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Preparation and Functional Characterization of Decellularized Bovine Tendon Scaffolds for Tendon Tissue Engineering

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ABSTRACT

The study was conducted to develop a naturally derived tendon tissue engineered scaffold with the preservation of the native ultra-structure, tensile strength and biochemical composition of the tendon extracellular matrix. Decellularization was achieved by using two different protocols viz. repeated freeze and thaw technique and 2% SDS. In vitro evaluation of the graft was done by H&E staining, Masson-Trichome staining, Alcian blue staining, DNA quantification analysis and Scanning Electron Microscopy (SEM). The in vitro evaluation suggested that SDS was better than freeze and thaw technique in terms of effective decellularization. The cell removal was better in SDS group than freeze and thaw as depicted by H&E staining and DNA quantification. Also the structure and alignment of collagen fibers were preserved in SDS group. The intrinsic ultra structure of tendon tissue was well preserved based on scanning electron microscopy examination.

Keywords: Decellularization, proteoglycans, scaffold, tendon, bovine

Tendon tissue is a type of connective tissue which physically binds muscles to skeletal structures permitting locomotion and enhancing joint stability (Wang, 2006). Tendon has a multi-unit hierarchical structure of collagen molecules, fibrils, fibre bundles, fascicles and tendon units designed to resist tensile loads (Hoffmann and Gross, 2007). Tendon injuries are difficult to manage due to impaired healing and frequently result in long-term pain and discomfort, which places a chronic burden on health care systems (Sharma and Maffulli, 2006). Tissue engineered tendon may be used as a graft material in extensive tendon injuries. Since tendon reconstruction requires highly stable scaffolds, the implantation of a natural extracellular matrix (ECM) might provide a promising therapeutic strategy. ECM from tendons has been studied for tissue engineering and regenerative medicine applications (Cartmell and Dunn, 2000). Advantages of a natural ECM as a scaffold are its matching biomechanical properties as well as suitable biochemical and structural composition to guide cell growth. In addition, there are growth factors entrapped within the natural ECM, which provide adequate tenogenic stimuli accelerating cell differentiation (Ker *et al.* 2011). Hence, preservation of

the native ultra structure and biochemical composition of tendon ECM during the process of tissue decellularization is highly desirable. Typical growth factors supporting tenogenic differentiation are the growth and differentiation factors, (GDF) 5, 6 and 7 (Wolfman *et al.* 1997), fibroblast growth factor-2, FGF-2 (Jung *et al.* 2009) and the bone morphogenetic protein, BMP 2 (Hoffmann *et al.* 2006). It is well known that tendon is a dense connective tissue so it is difficult to remove the cell components. The decellularization protocol is used to efficiently remove all cellular and nuclear materials while minimizing any adverse effect on the composition, biological activity and mechanical integrity of the remaining ECM. Several decellularization strategies, suitable for removing antigenic cellular components, have been developed. Most of them are based on repeated freezing, vigorous mechanical agitation and the use of detergents, chelating agents, zwitterionic detergents, alkali or acid treatment and various enzymatic treatment such as trypsin and nucleases, alone or in combination (Ingram *et al.* 2007; Deeken *et al.* 2011; Gilbert, 2012) and rapid freeze and thaw (Sass *et al.* 2009). Sodium dodecyl sulphate (SDS), an ionic detergent, destroys the cell membrane and denatures proteins (Seddon *et al.* 2004*).* It was found that 2% SDS removed a mean 83% of DNA from the tendon scaffolds and 1% SDS resulted in a 76% reduction (Youngstrom *et al.* 2013)

MATERIAL AND METHODS

The Achilles tendon from buffalo aged about 1.5- 2 yr was harvested randomly and aseptically from a slaughter house within 3 hours of slaughter. The Achilles tendon was exposed by opening the skin and the tendinous portion between the insertion to bone and the muscle-tendon junction was harvested. The harvested tendon was frozen at - 80°C until processing.

The tendon explants were cut in uniform length and thickness and were randomly assigned into three different treatment groups, each consisting of 10 explants. The one group explants were immersed only in PBS for 24 hr and kept as control. Other group explants were exposed to 5 freeze-thaw cycles. The tendon slices were put into a metal beaker and placed in liquid nitrogen for 2 min. After each freezing cycle, explants were thawed at 56°C for 10 min. Following three washings with PBS with 30 min each, the tendon slices were incubated in RNase 100µg/mL and DNase 150 IU/mL at 37°C. Then tendons were rinsed for 30 min in PBS at room temperature. The third group explants were immersed in 250 mL of decellularization solution containing 2% SDS w/v (Sigma,USA). These explants immersed in 2% SDS solution were kept on horizontal shaker at 37°C to allow adequate perfusion of the tendon matrices. Tendon samples were rinsed in PBS six times to remove residual detergent before incubation with 0.05% trypsin-EDTA (Gibco, USA) for 10 min and washed with water. Additional treatment steps included incubation in DNase for 30 min and 95% ethanol for 2 hr at 4°C, separated with and followed by three 10 min washes in water.

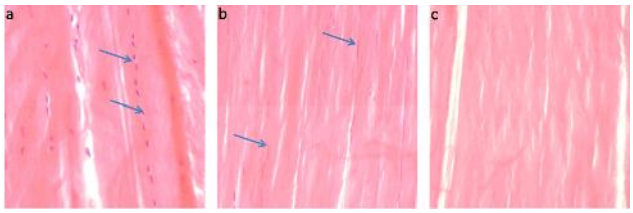

Fig. 1. Phoromicrograph showing: (a) Native tendon; nuclei (arrows) exhibiting a characteristic elongated morphology, (b) F&T treated; nuclear remains (arrows) of resident were evident, (c) SDS trated; the cellular stuctrures had disappeared, leving empty spences between the collagen fiberrs with vcery little remaining cell debries. (H&E stain; 20x)

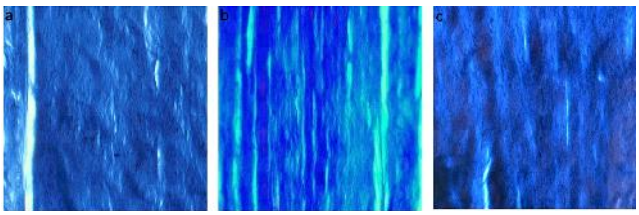

Fig. 2. Phoromicrograph showing: (a) Native tendon; the crimpiness and tightly arranged collagen fibers, (b) F&T treated; collagen fibers were loosely arranged and crimpiness was less as compared to control, (c)SDS treated; collagen fibers are compactly arranged like control group. (Masson Trichome staining; 20x).

Histology

For histology, the samples were fixed in 4% formaldehyde for 24-48 hr at room temperature and embedded in paraffin, and then longitudinally cut into 5µ thick sections. The sections were mounted on slides and stained with Haematoxylin and Eosin (H&E) stain as per the standard procedure (Luna, 1968). H&E was used to examine the overall histological structure of tendon tissue (Torres *et al.* 2000; Awad *et al.* 2003), the organisation of tissue fibers (Carpenter and Hankenson, 2004) and the presence of the cells in the construct. Special staining for collagen was done by using Masson's trichrome stain (Masson, 1929). Distribution of proteoglycans within the sample was visualized through the use of Alcian blue stain (Alam, 2011)

DNA quantification assays

A quantification analysis was done to evaluate the efficacy of removal of all cellular contents from tendon slices. All tendon slices were washed with distilled water for 5 min. DNA extraction was done by conventional phenol/chloroform DNA extraction procedure. After the extraction of DNA, the DNA was measured using Nanodrop (Thermo scientific, USA).

Scanning Electron Microscopy (SEM)

The samples were washed in PBS, fixed in 2.5% glutaraldehyde in PBS overnight at 4°C. The samples were washed three times with 0.1M PBS for 15 min each and then dehydrated in graded series of ethanol (in PBS). The tissues were first incubated in 30, 50, 70, 90 and 100% ethanol for 15min each. The tissue samples were then dried in a Critical Point Dryer using CO_2 as the transitional fluid and mounted on scanning electron microscope specimen holders. The specimens were sectioned longitudinally and transversely and then coated by hexa-methyl-di-silazane (HMDS) to improve the quality of SEM images. The samples were vacuumed and gold-coated on a Cu mount and were then examined by a scanning electron microscope (Jeol, ISM 840 model) (Meimandi-Parizi *et al.* 2013). Different magnifications from 50 to 15,000 were used to analyze the morphological and morphometrical characteristics of the tissue samples.

Statistical analysis

All values were recorded as mean ± standard deviation (SD). A one way analysis of variance (ANOVA) with a Tukey's *post hoc* test was performed to determine statistical difference between the different groups in DNA quantification.

RESULTS

The decellularization protocol adopted using non-ionic detergent and rapid freeze and thaw technique led to efficient reduction of all cellular and nuclear material with no adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM. The gross appearances of decellularized tendon were almost unchanged except in freeze and thaw group, where it was observed to be whiter. It was grossly appreciated that the collagen fibres have loosened in arrangement.

Histology

H&E staining demonstrated successful decellularization of the processed sample (fig 1). Histology of native tendon histology showed tenocyte nuclei exhibiting a characteristic elongated morphology, in parallel alignment with collagen fibrils in lacunae. Tenocytes were arranged in parallel rows along the longitudinal axis of tendons, with gap junctions linking flattened cytoplasmic processes that extended through the extracellular matrix between cells (Fig. 1a). Nuclear remains of resident tenocytes were evident, as blue staining in the H&E sections due to membrane disruption from repetitive freeze/thaw cycles (Fig. 1b). A marked reduction in number of cells and cell debris as reflected in fig. 1c of 2% SDS treatment was evident in H&E staining. The cell nuclei showed a marked reduction. The empty spaces between the collagen fibres were also evident.

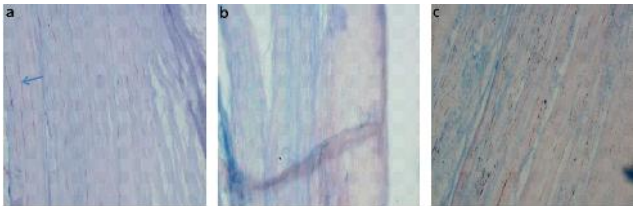

Fig. 3. Photormicrograph Alcian blue staining: (a) native tendon; bluish matrix of proteoglycans between the compactly arranged regular arrays (arrow) and collagen fivrils, (b) F&T treated; the matrix colour faded put to decrease in protogelycans with less rose color tenocytes, (c) treated; less proteoglycans compared to control (Alcian blue; 20x)

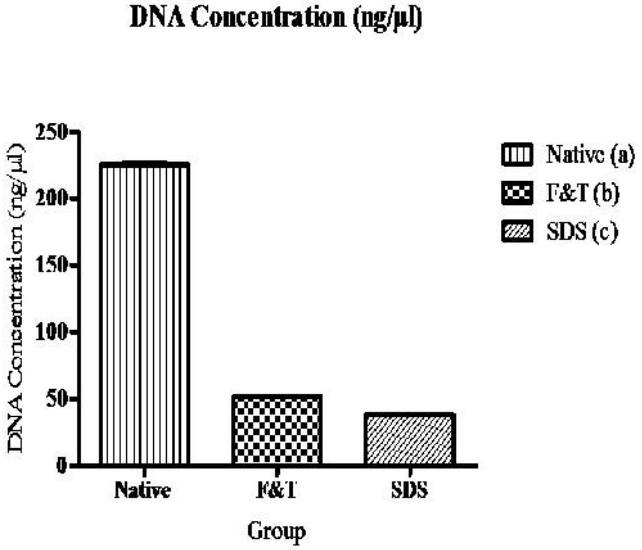

Fig. 4. Mean ± SD values of DNA content (ng/µl) after decellularization.

Masson trichome staining revealed the crimpiness of the native collagen (Fig. 2a). Staining revealed that the cellular structures had disappeared, leaving empty

spaces between the collagen fibers with very little remaining cell debris in freeze and thaw group. Collagen fibers were loosely arranged (Fig. 2b). The characteristic deep blue colouration of collagen of SDS group after staining with Masson's trichrome was apparent, consistent with maintenance of collagen content (Fig. 2c).

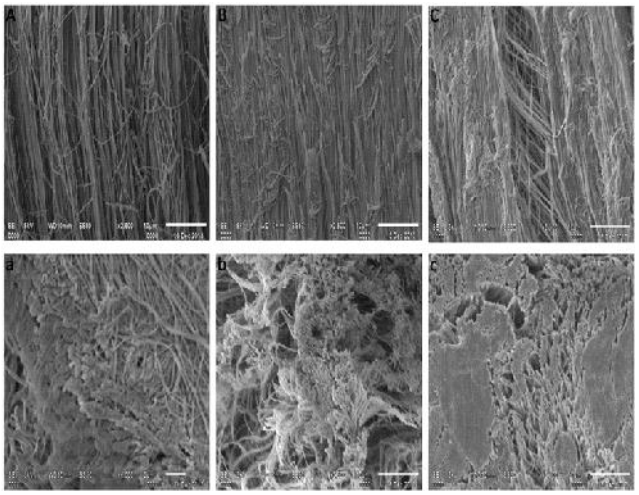

Fig. 5. Longitudinal and cross sectional scanning electron microscopy images of tendon showing: A, a Native tendon; the fibers appeared more organized, and wavy B,b F&T treated; the collagen fibers with very little residual cell debris, C,c SDS treated; the compacat arrangemt pf collagen fibers was pressved and dhowed structural resemblance to the native tendon. A, B, C- Longitudinal Section ; a, b, c – Cross section

Alcian blue staining exhibited intense GAG staining of native tendon (Fig. 3a). Alcian blue staining of freeze and thaw treated group showed blue stained proteoglycans with rose coloured nuclear remnants between them (Fig. 3b). SDS treatment maintained collagen content and resulted in minimal GAG loss and maximal DNA removal (Fig. 3c). The scaffolds decellularized with 2% SDS maintained the collagen and GAG content, comparable to that of native tendon.

DNA Quantification assay

To evaluate the efficacy of decellularization, DNA quantification was done in all three groups. It was found that native tendon retained significantly highest ($P<0.001$) amount of DNA (225.3±2.811 ng/µL) as compared to all the groups (Fig. 4). Comparison between the two test groups revealed significantly ($P<0.01$) lower values of DNA concentration in SDS (38.6375±0.089 ng/µL) as compared to F&T treatment (52.3125±0.950 ng/µL).

Scanning Electron Microscopy (SEM)

The scanning electron microscopy pictures of the native tendon and processed tendon samples are presented in fig. 5. SEM was used to examine the architecture of the collagen fibril meshwork within the tendon after decellularization and compared with fresh native tendon. The fibers appeared more crimped and wavy (Figs. 5A & a). Tendon treated with rapid freeze and thaw technique showed loosening of collagen fibers. The cellular structures disappeared, leaving empty spaces between the collagen fibers with very scanty residual cell debris (Figs. 5B & b). The integrity and density of collagen fibres in SDS treated group was maintained. The matrix morphology was preserved (Figs. 5C & c). Collagen fibers were compactly arranged. SDS showed structural resemblance to the native tendon.

DISCUSSION

The current study combined and modified a series of existing decellularization methods for the decellularization of bovine tendon, with the aim of producing a suitable scaffold. The decellularized constructs were then evaluated on basis of the DNA content, collagen network, GAG content and gross structure. The aim was to test different decellularization protocols and compare their ability to decellularized bovine Achilles tendon while maintaining collagen ultra-structure and minimizing loss of GAG content. The hypothesis was that a treatment protocol using SDS would remove the majority of cellular debris without reducing collagen content or compromising structural organization of the decellularized tendon scaffold, preserving scaffold topography and mechanical properties. The findings demonstrated that effective decellularization of the bovine tendon preserved the biological properties. The preservation of the gross architecture, collagen meshwork and GAG content with the visco-elastic behaviour of the construct indicated that decellularization methods were able to bring about ideal qualities of scaffolds. Both the protocols of decellularization have led to decrease in number of cells, which was confirmed on H&E staining, Masson's trichrome staining and DNA quantification. Similar methods have been adopted by earlier researchers to examine the efficacy of decellularization procedures (Youngstrom *et al.* 2013; Burk *et al.* 2014). Removal of cells was more prominent in SDS than freeze and thaw treatment. Cartmell and Dunn (2004) published a study on decellularization of patellar tendon grafts with SDS and found 70%–90% reduction of intrinsic cells and similar biomechanics despite morphological changes in

the tissue. Use of freeze-thaw cycles enhances decellularization effectiveness in large tendons, while not being effective alone, especially in terms of DNA removal (Ning *et al.* 2012). The retention of collagen as expressed by Masson's trichrome stain was preserved in both treatment groups. Freeze and thaw technique showed comparatively a lighter staining for Masson's trichrome. GAG was preserved in both groups. The 2% SDS treatment maintained collagen content and resulted in minimal GAG loss and maximal DNA removal. The proteoglycans are more or less preserved in this study also. Untreated tendon underwent structural comparison with scaffolds decellularized with 2% SDS. The characteristic deep blue colouration of collagen after staining with Masson's trichrome was apparent even in decellularized tendon, consistent with maintenance of collagen content. Low concentrations of TnBP and SDS have shown utility in preliminary decellularization study of rattail tendon (Cartmell and Dunn, 2000). SDS has effectively removed cellular debris from connective tissues when other methods have failed (Elder *et al.* 2009) and has been used successfully in whole-organ (Ott *et al.* 2008; Uygun *et al.* 2010) and even multi organ (Park and Woo, 2012) decellularization.

The SEM results of SDS group showed that the arrangement of collagen fibers was more closely packed and organized. SEM images did not demonstrate disruption of collagen alignment or fiber thickness at the micrometer level; though a slight reduction in fibril density was evident in the SDS-treated group that may prove beneficial for seeding scaffolds with cells (Youngstrom *et al.* 2013). The present study showed that tendon treated with repeat freeze and thaw showed some disruption of collagen fibers than native tendon. This is in conjunction with the findings of Chen *et al.* (2011) histological observation showed that the following changes happened as the number of freezing-thawing increased: the arrangement of tendon bundles and collagen fibrils became disordered until ruptured, cells disrupted and apparent gaps appeared between tendon bundle because the formation of ice crystals.

To conclude, bovine acellular tendon scaffolds were successfully generated by using both freeze and thaw technique and 2% SDS treatment, and the *in vitro* evaluation suggested that SDS was better than freeze and thaw technique in terms of effective decellularization. The cell removal was better in SDS group than freeze and thaw as depicted by H&E staining and DNA quantification. The structure and alignment of collagen fibers was also preserved in SDS group.

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