Evaluation of decellularized extracellular matrix of skeletal muscle for tissue engineering

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Objective: We evaluated the effectiveness of enzyme-detergent methods on cell removal of mouse skeletal muscle tissue and assessed the biocompatibility of the decellularized tissues by an animal model.

Methods: The mouse latissimus dorsi (LD) muscles underwent decellularization with different enzyme-detergent mixtures (trypsin-Triton X-100, trypsin-sodium dodecyl sulfate (SDS), trypsin-Triton X-100-SDS). The effectiveness of decellularization was assessed by histology and DNA assay. The content in collagen and glycosaminoglycan was measured. The biomechanical property was evaluated in uniaxial tensile tests. For biocompatibility, the decellularized muscle specimens were implanted in situ and the tissue samples were retrieved at day 10, 20, and 30, to evaluate the host-graft inflammatory reaction.

Results: Extensive washing of the mouse LD muscles with an enzyme-detergent mixture (trypsin and Triton X-100) can yield an intact matrix devoid of cells, depleted of more than 93% nuclear component and exhibiting comparable biomechanical properties with native tissue. In addition, we observed increased infiltration of inflammatory cells into the scaffold initially, and the presence of M1 (CD68)-phenotype mononuclear cells 10 days after implantation, which decreased gradually until day 30.

Conclusions: The enzyme-detergent method can serve as an effective method for cell removal of mouse skeletal muscle. In short-term follow-up, the implanted scaffolds revealed mild inflammation with fibrotic tissue formation. The decellularized extracellular matrix developed herein is shown to be feasible for further long-term study for detailed information about muscle regeneration, innervation, and angiogenesis in vivo.

Keywords: Decellularization, Extracellular matrix, Biocompatibility

INTRODUCTION

Autologous muscle flaps, used to provide coverage of vital structures, play an important role in soft tissue reconstruction. However, the disadvantages of this treatment method include donor site availability and morbidity (1, 2). This reconstruction method cannot be performed for large defects in patients with massive soft tissue loss due to trauma, congenital deficiency, or tumor ablation. Tissue-engineered skeletal muscle has the potential to reduce donor-site morbidity and supply unlimited tissue for reconstructive surgery (3).
A major approach in tissue engineering of skeletal muscle involves the use of engineered matrices, with or without cell seeding, to support the growth and differentiation of myogenic cells (1-3). The engineered scaffolds are often produced from synthetic or natural materials, such as alginate (4), collagen (5, 6), and hyaluronan (7). The complex composition and microstructure of the extracellular matrix (ECM) of muscle cells makes it difficult to closely mimic the native cellular environment with an engineered matrix (8, 9).

The use of a decellularized ECM as a scaffold for the development of a tissue-engineered skeletal muscle has had some preliminary promising results and has gained much attention as an alternative to engineered scaffolds (1, 3). The concept of using a decellularized ECM as a scaffold in tissue engineering arises from the fact that tissue cells adapt to their surrounding microenvironment, and a better tissue response may be expected if a nearly “native” microenvironment is provided (1, 8-10). With its superior biocompatibility and tissue-guiding ability, decellularized ECM has been widely applied in the engineering of tissue substitutes for organs such as the bladder, urethra, trachea, heart valve, skin, and small intestine (10-15).

Liu et al used an acellular muscle graft, prepared using chemical methods, to repair an extended nerve defect in a rat model (16). Gamba et al used an acellular diaphragm matrix to repair an external oblique muscle defect of the abdominal wall in a rabbit model. However, they did not observe positive electromyography findings or myogenesis after 9, 40, and 90 days (2). Borschel et al cultured C2C12 myoblasts on a mouse extensor digitorum longus muscle acellular matrix in differential medium for three weeks, and observed cell-matrix integration with myofibril differentiation. Isometric force measurement showed a length-tension relationship (17, 18). Conconi’s group seeded cultured myoblasts on homologous acellular matrix. These myoblast-seeded patches were then transplanted to repair oblique abdominis muscles. The repaired tissue showed well-preserved muscle structure, abundant blood vessels and myoblasts, and single motor-unit potentials for approximately nine days. This cell-seeded matrix was also used to repair a full-thickness defect of the abdominal wall in female Lewis rats. The implants integrated with the host tissue and maintained their original dimensions for nine months (3, 19). Turner et al reported that a small intestinal submucosa (SIS) ECM scaffold implanted for six months in dogs at the site of gastrocnemius musculotendinous injury resulted in scaffold-localized tissue formation that exhibited the ability to contract in response to direct in vitro stimulation (20).

Similar results were obtained with SIS implantation in a rodent with volumetric muscle injury of the abdominal wall in situ (21). Merritt et al reported that implantation of a decellularized muscle matrix alone did not facilitate functional recovery for up to 42 days post injury. However, injecting mesenchymal stem cells into the scaffold at seven days after the creation of the surgical defect resulted in 85% recovery of contralateral muscle force at 42 days (22). Moreover, Machingal et al indicated approximately 72% of tetanic force recovery at two months of follow-up in a rat latissimus dorsi (LD) muscle defect model after implantation of a porcine bladder acellular matrix-rat myoblast construct cultured in a bioreactor for 1 week (23). Daly et al developed a rabbit compartment syndrome model, and following implantation of porcine small intestine submucosa acellular matrix in situ, they noted gradual myogenesis and muscle regeneration after one to three months in rabbits that underwent fasciotomy (24).

Although the implantation of decellularized skeletal muscle is a promising method for muscle tissue reconstruction, the effectiveness of these implants varies (2, 19-22). The different methods applied for achieving decellularization are believed to be the main cause for this inconsistency. The decellularization process usually involves a series of different physical, chemical, and enzymatic treatments (25). However, these treatments may, to some extent, damage the ECM and alter its biochemical, mechanical, and structural properties.

Therefore, optimized decellularization protocols need to be developed according to specific requirements. Qing et al studied the optimized decellularization protocol using a rat skeletal muscle and suggested that oscillatory treatment of rat skeletal muscle at 4°C with 1% SDS for 72 h can yield acellular matrices with an intact ECM, complete removal of muscle fibers (26). Gillies et al demonstrated that using latrunculin B, hypertonic saline solution, and DNase I, can effectively decellularize the mouse tibialis anterior muscle without altering its ECM composition or mechanical properties (27).

In the present study, we investigated the effectiveness of enzyme-detergent methods for mouse skeletal muscle decellularization and the degree of decellularization was evaluated by histological and DNA assay. The scaffold characteristics were evaluated by scanning electric mi-
Decellularized skeletal muscle ECM
croscopy, biochemical assay, and biomechanical test. In addition, decellularized muscle matrix was implanted in situ in a mouse model and observed 10, 20, and 30 days after implantation to evaluate the inflammatory response and its biocompatibility.

MATERIAL AND METHODS

**Harvesting and decellularization of mouse latissimus dorsi muscle**

Male ICR mice (Institute cancer research, Bltw:CD1) aged 8 to 12 weeks were purchased from the Charles River laboratory (BioLASCO Taiwan, Taipei, Taiwan) for skeletal muscle decellularization. The mice were handled according to the guidelines of the Institutional Animal Care and Use Committee. Donor mice were anesthetized with Zoletil 50 (50 mg/kg, Virbac, Taipei, Taiwan), followed by skeletal muscle removal. The decellularization treatments were divided into four groups as shown in Table I. Agitation and rolling were performed during solution incubation to improve mixing of the enzyme or detergent. All specimens were then preserved in PBS at -20°C before examination and implantation.

**Histology**

Muscle tissue was embedded in paraffin after being fixed in 10% buffered neutral formalin. Embedded tissue was sectioned at a thickness of 5 μm along the parallel or perpendicular direction of the fibers. Tissue staining with H&E and the modified VerHoff Van Gieson stain (EMS, Hatfield, PA, USA) was used to identify regions of collagen and elastin containing ECM, muscle fibers, and other cytoplasmic cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) to assess the extent of cell removal.

**DNA quantification**

The DNA from either native or decellularized tissue samples of approximately 20-30 mg was isolated, purified, and rehydrated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the recommended protocol. Each assay was performed in triplicate.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>B1</td>
<td>0.1% trypsin/0.01% EDTA 24 h&lt;br&gt;0.1% aprotinin/PBS wash 30 min with sonication&lt;br&gt;1% Triton X-100 1 week*&lt;br&gt;0.1% aprotinin/PBS wash 30 min with sonication&lt;br&gt;DNAase (50 U/ml)/RNAase (1 U/ml) in hypotonic buffer 3 h&lt;br&gt;PBS wash with sonication 30 min x 3 times</td>
</tr>
<tr>
<td>B2</td>
<td>The same as B1 except 1% Triton X-100 1 week replaced by 2 weeks*</td>
</tr>
<tr>
<td>C</td>
<td>The same as B1 except 1% Triton X-100 replaced by 0.1% SDS*</td>
</tr>
<tr>
<td>D</td>
<td>0.1% trypsin/0.01% EDTA 24 h&lt;br&gt;0.1% SDS 1 week*&lt;br&gt;0.1% aprotinin/PBS wash 30 min with sonication&lt;br&gt;1% Triton X-100 1 week*&lt;br&gt;0.1% aprotinin/PBS wash 30 min with sonication&lt;br&gt;DNAase (50 U/ml)/RNAase (1 U/ml) in hypotonic buffer 3 h&lt;br&gt;PBS wash with sonication 30 min x 3 times</td>
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* Triton X-100 and SDS solution were changed every 2 days.

**Collagen assay**

Total collagen content was determined using the Sircol Collagen Assay kit (Biocolor, Carrickfergus, UK). Briefly, about 20 mg of dry tissue sample were digested with 1 mL pepsin (0.1 mg/1 ml Pepsin, 0.5 M Acetic acid, pH 2) and placed at 4°C overnight. Digested samples were then assayed following the manufacturer’s protocol. Each assay was performed in triplicate.

**Glycosaminoglycan assay**

Glycosaminoglycan (GAG) concentrations were measured using the Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor Ltd). ECM, 25-30 mg dry weight, was digested using 0.1 mg/ml proteinase K with 10 mM Tris (pH 8.0, 50 mM NaCl, and 1 mM EDTA) for 24 h at 50°C. Digested samples were then assayed following the manufacturer’s protocol. Each assay was performed in triplicate.
Scanning electron microscopy analysis

For groups A, B1, B2, muscle tissue was fixed in 4% paraformaldehyde for 45 min at room temperature and subsequently dehydrated using a graded ethanol series. Samples were then snap-frozen in liquid nitrogen and broken into three slices (2 mm thickness), transverse to the direction of fibers, and lyophilized. Lyophilized muscle slices were sputter-coated with gold using an Emitech K575 Sputter Coater (EM Technologies, Kent, UK) and examined using a Philips XL30 Scanning Electron Microscope (Philips-FEI, Eindhoven, The Netherlands).

Biomechanical test

Uniaxial tensile test was performed to determine the stress-strain response of the native and decellularized muscle. Control, B1, and B2 group samples were cut by surgical scissors along its line of axis (longitudinal direction) into 20 × 2 mm with a gauge length of 5 mm. The thickness of the test sample was recorded. The samples, set to their natural (no-load) length, were mounted on custom-made jaw holder on the displacement controlled tensile testing machine with a 500 lb load cell (self-assembly mini-MTS, 0.1 lb resolution), while submerged in a plexiglass cylinder that was filled with saline solution at room temperature. Pre-conditioning was performed to a maximum force of 10% of the break strength over five cycles and the sections were then tested to failure at a strain rate of 0.5 mm/min. The tangent modulus and failure strength/strain were recorded.

Implantation

Bilateral LD muscle defects of approximately 2 × 1 cm² in size were created in six mice under anesthesia. We then harvested another 12 pieces of mouse LD muscle for decellularization. Decellularized ECM specimens of LD muscle for groups B1 and B2, were then studied. Six specimens were bilaterally implanted in situ in three mice in each group. The matrix patch was placed at the defect site, fixed, and immobilized with interrupted 6-0 silk sutures at the insertion of the muscle. The back wound was irrigated with normal saline and approximated with 3-0 nylon. The mice were sacrificed and muscle samples were retrieved on days 10, 20, and 30 after surgery. The sample sections were examined for the inflammatory reaction at the matrix-native muscle interface and the inflammatory cell profile by H&E and immunohistochemical staining.

Statistical analysis

All results were evaluated using a two-tailed Student's t-test (SPSS15.0, AsianAnalytics, Taipei, Taiwan) and are presented as mean ± S.E. A p<0.05 was regarded as statistically significant.

RESULTS

Histological evaluation of decellularized matrices and DNA quantification

Histological staining of tissue sections was performed to evaluate the efficiency of decellularization. In fresh-frozen control tissues, intense cellular remnants, particularly nuclear materials, were clearly seen in H&E-, Van Gieson's-, and DAPI-stained sections (Figs. 1a, f, and k). The treatment groups of B1, B2, C, and D all demonstrated a depletion of recognizable cell components compared to control group in H&E staining (Figs. 1b-e). The clearance of nuclear components was further evident in Dapi staining (Figs. 1l-o). In the Van Gieson's staining, the extracellular structure of muscle tissue in group B1 and B2 was comparable to the controls while there was mainly fibrous structure preserved in the groups C and D. Figure 2 shows that the DNA contents in the decellularized ECMs was significantly reduced from 1.40 ± 0.11 μg/mg dry weight in untreated muscle to 0.11 ± 0.01, 0.09 ± 0.01, 0.19 ± 0.02, and 0.06 ± 0.02 μg/mg dry weight in groups B1, B2, C, and D, respectively, where p<0.05 compared to the native control for all cases.

Collagen and glycosaminoglycan (GAG) content

Figure 3a shows that the collagen contents of the decellularized specimens treated with Triton X-100 for one week and two weeks (B1: 3.13 ± 0.15 μg/mg dry weight, B2: 2.99 ± 0.34 μg/mg dry weight) were comparable to control group (3.07 ± 0.13 μg/mg dry weight) but were significantly reduced in groups C and D. (C: 2.12 ± 0.30 μg/mg dry weight, D: 2.25 ± 0.07 μg/mg dry weight, p<0.05). Total GAG content significantly decreased from 3.98 ± 0.18 μg/mg dry weight in control group to 2.37 ± 0.18, 2.48 ± 0.32,
Decellularized skeletal muscle ECM

2.02 ± 0.26 and 2.09 ± 0.38 μg/mg dry weight in group B1, B2, C, and D, respectively (Fig. 3b).

Scanning electron microscopy

Scanning electron microscopy (SEM) images obtained from groups A, B1, and B2 showed that, unlike untreated muscle which showed a densely packed structure, the decellularized muscles exhibited a clear fibrous structure with no cells observed between the fibrils. The thicker wavy fiber bundles are believed to represent collagen interwoven with more slender elastic fibers to form a porous network (Fig. 4).

Biomechanical test

The uniaxial tensile test showed there was no significant difference in the tangent modulus between groups A, B1, and B2 (0.6 ± 0.01 MPa, 0.63 ± 0.01 MPa and 0.52 ± 0.03 MPa, p>0.05). The failure strength was close in group A, B1, and B2 (0.631 ± 0.04, 0.627 ± 0.06, 0.581 ± 0.09 MPa, respectively; p>0.05). The extensibility was larger in group B2 (failure strain 1.14), compared to groups A (0.91) and B1 (0.91) but there was no significant difference (Fig. 5). The mechanical properties of the decellularized ECM of groups C and D were not tested because of the apparent damage of the ECM structure.

Implantation

Figures 6a-c illustrate the harvesting of the LD muscle, the decellularized matrix, and the implanted tissue. The H&E staining micrographs of implanted matrix in group B1 retrieved at 10, 20, and 30 days postoperatively are shown in Figures 7a, b, and c, respectively. Moreover, H&E staining micrographs for group B2 are shown in Figures 7e, f, and g. Inflammatory cells, composed of mononuclear cells and multinucleated foreign-body giant cells, were seen infiltrating mainly the acellular tissues in the Triton X-100 one-week and two-week treatment groups. There was no integration between the acellular matrix and native muscle tissue at the interface. The layer of inflammatory cells decreased in thickness at 20 days after surgery, and the depth of the...
inflammatory cell infiltration into the outer layers of the native muscle tissue was greater than that observed at ten days after surgery. Neither fibroblasts nor neocollagen fibrils were observed at the interface. Integration between acellular matrix and underlying muscle tissue with neocollagen fibril formation was identified at 30 days after surgery. Obvious infiltration of inflammatory cells was noted in the group treated with Triton X-100 for two weeks, but no myofibril formation was seen. The amount profile of inflammatory cell in group B2 is presented in the Figure 8. The neutrophils were the primary inflammatory cells at day 10, followed by histiocytes and lymphocytes. After 30 days, the lymphocytes replaced the neutrophils as the major inflammatory cells at the interface of decellularized matrix and native muscle. Immunohistochemical staining indicated positive results for CD 68 macrophages in both the Triton X-100 one-week (Fig. 7d) and two-week (Fig. 7h) treatment groups at 10 days after surgery. However, the number of CD 68 macrophages gradually decreased until day 30.

### DISCUSSION

Several types of natural scaffolds, such as porcine small intestinal submucosa, acellular dermal matrix, and collagen, are currently used to repair abdominal wall defects...
Decellularized skeletal muscle ECM

in different animal models (5, 6, 20, 21, 28, 29). Among these, acellular matrices obtained by detergent-enzymatic methods have great potential for tissue regeneration, because many of the primary molecular structures of ECM are highly conserved among various species, and the immunogenic response of the host tissue is therefore expected to be low (3, 10). Ueda et al used gel electrophoresis (SDS-PAGE) to demonstrate the complete antigen clearing from the porcine heart valve by combination treatment with SDS and Triton X-100 (10). Gilbert et al showed that the ECM possesses all the characteristics, including highly ordered and hierarchical organization, favorable for tissue regeneration, and is therefore a good candidate for tissue regeneration (30). The ECM also provides a supportive medium for blood or lymphatic vessels as well as nerves (31).

For the acellular scaffold, the first important issue is to verify the effectiveness of cell removal and the second is to identify the optimal decellularization protocol, according to morphological, biochemical, and mechanical properties that will facilitate the appropriate differentiation and maturation of myoblasts for the formation of myotubes and myofibers. Zhang et al showed 93% of DNA clearance ratio by using 0.05% trypsin and 1% Triton X-100 (32). The method of Gillies (latrunculin B, hypertonic saline solution, and DNase I) achieved 96% DNA clearance ratio of mouse tibialis anterior muscle; however, when he repeated the method of Stern et al (0.05% trypsin 1 h + Triton X-100 5 days), only about 42% (from 2.92 ± 0.14 to 1.71 ± 0.17 μg/mg dry weight) of the nuclear components was removed (27, 33). The efficiency of trypsin and Triton X-100 on cell removal is mixed (30, 34-38). Effective cell removal (3.0 ± 0.4% residual DNA content) with Triton...
Lin et al

X-100 for pericardium decellularization has been reported by Mendoza-Novelo et al (39). Also, SDS is regarded as very effective for cell removal (30). Woods et al found that for the cell nuclei removal of porcine bone and anterior cruciate ligament, Triton X-100-SDS treatment was more effective than those of Triton-Triton or Triton-TnBP (tributyl phosphate) treatments (37). In our study, DNA clearance ratios of 92.2%, 93.3%, 86.4%, 95.7% in groups B1, B2, C, and D, respectively, can be achieved. Among them, treatments by trypsin + Triton X-100 (groups B1 and B2) and Triton X-100-SDS (group D) seemed to be more effective in cellular component removal in mouse skeletal muscle. The SEM findings in groups B1 and B2 indicate the presence of gaps between fibrous bundles in decellularized ECM of approximately 5 μm to 10 μm in size, which is suitable for cell adhesion and migration. It is unlikely that any specific combination of reagents will be suitable for the 100% removal of all cell components from different tissues. A previous study suggested that an ECM with most or all of the visible cellular material removed is safe for implantation (40).

The collagen content of a decellularized matrix in the trypsin-Triton X-100 groups (groups B1 and B2) is nearly completely preserved compared to an approximate 27% to 31% reduction in the trypsin-SDS group (group C) and the trypsin-Triton X-100-SDS group (group D). The preservation of collagen content in the trypsin-Triton X-100 groups is consistent with our SEM observation, which reveals intact collagen bundles. Schenke-Laylanda et al found prolonged exposure to trypsin/EDTA did not affect the collagen content in a decellularized heart valve (30, 35). Woods et al revealed Triton X-100 had no effect on the collagen content of an ECM obtained from the anterior cruciate ligament (30, 37). As for the effect of SDS, some have suggested that SDS treatment tends to cause the loss of collagen integrity but not the collagen content (30, 35). For the pericardium, it has been shown that decellularization with SDS causes irreversible swelling and denaturation compared to native tissue (41, 42).

Different from the collagen content, we observed substantial reduction (~28-47%) in the GAG content for all of the four groups of decellularized matrix. The decrease in GAG content may be due to the removal of GAGs associated with cell membrane. It has been reported that approximately 30% of GAGs are associated with cell membranes (30). Therefore, it is likely that these GAGs may be removed along with the disruption of sarcolemma and cell membrane during the decellularization process (30). Many experimental results have indicated that prolonged exposure to trypsin/EDTA may cause the decrease in GAG content (30, 34, 35). Nevertheless, the effects of Triton X-100 on the GAG content of a decellularized ECM is indefinite (30, 36-38). Mendoza-Novelo et al showed that the GAGs content in a decellularized pericardium treated by Triton X-100 is about 40% lower than native tissue (39). However, there was no difference in the sulfated GAG content in the ACL after treatment with Triton X-100 for four days (37). Previous literature suggests that SDS treatment tends to cause a decrease in GAG content (30, 35). Disparate results have been observed by using the same reagents in various tissues, such as porcine heart valves, pericardial tissue, liver or adipose tissues (10, 31, 35, 43, 44, 45).

The present mechanical tests show that the mechanical properties, including tangent modulus, failure stress and strain, of the decellularized ECM in groups B1 and B2 are very similar to the mechanical properties of the native tissue. Qing et al revealed results showing no significant difference in maximal breaking load and maximal extensional displacement between control and decellularized groups (26). Gillies also found that the stress values for strain of 10% to 100% and tangent modulus at 100% strain were not significantly different between untreated and decellularized samples (27). However, prolonged exposure to trypsin/EDTA may cause a decrease in tensile strength up to 50% (30, 34, 35). Mendoza-Novelo et al suggested the
Decellularized skeletal muscle ECM
decrease in GAG content of the Triton X-100 decellularized pericardium was related to the changes of its tensile properties (39). Decrease in tensile strength of pericardium after the treatment with SDS was noted (41, 42).
To test the biocompatibility, we implanted group B1 and B2 acellular matrices into the back of ICR mice. Despite the thorough removal of cellular components and nuclear materials, there was an innate inflammatory reaction, particularly a mononuclear response. In addition, immunohistochemical staining for CD68 yielded positive results at the incipient stage (10 days after surgery). Since the M1 and M2 phenotype profile was associated with chronic inflammation and constructive remodeling (46), respectively, the gradual decrease in M1 macrophages in the earlier stage may indicate the initiation of tissue remodeling.
This study is a preliminary evaluation of the effects of enzyme-detergent treatment on the compositional and structural properties of decellularized muscle tissue. Implantation tests for the decellularized ECM treated with trypsin-Triton X-100 showed mild inflammatory reaction in the short-term period of 30 days as compared to other studies. However, the current study has certain limitations. It lacked a detail characterization of durability and remodeling, including muscle regeneration, angiogenesis, and nerve innervation. Furthermore, a successful regeneration will require ingrowth of the right cell types and our attention will focus on seeding stem cell on the matrix in future studies.

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