



**International Journal of Biology, Pharmacy
and Allied Sciences (IJBPAS)**

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

TISSUE ENGINEERING AND HISTOLOGY OF OSTRICH TENDON

SAEED HOSSEINI, SHAPOUR HASANZADEH*, RASOUL SHAHROOZ

Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

*Corresponding Author E Mail: s.hasanzadeh@urmia.ac.ir

ABSTRACT

Tendons are soft connective tissues, which connect muscle to bone forming a musculotendinous unit, whose primary function is to transmit tensile loads generated by muscles to move and enhance joints stability. The main goal of tissue engineering design and create a structure similar to the natural structure of a tissue in vivo for repair tissue damages. Tissue transplantation, there are always problems, including a shortage of suitable tissue and immunological rejection of grafted tissue is in tissue engineering is one of the newest methods to reduce the immune response to transplanted tissue, is tissue decellularization methods. After fixation and routine tissue process, to evaluate the structure of tissue H&E stained, collagen quality assessment xenograft Picrosirius red stained , review and tracking the decellularization, detected nuclei trace and any DNA remaining 4', 6'diamino-2-phenylindole-2HCl (DAPI) staining was used. Evaluation of properties of decellularization tendon structure and comparison with ostrich tendons found this xenograft is the appropriate for graft. Results by Mann-Whitney ($P \leq 0.05$) were performed with SPSS software. Compared with ostrich tendons and decellularization tendon, the tissue integrity is somewhat reduced cohesion between collagen fibers were observed in some places a few miles away. Quality collagen stained by Picrosirius red that there is no difference between them. In assessing the amount of decellularization following DAPI staining completely cell-free of the three-dimensional scaffold and remains free of nuclear DNA.

Keywords: Xenograft, Achilles tendon, scaffold, decellular, Ostrich

INTRODUCTION

Tendons are dense connective tissues consisting of parallel collagen fibers embedded within an extra-cellular matrix. Tendons connect muscle to bone and transmit tensile force generated by muscles to move and stabilise joints. They must be capable of resisting high tensile forces with limited elongation [1]. The Achilles tendon is one of the longest and strongest tendons in the body. Achilles tendon ruptures can occur in a normal tendon if an excessive load is applied, that is the most frequently ruptured tendon in the lower limb and accounts for almost 20% of all large tendon injuries [1]. The structure of the tendon contains dense regular connective tissue, mainly type I collagen fibers.

For tendon regeneration, The collagen fiber architecture of an extra cellular matrix (ECM) plays a critical role in determining its biomechanical behavior [2, 3]. Tendon disorders are frequent, and are responsible for much morbidity both in sport and the workplace [4]. Tissue engineering techniques using novel scaffold materials offer potential alternatives for managing tendon disorders. Tissue engineering strategies to improve tendon repair healing include the use of scaffolds, growth factors, cell seeding, or a combination of these approaches for tissue

regeneration [5]. proposed that xenograft are highly attractive as they carry small risk of infectious disease, do not compromise the patient's remaining tissues and may have the same structure as the component being replaced [6]. Xenogeneic and allogeneic cellular antigens are, by definition, recognized as foreign by the host and therefore induce an inflammatory response or an immune-mediated rejection of the tissue. However, components of the ECM are generally conserved among species and are tolerated well even by xenogeneic recipients [7]. The tissues from which the ECM is harvested, the species of origin, the decellularization methods and the methods of terminal sterilization for these biologic scaffolds vary widely. Each of these variables affects the composition and ultrastructure of the ECM and accordingly, affects the host tissue response to the ECM scaffold following implantation.

The ostrich (*Struthio camelus*), the largest extant bird, is a highly cursorial animal and is acknowledged as the fastest biped with the greatest capacity for long-endurance running [8]. The musculoskeletal morphology of the ostrich pelvic limb has been the subject of various studies. Muscle distribution further enhances the swing dynamics of the limb.

The largest muscle in the pelvic limb was the ankle extensor gastrocnemius, which on average accounted for 18% of the total muscle of the pelvic limb. This muscle could be separated into three distinct heads, medial, lateral and caudal, decreasing in size in that order. The heads fused in the mid to distal region of the tibiotarsus and inserted onto a large, wide tendon that passed caudally over the ankle and accounted for some 20% of the total tendon mass in the limb. With the exception of gastrocnemius the usual trend was for larger muscles to be located more proximally in the limb, above the knee joint. [8, 9].

The aim of this study was to investigate histological ostrich tendon and following that tissue engineering of ostrich's tendon to offer for usage in regenerative medicine to repair tendon injury.

MATERIALS AND METHODS

Histological Study

Histological analysis was performed to evaluate tissue integrity and decellularization. Engineered tendons and ostrich tendon as positive controls were fixed, paraffin-embedded and sectioned following standard protocols. Specimens were then stained for performed to analyzed of tissue integrity H&E and Masson's Trichrome, cell removal (DAPI) and

collagens (picrosirius red), (n=3) and mounted on glass slides to microscopic examination and photographed on a light microscope Olympus (model CX31RBSFA) or BX51P polarizing microscope for DAPI and picrosirius staining [10, 11].

Decellularization Assay

A decellularization protocol generally begins with lysis of the cell membrane using ionic solutions, detergents using solubilization of cytoplasmic and nuclear cellular components, and finally removal of cellular debris from the tissue.

One or two months-old ostrich have been slaughtered, that foot as tissue and carcass wastes were referred to the Laboratory in cool box. Digital flexor tendons were retrieved aseptically through a paratendinous incision of approximately 5×1×0.5 cm, transferred to a sterile container, cut and shaped immediately and preserved in sterile phosphate buffered saline (PBS) solution at -20 until further processing.

Ionic detergents are strong detergents that can completely disrupt cell membranes and fully denature proteins. SDS is among the most commonly used ionic decellularization agents because the effectively solubilize cytoplasmic membranes, lipids, and DNA. For decellularization the preserved ostrich tendons were thawed at room temperature.

Decellularization protocol was, soaking in aqua dest (DW) for 24 h, 1% SDS solution for 24 h, DW for 12 h this process was repeated twice, and 70% ethanol for 24 h. Following that, tendons were washed extensively in sterile PBS [12].

Statistical analysis

Comparisons between the processes were performed using the Mann–Whitney U-test was used for pairwise comparison. Statistical analyses were performed with SPSS ver.19 statistical software. The data sets when appropriate using a significance of ($p < 0.05$).

RESULTS

Histological examination

After fixation and routine tissue process, to evaluate the structure of tissues H&E and Masson's Trichrome were stained. Ostrich tendon tissue showed a type of dense regular connective tissue which less Vascularity and Tendon has a multi-unit hierarchical structure of collagen type I and III, fibrils, fiber bundles, fascicles and tendon units. Ostrich tendon has a crimped, waveform appearance. Ostrich tendon has stretched nuclei with dark strong appearance (figure 1).

The decellularization protocol resulted in completely cell-free, that was endorsement with DAPI staining (figure 2) connective tissue matrices that maintained. H&E and Masson's Trichrome stained light

microscopic sections of SDS-treated tendons demonstrated the absence of cell nuclei and tendon fibers, indicating that no intact cells remained within ECM (figure 3). Masson's Trichrome stain verified the preservation of collagen as compared with control, although staining showed a decreased density of integrity within the SDS-treated tendons compared with ostrich tendons as control. Control compared with tendon after the decellularization process, the consistency and density somewhat eased slightly in some areas are observed between the different disciplines of collagen. Compared to the level of mean difference ($P \leq 0.05$) showed a significant difference ($P = 0.004$).

In order to assess the quality of collagen grafts after SDS treated were stained picrosirius red (figure 4). First, after staining with picrosirius red, by polarizing microscope slides were examined nm540 Olympus-BX51-wavelength (red and disciplines collagen fibers of good quality with low quality will see a green color). result of assess the quality of collagen by staining picrosirius red were showed tendon scaffold mainly of type I collagen is composed of 95% natural structure was seen in red with polarizing light And only a small amount of green was about 5%. Decellularized by statistical comparison with

the control group, the mean difference in level decellularized ($P \leq 0.05$) showed no significant difference ($P = 0.58$).

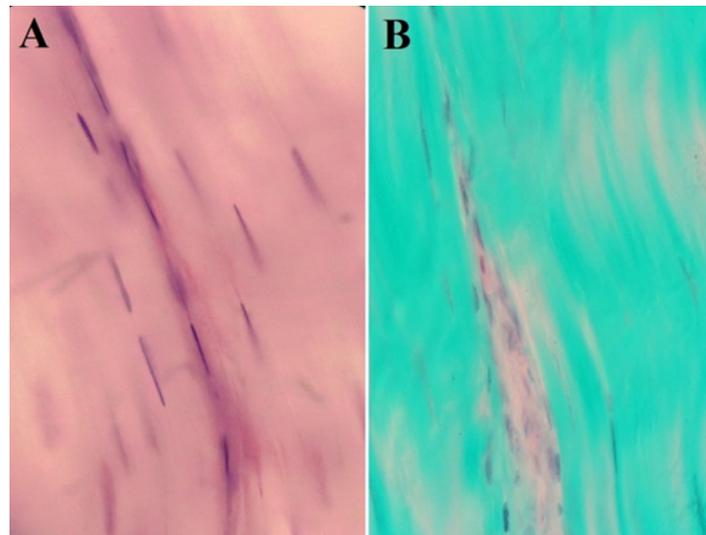


Figure 1: Ostrich tendon tissue structure (control), (A) density and arrangement of collagen fibers. Arrow indicates the tenocyte in endotenon, stained H&E. (B) represents the collagen fiber stained green with Masson's Trichrome, and Arrow indicated the muscle in vessel that stained pink, magnification (1000 X)

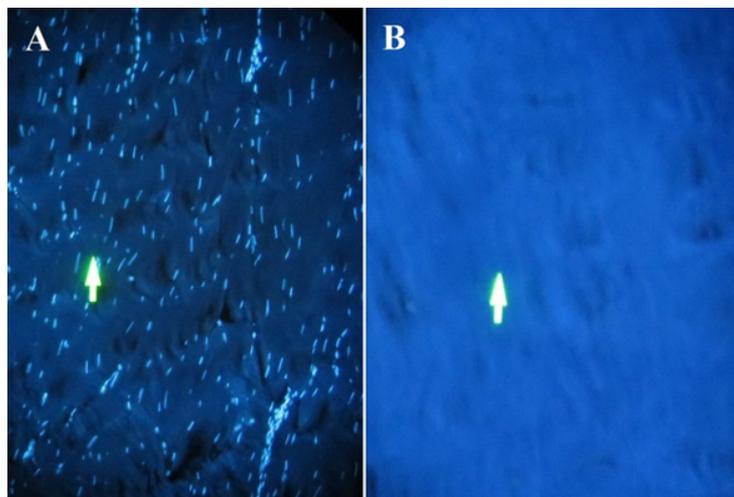


Figure 2: Ostrich tendon (control), (A) Ostrich tissue structure in DAPI staining showed that the DNA has been detected, the blue represents the cores drawn parallel with the longitudinal. Arrow refers to the core tenocyte the ostrich tendons, zoom (400X). (B) Tendon tissue structure in the decellularization shows DAPI staining was performed to detect DNA, which is seen as a blue dot indicating any nuclear debris is found. Arrow refers to the core scaffold without nuclei, magnification (400X).

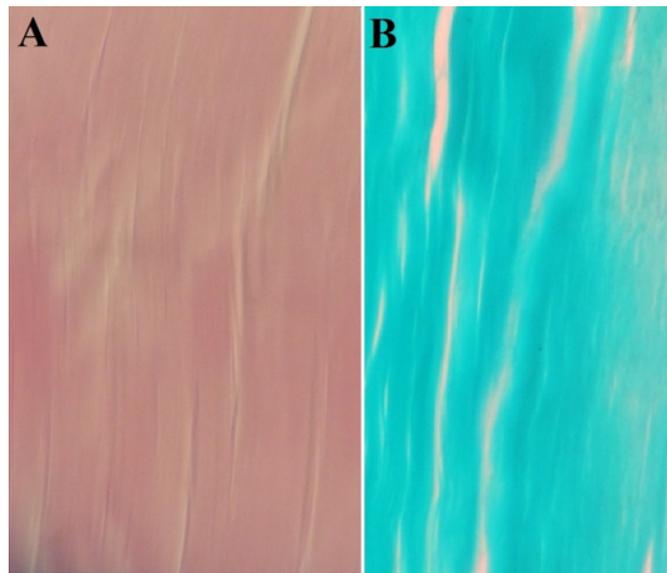


Figure 3: Decellularization tendon tissue structures, which reflects the density and porosity string and create distance between collagen fibers, refers. The arrow represents the distance created. Compared to the average level difference ($P \leq 0.05$) significant difference compared with the control group, H & E and Masson's Trichrome staining, magnification (400X).

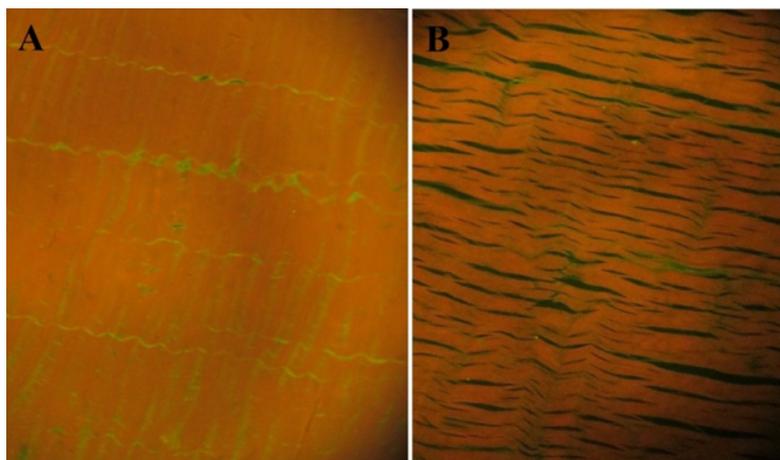


Figure 4: Tissue structure quality Assessment with Picrosirius red. (A) Ostrich quality tissue structure (control) showed, collagen has a good quality. (B) Quality tendon tissue decellularization shows the statistical comparison with the control group, the mean difference in level ($P \leq 0.05$) no significant difference (red good quality and green color is collagen with less quality), Arrow indicates low-quality collagen in tendon ostrich, magnification (400X).

DISCUSSION

Tendons are soft connective tissues, which connect muscle to bone forming a musculotendinous unit, whose primary function is to transmit tensile loads generated by muscles to move and enhance joints stability. Ostrich tendon has a Crimped,

waveform appearance that plays an important role in its mechanical properties[13]. The angle and length of the “crimp pattern” depends on the type of tendon, its anatomical site within the body and its location within the tendon tissue. The differences in the “crimp pattern” affect tendon's mechanical

properties, and fibers which have a small crimp angle are mechanically weaker than those with a larger crimp angle [14]. The biomechanical properties of tendons are mainly attributed to the high degree of organization of the tendon extracellular matrix, primarily composed of collagen type I, arranged in triple-helical molecules bundles that have different dimensions and which are aligned in a parallel manner in a proteoglycan matrix[3]. The scaffold should encourage cellular recruitment and tissue ingrowth. Early in the repair process, the scaffold should maintain its mechanical and architectural properties to protect cells and the new, growing tissue from strong forces and early inflammatory events[15]. Tendon extracellular matrix are mainly composed of type I collagen, so scaffolds based on collagen derivatives are highly biocompatible, then collagen derivatives also exhibit superior bio-functionality: they better support cell adhesion and cell proliferation. In order to be utilized successfully as a biomaterial, native extracellular matrix must first be decellularized to remove any allogenic cells and to prevent adverse immunological reactions. Native scaffolds are bioactive and promotes cellular proliferation and tissue ingrowth[16]. Treatment with 1% SDS for 24 h resulted in

an acellular ostrich tendon matrix with retention of near normal structure, cell removal using SDS suggested this treatment is potentially useful for removing cells from tendon allografts or xenografts without compromising the graft structure or mechanical properties[7, 12, 17, 18]. One of the most important factors in cell proliferation and migration of pores in the scaffold are interdisciplinary. Expressed porosity and microscopic distances within the scaffold attachment factor, cell proliferation and migration[19]. According to the scaffold or matrix between cells in normal tendon is very compact and tight spaces created between tendon fibers, scaffolds after decellularization process, receive and impart the required elements and waste in the process of cell proliferation and differentiation cell compared to normal tendon will be easier and better. In order to function as a living tissue, it is essential that the acellular scaffold is recellularized either in vivo or in vitro prior to implantation, so that remodeling of the scaffold to maintain the correct ultrastructural and physical properties can occur. Recellularization in vitro allows for further conditioning of the graft prior to implantation, and hopefully a more successful outcome[20].

CONCLUSION

The potential benefits provided by biologic scaffold materials for replacement and reconstruction of damaged or missing tissues and organs are noteworthy. Histological analysis for evaluate tissue integrity and decellularization showed engineered ostrich tendon method described herein, the effects of this method upon matrix structure/composition, and the subsequent host response are intended as guidelines in the design and manufacture of effective ECM based bioscaffolds.

ACKNOWLEDGEMENT

This research was funded by Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

REFERENCES

- [1] T.M. Chen, Rozen W.M., Pan W.r., Ashton M.W., Richardson M.D., and Taylor G.I., "The arterial anatomy of the Achilles tendon: anatomical study and clinical implications," *Clinical Anatomy*. 22(3): p. 377-385. 2009.
- [2] S.F. Badylak, Freytes D.O., and Gilbert T.W., "Extracellular matrix as a biological scaffold material: structure and function," *Acta biomaterialia*. 5(1): p. 1-13. 2009.
- [3] S.F. Badylak and Valentin J.E., "Extracellular Matrix Bioscaffolds for Orthopaedic Applications," *The Journal of Bone & Joint Surgery*. 92(5): p. 1317-1318. 2010.
- [4] M. Kongsgaard, Aagaard P., Kjaer M., and Magnusson S., "Structural Achilles tendon properties in athletes subjected to different exercise modes and in Achilles tendon rupture patients," *Journal of Applied Physiology*. 99(5): p. 1965-1971. 2005.
- [5] J.T. Shearn, Kinneberg K.R., Dymant N.A., Galloway M.T., Kenter K., Wylie C., and Butler D.L., "Tendon tissue engineering: progress, challenges, and translation to the clinic," *J Musculoskelet Neuronal Interact*. 11(2): p. 163-73. 2011.
- [6] J.M. Wisbeck, Parks B.G., and Schon L.C., "Xenograft scaffold full-wrap reinforcement of Krackow achilles tendon repair," *Orthopedics*. 35(3): p. e331-4. 2012.
- [7] T.W. Gilbert, Sellaro T.L., and Badylak S.F., "Decellularization of tissues and organs," *Biomaterials*. 27(19): p. 3675-3683. 2006.
- [8] N.U. Schaller, Herkner B., Villa R., and Aerts P., "The intertarsal joint of the ostrich (*Struthio camelus*): Anatomical examination and function of passive structures in locomotion," *J Anat*. 214(6): p. 830-47. 2009.
- [9] J. Rubenson, Lloyd D.G., Besier T.F., Heliam D.B., and Fournier P.A., "Running

in ostriches (*Struthio camelus*): three-dimensional joint axes alignment and joint kinematics," *J Exp Biol.* 210(Pt 14): p. 2548-62. 2007.

[10] A. Azhim, Ono T., Fukui Y., Morimoto Y., Furukawa K., and Ushida T. Preparation of decellularized meniscal scaffolds using sonication treatment for tissue engineering. in *Engineering in Medicine and Biology Society (EMBC), 2013 35th Annual International Conference of the IEEE.* 2013.

[11] A. Ofenbauer, Sebinger D.D.R., Prewitz M., Gruber P., and Werner C., "Dewaxed ECM: A simple method for analyzing cell behaviour on decellularized extracellular matrices," *Journal of Tissue Engineering and Regenerative Medicine.* 9(9): p. 1046-1055. 2015.

[12] T.J. Keane, Swinehart I.T., and Badylak S.F., "Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance," *Methods.* 84: p. 25-34. 2015.

[13] F. Bassetto, Volpin A., and Vindigni V., "Regenerative Medicine for Tendon Regeneration and Repair: The Role of Bioscaffolds and Mechanical Loading," *Biomaterials Science and Engineering.*

[14] J. Wilmink, Wilson A., and Goodship A., "Functional significance of the

morphology and micromechanics of collagen fibres in relation to partial rupture of the superficial digital flexor tendon in racehorses," *Research in veterinary science.* 53(3): p. 354-359. 1992.

[15] U. Cheema, "Standardisation in Cell and Tissue Engineering: Methods and Protocols." 2013.

[16] B.C. Pridgen, Woon C.Y., Kim M., Thorfinn J., Lindsey D., Pham H., and Chang J., "Flexor tendon tissue engineering: acellularization of human flexor tendons with preservation of biomechanical properties and biocompatibility," *Tissue Engineering Part C: Methods.* 17(8): p. 819-828. 2011.

[17] G. Schulze-Tanzil, Al-Sadi O., Ertel W., and Lohan A., "Decellularized Tendon Extracellular Matrix—A Valuable Approach for Tendon Reconstruction?," *Cells.* 1(4): p. 1010-1028. 2012.

[18] A.D. Bhrany, Beckstead B.L., Lang T.C., Farwell D.G., Giachelli C.M., and Ratner B.D., "Development of an esophagus acellular matrix tissue scaffold," *Tissue engineering.* 12(2): p. 319-330. 2006.

[19] D.W. Huttmacher, "Scaffolds in tissue engineering bone and cartilage," *Biomaterials.* 21(24): p. 2529-2543. 2000.

[20] T. Martinello, Bronzini I., Volpin A., Vindigni V., Maccatrozzo L., Caporale G., Bassetto F., and Patruno M., "Successful

recellularization of human tendon scaffolds using adipose-derived mesenchymal stem cells and collagen gel," *Journal of tissue engineering and regenerative medicine*. 2012.