissue samples and are denoted as post-processed umbilical cord tissues. The SEM images of the post-processed samples reveal that the structural integrity of collagen fibers within the ECM is maintained (Figure 2). The diameter of the UCT fibers increased from approximately 61nm pre-processing to 65 nm post-processing. This increase could be due to slight swelling from the saline rinse and is not statistically significant. The average diameter of healthy mid-zone articular cartilage is around 67nm (Figure 3), similar in size and formation to the processed WJ (Figure 5). This characteristic further confirms minimal manipulation of pre- and post-processed tissue.

Collagen is the most prevalent structural macromolecule within the extracellular matrix, arranged in a cross-fiber network [14-16]. The strategic linkage of collagen fibers allows the network to provide protection, cushioning, and structural support, identical to the role of WJ in the umbilical cord [12]. The structure of the collagen fibers remains intact after processing, affording the WJ allograft the intended function of providing cushioning and structural support to the site of the structural tissue defect [17,18]. The structural composition of collagen before processing the umbilical cord tissue samples is shown in Figure 1. It is evident when comparing Figures 1 and 2 that the structural properties

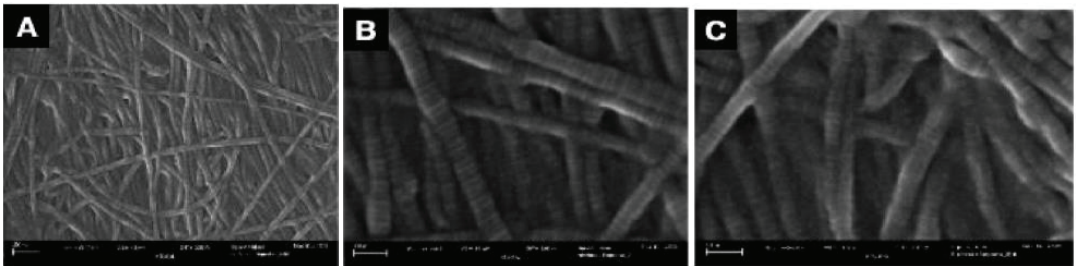

Figure 1 SEM micrographs of pre-processed umbilical cord tissue samples.
(A) SEM image of cross-linked collagen structures. (Scale bar: 300nm).
(B) SEM image of collagenic structure fibers. (Scale bar: 100nm).
(C) SEM image of collagenic structure fibers (Scale bar: 100nm).

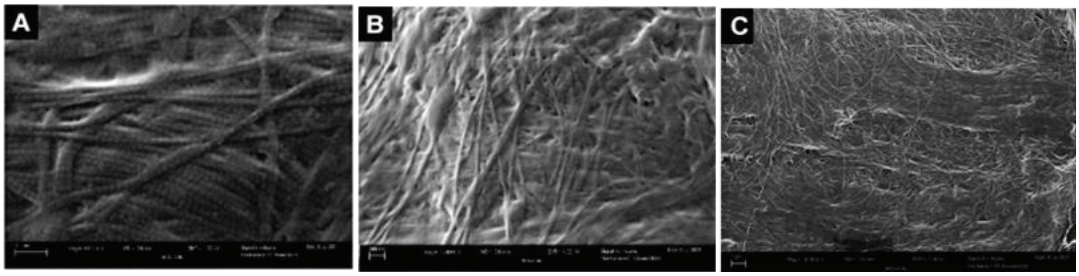

Figure 2 SEM micrographs of post-processed umbilical cord tissue samples.
(A) SEM image of preserved cross-linked collagen structures. (Scale bar: 300nm).
(B) SEM image of preserved random directional structural composition of collagen fibers. (Scale bar: 300nm).
(C) SEM image of multidirectional linkage of collagen fibers (Scale bar: 1μm).

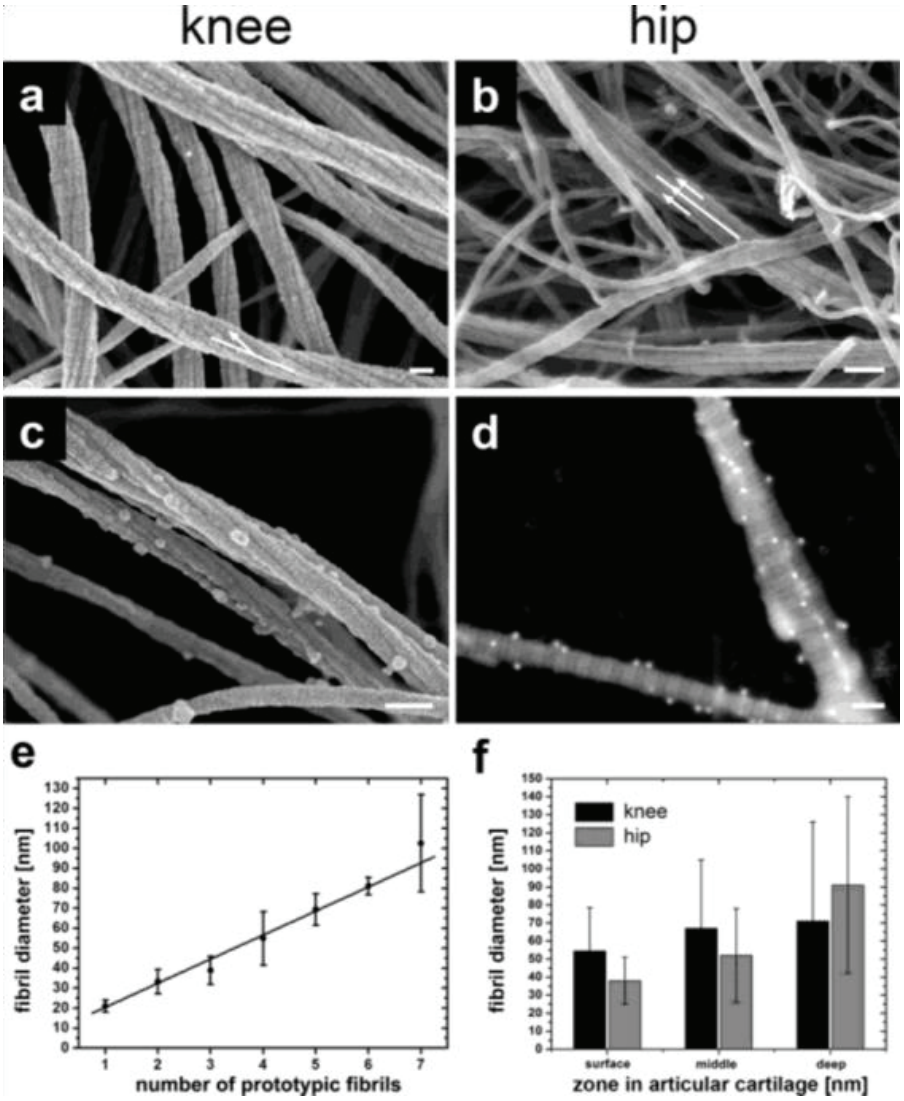

Figure 3 Literature comparison of SEM images of normal human articular cartilage after enzymatic depletion of the proteoglycan moiety and chondrocytes.
(a) SEM image of the collagen fiber meshwork from knee surface articular cartilage shows (i) the 67 nm D-band periodicity, (ii) the hierarchical organization of 5–7 threads of prototypic fibrils forming an individual collagen fiber; note that each prototypic fibril exhibits the 67 nm D-band periodicity; and (iii) a twisting of the prototypic fibril along the long axis of roughly about 400 nm (white arrow).
(b) SEM image of hip surface articular cartilage with untwisted fibers (white arrows).
(c) Collagen fibers were labeled by collagen II antibodies with 18-nm gold particles attached and directly inspected in cartilage by SEM.
(d) Imaging of 18-nm gold particles using the backscattering electron (BSE) mode in the SEM on extracted collagen type II fibers.
(e) Graph shows the increase of collagen fiber diameter with the number of prototypic fibrils.
(f) Comparison between fiber diameters in each zone in hip articular cartilage and knee articular cartilage. Scale bars, 100 nm (a to d) [8].

of the WJ before and after processing have been retained and are consistent with FDA guidelines defining minimal manipulation of WJ allografts.

The multidirectional collagen network observed in WJ appears to be structurally comparative to the collagenic organization found in the extracellular matrix of cartilage [16,19]. Previously published research indicates that WJ cells have excellent chondrogenic differentiation capabilities, which could be beneficial in cartilage tissue engineering [19]. Figure 4 illustrates the mesh network of collagen in human articular cartilage. The anatomic structural similarities between the two collagen networks indicate that human umbilical cord tissue allografts are comparatively homologous in structure to the extracellular matrix found in cartilage. The breakdown of collagen (Figure 4C) results in the deterioration of cartilage [8]. Patients with structural tissue defects typically experience impairments in functional outcomes and quality of life. While applications of human umbilical cord tissue allografts have been used extensively in the clinical treatment of cartilage tears, there is also potential for allografts to be used in the pathology of intervertebral discs, as well as muscle tears [20]. As a non-surgical option, applying homologous WJ tissue allografts to structural tissue defects warrants clinical consideration.

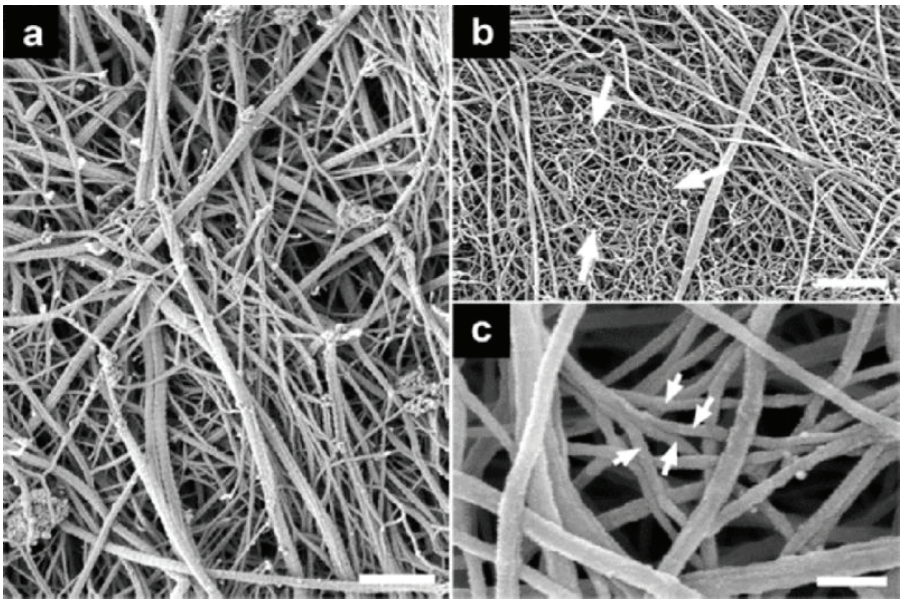

Figure 4 Literature comparison of SEM images illustrating articular cartilage in osteoarthritic patients.
(a) SEM image of grade 3 osteoarthritic cartilage (knee), exhibiting breakdown of thicker collagen fibers with a diameter of 40–60 nm into thinner fibers down to bundles made of only one prototypic fibril of 18 ± 5 nm in diameter.
(b) SEM image of grade 3 osteoarthritic cartilage (knee) shows the end-stage of fiber breakdown, that is a wool-like structure (white arrows) with filaments exhibiting a diameter of d = 13 ± 2 nm.
(c) Degrading articular cartilage larger fibers split into smaller sized fibrils that are often arranged as a highly entangled fibrillar meshwork (white arrows). Scale bars, 500 nm (a and c); 100 nm (b).
Reprinted from Gottardi R, et al. (2016), Supramolecular Organization of Collagen Fibrils in Healthy and Osteoarthritic Human Knee and Hip Joint Cartilage. PLOS One, 11(10): e0163552.
doi:10.1371/journal.pone.0163552.

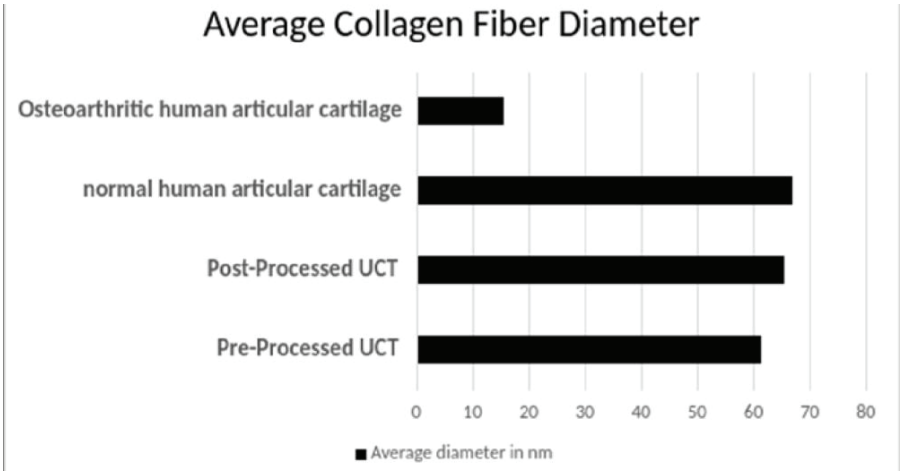

Figure 5 Fiber diameters were determined from SEM photos of post and pre-processed UCT tissues and compared with diameters found in the supramolecular organization of collagen fibrils in healthy and osteoarthritic human knee and hip joint cartilage study.

Despite WJ being a non-vascular, dense irregular connective tissue, there is also the promise of homologous applications for WJ in vascularized tissue. In figure 6C, the organization of the collagen fibril network in skeletal muscle closely resembles the multidirectional collagen fibers seen in the post-processed umbilical cord tissue samples (Figure 2).

The extracellular matrix of skeletal muscles comprises a three-dimensional architecture consisting primarily of collagen, glycoproteins, and elastin [21]. The comparative composition and organization of collagen between post-processed WJ and skeletal muscle extracellular matrices suggest that WJ can serve as a homologous allograft in structural tissue defects of muscular origin [21,22]. Forty percent of the human body weight is composed of skeletal muscle [22]. The application of WJ as a homologous allograft in patients with structural tissue defects in skeletal muscle

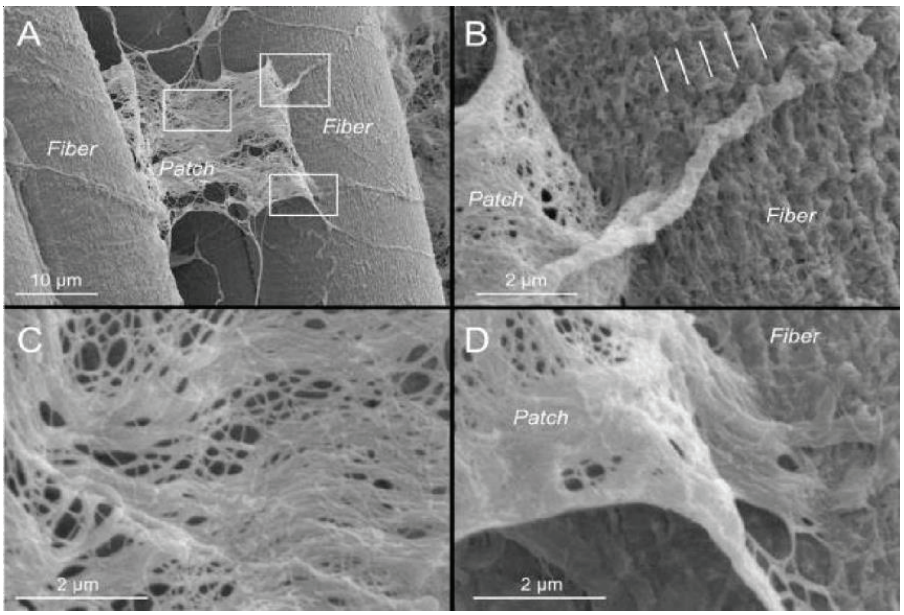

Figure 6 Literature comparison of SEM images illustrating collagen presence in skeletal muscle collagen types II, III, V, VI, and XII have been isolated from WJ.
(A) Sections of ECM separated from muscle fibers during sample preparation. Sections are represented by white rectangles. (Scale bar: 10µm).
(B) Longitudinal ECM organization is seen on the fiber surface, as noted by the rectangular lines. (Scale bar: 2µm).
(C) Collagen fibril network organization observed through the central region of an ECM patch. (Scale bar: 2µm).
(D) ECM patch appears firmly connected to the skeletal muscle fiber surface. (Scale bar: 2µm) [21].

provides an opportunity for future observational and prospective clinical studies. If shown efficacious, the clinical applications of human umbilical cord tissue allografts will be extensive in both vascularized and non-vascularized tissue. The utilization of umbilical cord tissue allografts in regenerative medicine will be continually enhanced with additional discoveries of homologous use applications in clinical research data.

Conclusion

Through observational, collagenic structural comparison from SEM images, WJ demonstrates the potential to be used as an architectural scaffold for ECM supplementation in cartilage-based tissue structures and skeletal muscle tears. Even after processing, WJ retained its pre-processing structural characteristics. Further, the SEM images demonstrate comparative microarchitectural characteristics in post-processing WJ samples and ECM in articular cartilage and peri-muscle fiber fascia. The shared SEM characteristics are consistent between both tissue types, supporting homologous use.

In future work, we intend to use proteomic analysis of collagen fibers to confirm the anatomical comparison between human umbilical cord tissue allografts, skeletal muscle, and cartilage. This analysis would be coupled with high-resolution confocal antibody staining of umbilical cord tissue to allow for differentiation between the different types of collagen in the extracellular matrix. Future case studies documenting umbilical cord tissue allograft efficacy could include applications in orthopedics, gerontology, sports medicine, general family medicine, and operating rooms. These advancements and commercial coverage of WJ allografts would increase physician and patient access to commercially available umbilical cord tissue allografts. Ease of access to perinatal birth tissue allografts like Wharton's Jelly could dramatically improve community health, and reduce global surgical healthcare costs.

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Supplementary materials

Not applicable.

Author contributions

Conceptualization, J.M.D., J.S., and T.C.B.; methodology, J.M.D.; software, T.C.B.; validation, J.M.D., J.S., and T.C.B.; formal analysis, J.R.P..; investigation, J.M.D., and T.C.B.; resources, T.C.B.; data curation, J.M.D., J.S., and T.C.B.; writing—original draft preparation, J.M.D.; writing—review and editing, J.R.P.. and T.C.B.; visualization, J.S. and T.C.B.; supervision, J.R.P. and T.C.B..; project administration, J.S.;

funding acquisition, T.C.B. All authors have read and agreed to the published version of the manuscript."

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Informed consent statement

Informed Consent was obtained from all consenting mothers during the donation of the umbilical cords.

Data availability statement

Not applicable.

Conflicts of interest

Justine Davis, and Tyler Barrett are associated with Regenative Labs. Regenative Labs was involved in the design of the study, analysis, and manuscript authorship. Regenative Labs influenced the decision to publish.

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