An Acad Bras Cienc (2021) 93(2): e20190942 DOI 10.1590/0001-3765202120190942
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CELLULAR AND MOLECULAR BIOLOGY

Effects of chemical and physical methods on decellularization of murine skeletal muscles

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Abstract: Volumetric muscle loss causes functional weakness and is often treated with muscle grafts or implant of biomaterials. Extracellular matrices, obtained through tissue decellularization, have been widely used as biological biomaterials in tissue engineering. Optimal decellularization method varies among tissues and have significant impact on the quality of the matrix. This study aimed at comparing the efficacy of four protocols, that varied according to the temperature of tissue storage and the sequence of chemical reagents, to decellularize murine skeletal muscles. Tibialis anterior muscles were harvested from rats and were frozen at -20°C or stored at room temperature, followed by decellularization in solutions containing EDTA + Tris, SDS and Triton X-100, applied in different sequences. Samples were analyzed for macroscopic aspects, cell removal, decrease of DNA content, preservation of proteins and three-dimensional structure of the matrices. Processing protocols that started with incubation in SDS solution optimized removal of cells and DNA content and preserved the matrix ultrastructure and composition, compared to those that were initiated with EDTA + Tris. Freezing the samples before decellularization favored cell removal, regardless of the sequence of chemical reagents. Thus, to freeze skeletal muscles and to start decellularization with 1% SDS solution showed the best results.

Key words: extracellular matrix, skeletal muscle, decellularization, biomaterial.

INTRODUCTION

Skeletal muscles correspond to 50% of the body mass and present high regenerative capacity (Juhas & Bursac 2013). However, their self-renewal ability is decreased after orthopedic and peripheral nerve damages, irreversible muscle atrophy and volumetric muscle loss (VML) (Wu et al. 2012). VML is the immediate loss of muscle fibers and can lead to inadequate mechanical and functional performances of the remaining musculature.

Reconstruction of VML commonly requires transplant of autologous muscle grafts, which present several drawbacks, including insufficient donor tissue, particularly in case of severe injuries, loss of function and high morbidity at the donor site (Jana et al. 2016). Alternatively, implant of biological biomaterials, composed of tissue's extracellular matrix (ECM), has been investigated. Decellularization protocols are tissue and organs dependent and directly impact the quality of the resulting ECM (Leonel et al. 2017). Ideal ECM-based scaffolds should mimic the structure, biochemical and biomechanical cues of the tissue to be reconstructed (Lee et al. 2017).

Decellularized tissues have been used alone or loaded with stem and/or progenitor cells (Parmaksiz et al. 2016), in several applications, including regeneration of skin (Chen et al. 2004), ligaments (Endress et al. 2012) and tendons

(Yin et al. 2013). In the case of skeletal muscles, allografts (ECM from donors of the same species as the recipient) and xenografts (from donors of species other than the recipient) have been analyzed *in vitro*, *in vivo* and clinically (Sicari et al. 2014, Porzionato et al. 2015).

Tissue decellularization can be performed using chemical, physical, enzymatic methods, or with a combination of them. Chemical methods comprehend the use of ionic (sodium dodecyl sulfate;SDS),nonionic(TritonX-100)orzwitterionic (3-[(3-chola-midopropyl) dimethylammonio]-1- propanesulfonate; CHAPS) surfactants, acid (peracetic acid) and basic solutions (ammonium hydroxide; NH_4OH), to disrupt and to eliminate cells (Gilpin & Yang 2017). Physical methods include freeze-thaw cycles, which reduce the content of vital cells (Burk et al. 2014). Enzymatic processes often apply the proteolytic enzyme Trypsin and the nucleases DNase and RNase to facilitate nucleotides elimination after cell lysis (Boccafoschi et al. 2017).

All methods have pros and cons. For instance, SDS can lead to complete cell removal and eliminates up to 90% of DNA, but it may decrease the ECM glycosaminoglycans and growth factors content; Triton X-100 is less aggressive than SDS, but it does not eliminate cellular components that remain attached to the matrix (Gilpin & Yang 2017). Therefore, distinct solutions and methods have been combined to guarantee cell removal and to minimize deleterious effects to the ECM physical structure and composition.

This study aimed at comparing the effects of physicochemical methods on decellularization of murine skeletal muscles, which are an important tool for investigations in tissue engineering and translational medicine.

MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committees of the Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA n° 9553300114) and the Instituto Butantan (CEUA n° 2989051015), Brazil.

Fifty male Wistar rats, weighing 350 grams, 3 months-old, were used as donors of the right and left tibialis anterior muscles (TA) (n= 100). Animals were subjected to euthanasia: anesthesia was performed through intraperitoneal injection of Ketamine and Xylazine (100mg/Kg and 10mg/Kg, respectively), followed by an overdose of the before mentioned drugs (3x de concentration). This was followed by intravenous injection of 10% potassium chloride. After absence of heart beating, thoracic and respiratory movements and loss of mucous membranes coloring were observed, TA muscles were dissected and harvested.

Murine skeletal muscle decellularization

Harvested muscles were initially washed in running water and incubated in phosphate buffered saline (PBS) 1x + 0.5% penicillin and streptomycin (LGC Biotechnology, Brazil), for 3 times of 5 minutes each, under agitation on a shaker TS-2000A VDRL (Biomixer, USA).

Four protocols were tested. They varied according to (1) the sequence of chemical reagents that were used and (2) the storage temperature. i.e. frozen at -20ºC or kept at room temperature (RT).

Protocols were the following:

1- EDTA+Tris RT group: samples were incubated in 5mM ethylenediamine tetraacetic acid (EDTA; LGC Biotechnology, Brazil) associated with 50 mM Tris (LGC Biotechnology, Brazil) for 2 days; rinsed in 1x PBS + 0.5% antibiotics (Penicillin-Streptomycin, 3x, 5 minutes each), followed by incubation in 1% sodium dodecyl

sulfate (SDS; LGC Biotechnology, Brazil) for 4 days. New washing was carried out using 1x PBS + 0.5% antibiotics (3x, 5 minutes) and incubation in 1% Triton X-100 (LGC Biotechnology, Brazil) for 2 days. Throughout this process, samples were kept at room temperature (RT) (n= 25).

2- EDTA+Tris -20°C group: after the initial washing and before decellularization, samples were frozen at -20°C for 4 days (n= 25). Then, they were thawed, incubated in 1x PBS for 20 minutes at room temperature and decellularized according to the sequence of solutions described above.

3- SDS RT group: samples were incubated in 1% SDS for 4 days; rinsed in 1x PBS + 0.5% antibiotics (3x, 5 minutes each); incubated in 5 mM EDTA + 50 mM Tris for 2 days; rinsed again in 1x PBS + 0.5% antibiotics (3x, 5 minutes each) and, incubated in 1% Triton X-100 for 2 days. Samples were kept at room temperature (RT) throughout the process (n= 25).

4- SDS -20°C group: after harvesting, samples were washed and frozen at -20°C (n= 25). After 4 days, they were thawed, incubated in 1x PBS for 20 minutes at room temperature and decellularized according to the sequence of solutions described for protocol SDS RT.

Throughout these processes, samples remained immersed in 25 ml of solutions, under agitation, at room temperature, for 10 h, during the day and without agitation overnight. The solutions were changed twice a day.

At the end, all samples were rinsed in 1x PBS + 0.5% antibiotics (3x of 20 min), sterilized in 70% alcohol (Synth, Brazil) (3x of 30 minutes) and rinsed again in sterile 1x PBS + 0.5 % antibiotics (3x of 20 minutes).

Macroscopically, samples were analyzed according to translucency, shape, consistency in handling and final size. Microscopical analyses (histology and scanning electron microscopy), quantification of remaining DNA and immunohistochemistry were also performed.

Histology

Decellularized and control (non-processed) skeletal muscles (n= 4) were fixed in 4% PFA for 48 hours, incubated in alcohol 70% overnight, dehydrated in ethanol (80%, 90%, 95%, 100% twice, 30 min), incubated in xylene (2x of 30 min) (Dynamic, Brazil), included in paraffin (ERV-PLAST, EasyPath, Brazil) for 3h and sectioned into 5 µm thickness slices (microtome Leica RM 2125RT, Germany). Before staining, slices were incubated in xylene (2x, 15 minutes) and rehydrated in decreasing series of alcohol (100%, 100%, 95%, 70%, 3 minutes) and in running water (10 minutes).

Hematoxylin and Eosin (H&E), Masson's Trichrome and Picrosirius staining were performed to allow observation of muscle fibers and cell nuclei, and the organization and preservation of collagen fibers.

Sections were analyzed under a Nikon 80i light microscope (Japan) in a 50x objective (for H&E and Masson's Trichrome) and under polarized light, using a Carl Zeiss microscope (Germany) with 16x objective (for Picrosirius staining).

DNA quantification

Decellularized muscles were frozen at -80°C for assessment of residual double-stranded DNA (dsDNA), using *Quant-iT™ PicoGreen® dsDNA Reagent* assay (Life Technologies, Massachusetts, USA), according to manufacturer's recommendations.

Briefly, samples were fragmented, placed into 96 well plates and incubated with PicoGreen® solution in a humidified 5% CO_2 chamber, at 37°C, protected from light. After 18 hours, the solution was transferred to black plates (Black Plate 96 wells, Falcon®-Cary,

North Carolina, USA) and the fluorescence was measured using a spectrophotometer reader (SpectraMax® Paradigm®, Sunnyvale, California, USA), with wavelength of 480 nm excitation and 520 nm emission. A linear standard curve was stablished using the fluorescence data of known concentrations of DNA (1, 10, 100 and 1000 ng/ml); the regression equation derived from this curve was used to calculate the concentration of dsDNA. This assay was performed in quadruplicate and the results were statistically analyzed.

Scanning Electron Microscopy (SEM)

The SEM analysis was performed to evaluate the 3D structure of the native (control) and decellularized muscles (n=3). Tissues were fixed in Karnovsky's solution (2.5% glutaraldehyde and 4% PFA, 1:1) for 24 hours, rinsed in distilled water (5x of 5 minutes) using an Ultrasonic Washer (Unique, Brazil), incubated in 70% alcohol overnight, dehydrated in ethanol (80%, 90% and 100% 2x) and dried in CO_2 critical point apparatus (Leica EM CDP300, Germany). Samples were gold coated (coater Emitech K550, England) and analyzed in a scanning electron microscope (Leo 435 VP, England).

Immunofluorescence

Immunofluorescence was used to assess the presence of laminin, fibronectin and collagen types I and III, in control and decellularized muscles. Samples were initially soaked in O.C.T. (Optimal Cutting Temperature) compound (Sakura Finetek 4583; U.S.A.) and frozen at -150°C. Slices were fixed with cold acetone (Vetec, Brazil) for 10 minutes, dried at room temperature for 20 minutes and kept with 1x Tris-buffered saline (TBS) + 2% bovine serum albumin (BSA) for 1 hour and incubated with primary anti-laminin antibodies (1:200) (Abcam-ab11575, rabbit polyclonal, USA), anti-fibronectin (1:200) (Abcam-ab2413, rabbit polyclonal, USA), anti-collagen I (1:100) (Santa Cruz Biotechnology, sc-25974, goat polyclonal, CA, USA) and anti-collagen III (1:100) (Santa Cruz Biotechnology, sc-8779, goat polyclonal, CA, USA) overnight at 4°C in a humidified chamber.

Then, they were washed (3x, 5 minutes each) with TBS + 0.2% BSA at room temperature, incubated with the secondary antibody for 1 hour and protected from light. The secondary antibodies Alexa-Fluor 488 goat anti-rabbit (1:300) (Life Technologies, A -11008, USA) and Alexa-Fluor 488 donkey anti-goat (1:200) (Life Technologies, A-11055, USA) were used. Slices were washed in TBS solution + 0.2% BSA (3x, 5 minutes), incubated with DAPI (1:10.000) for 10 minutes, at room temperature, mounted in glycerol:PBS (1:1) and analyzed under confocal microscope (FV1000 Olympus IX81, Japan) with 200x and 400x objectives.

Statistical analysis

The concentration of dsDNA (PicoGreen® assay) was analyzed by two-way ANOVA (effect of decellularization protocols in different temperatures) with Tukey post-test, using the statistical program Graphpad (Version 6.0). Significance level of 5% was adopted ($p<0.05$).

RESULTS

Decellularized muscles were evaluated according to the following aspects: macroscopic features of the samples (size, translucence and consistency at handling), removal of cells, concentration of dsDNA, the ECM 3D structure and preservation of proteins.

Macroscopic analysis

Decellularized muscles of all experimental groups became translucent, acquired gelatinous

consistency and decreased 0.5 cm in size, in average. These alterations were observed after the second day of processing in samples of protocols SDS RT and SDS -20°C, and after five days, for samples belonging to protocols EDTA+Tris RT and EDTA+Tris -20°C (Figures 1a-e). There was no difference in the consistency of handling of the samples.

Histological analysis

Despite the translucent aspect of the ECM, samples from groups EDTA+Tris RT and EDTA+Tris -20°C still had cell nuclei and intact muscle fibers after 8 days of processing, although areas with disorganization of muscle bundles were observed. Conversely, protocols SDS RT and SDS -20°C favored the removal of cells and muscle fibers (Figure 2).

The protocols SDS RT and SDS -20°C also preserved the structural and organizational integrity of the skeletal muscle connective tissue, including endomysium and perimysium, as observed with Masson's Trichrome staining. The maintenance of collagen fibers was noted in all experimental groups through Picrosirius staining, in which the yellow-orange-red birefringence indicated thicker fibers and the green birefringence, the thinnest (Figure 2).

DNA quantification

Samples that were frozen before the beginning of the decellularization process, as well as those belonging to the SDS groups, presented a trend of less dsDNA concentration.

There was no statistical difference between the EDTA+Tris groups, although a trend of less DNA was observed in samples frozen at -20°C, as compared to those kept at room temperature (RT) ($p=0.06$) (10.75 ± 0.85 ng/ml; 53 ± 20.1 ng/ml, respectively) (Figure 3).

Likewise, there was no difference ($p=0.9$) between the SDS groups (SDS RT: 10.73 ± 6.55 ng/ml; SDS -20°C: 3.25 ± 0.75 ng/ml).

The comparison between the groups EDTA+Tris RT and SDS RT, as well as between EDTA+Tris -20°C and SDS -20°C also showed no statistical differences ($p= 0.06$ for samples at RT and $p=0.9$ for frozen samples).

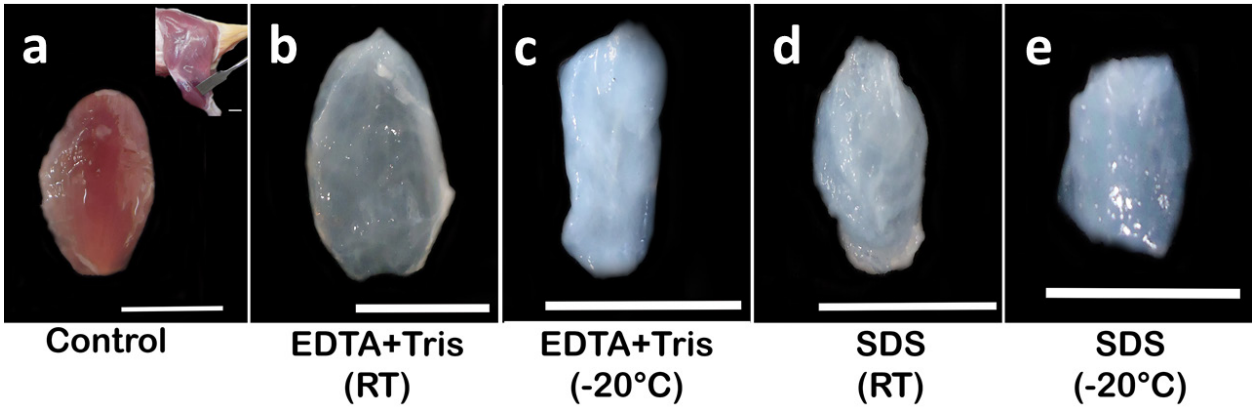

Figure 1. Macroscopic images of Wistar's tibialis muscle before (a) and after (b-e) decellularization, according to different protocols. Protocols EDTA+Tris RT and EDTA+Tris -20°C: samples decellularized with 5mM EDTA+ 50mM Tris, followed by 1% SDS and 1% Triton X-100, and kept at room temperature (RT) (b) or frozen at -20°C (c) before processing. Protocols SDS RT and SDS -20°C: samples decellularized with 1% SDS, followed by 5mM EDTA+ 50mM Tris and 1% Triton X-100, and kept at room temperature (RT) (d) or at -20°C (e) previously to decellularization. Change in translucence and size of the samples were observed.

Similarly, no statistical difference was observed with the comparison of groups EDTA+Tris -20°C and SDS RT (p=0.9).

Conversely, samples processed according to protocol EDTA+Tris RT presented higher amount of dsDNA, as compared to protocol SDS -20°C (p= 0.02).

Scanning electron microscopy

In control muscles, organization of muscle fibers, surrounded by connective tissue (endomysium and perimysium), was observed (Figure 4a). Samples prepared according to Protocols EDTA+Tris RT and EDTA+Tris -20°C, presented partial decellularization and had a few areas with intact microfibrils (Figures 4b-c).

Conversely, in samples belonging to protocols SDS RT and SDS -20°C, the elimination of muscle fibers was optimized, and the organization of endomysium and perimysium, preserved. Microfibrillar structure of the endomysium was detected after decellularization (Figures 4d-e).

Immunofluorescence

Proteins related to cell adhesion (laminin and fibronectin) and structural support (collagen types I and III) were preserved in the decellularized matrices processed according to the four protocols. Cell nuclei were not detected with the described protocol for DAPI staining, although remaining cells were evident under histological analysis (Figure 5).

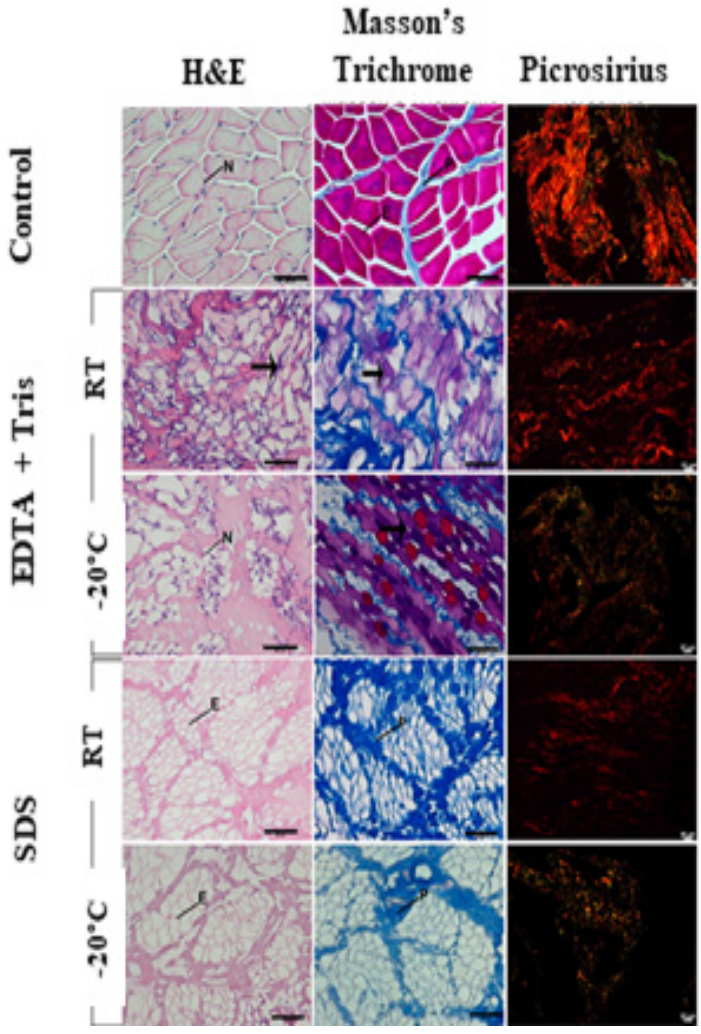

Figure 2. Histological images of the control and decellularized muscles. Samples of the groups EDTA+Tris RT and EDTA+Tris -20°C presented remnant cell nuclei and intact muscle fibers (black arrow). Conversely, samples of the groups SDS RT and SDS -20°C did not show cells and maintained the connective tissue structural features. Through microscopic polarized light (Picrosirius), thicker (yellow-orange-red birefringence) and thin (green birefringence) collagen fibers were detected. N: nucleus. E: endomysium. P: perimysium. H&E and Masson's trichrome scale bars: 50 µm. Picrosirius scale bars: 20 µm.

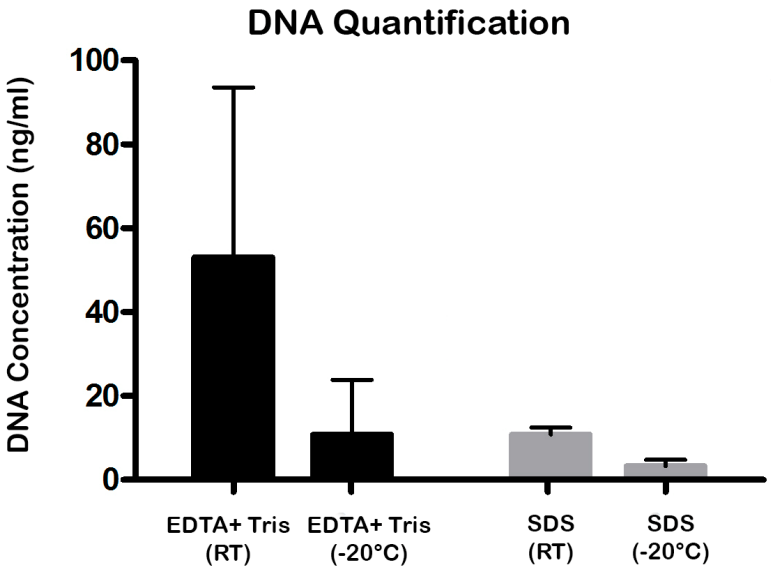

Figure 3. Analysis of the dsDNA content on skeletal muscles' ECM. Although there was a trend of less DNA in samples of the groups SDS RT and SDS -20°C, the differences were not statistically significant. Difference was detected only when groups EDTA+Tris RT and SDS -20°C were compared (p=0.02) (*p<0.05).

DISCUSSION

In volumetric muscle loss (VML), ECM implantation promotes muscle repair by providing physical and biomechanical structure to the injured tissue, host cell-mediated degradation of the biomaterial, recruitment of endogenous progenitors/ stem cells and modulation of the innate immune response (Dziki et al. 2016). ECM-based biomaterials can be applied alone or associated with cultured cells (stem cells or myogenic cells), which has been reported to favor VML repair in vivo (Quarta et al. 2017).

Skeletal muscles from different species have been decellularized through several methods, that differ among authors. For instance, canine skeletal muscles have been processed with a combination of physical (freezing at -80°C), chemical (association of chloroform/methanol; 2% deoxycholate; 1% Triton X-100; 0.1% peracetic acid/ 4% ethanol) and enzymatic methods (0.2% Trypsin/ 0.2% EDTA) (Wolf et al. 2012). Swine muscles have been processed using chemical (0.1% SDS; 1% Triton X-100; 0.1% peracetic acid/ 4% ethanol) and enzymatic reagents (0.02% Trypsin/ 0.05% EGTA; DNase), but with no physical methods (Zhang et al. 2016).

The effectiveness of chemical reagents may vary according to the cellularity, density, lipid content and thickness of tissues and/or organs to be decellularized (Crapo et al. 2011). Chemical solutions may lead to denaturation of the ECM proteins, which may compromise the morphology and composition of the matrix. This may ultimately affect the bioactivity of the ECM, after implantation.

In this study, four different protocols were tested to decellularize murine skeletal muscles, which represents an important model for translational medicine.

The chemical reagents used herein have been described in several other studies. Ethylenediamine tetraacetic acid (EDTA) is a chelator of Ca^{2+} and Mg^{2+}, which are essentials ions for cell/ECM interactions, thus facilitating cell removal (Gilbert et al. 2006). EDTA has been often applied in conjunction with Tris to improve cell lysis by osmotic shock, dissociation of DNA from other proteins and elimination of cell debris after lysis (Boccafoschi et al. 2017). These solutions are also used to remove cell debris. However, as they do not suffice to eliminate

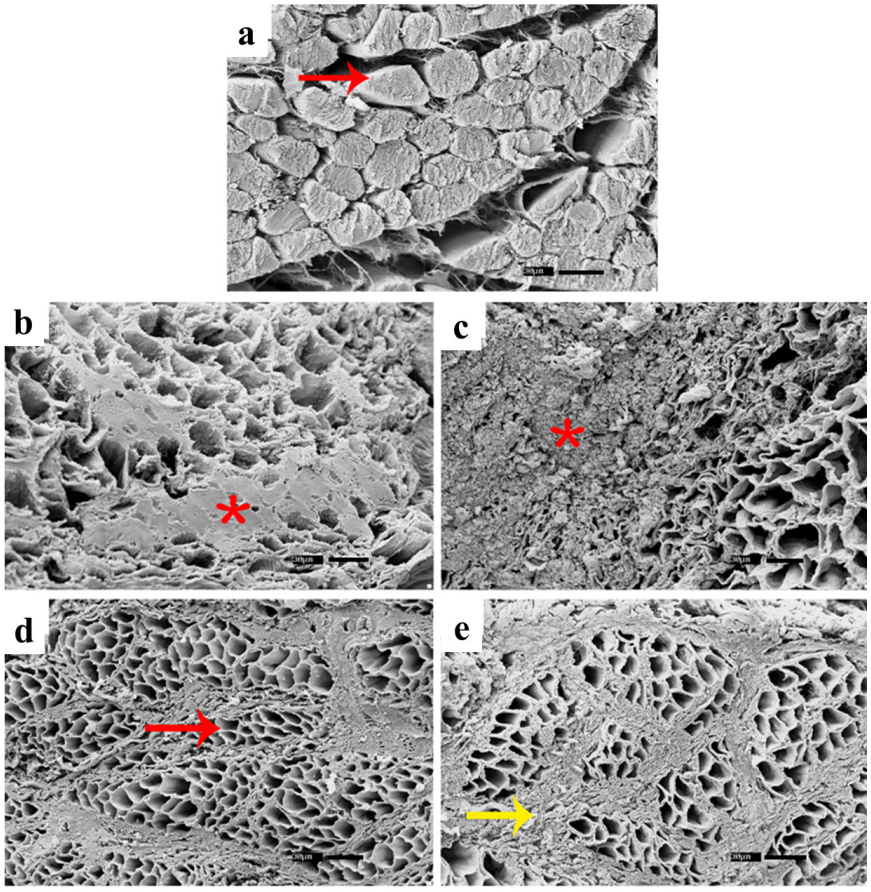

Figure 4. Scanning electron microscopy of the native (control) (a) and decellularized (b-e) muscles. The presence of intact muscle fibers was observed in samples of the groups EDTA+Tris (b-c), whereas of the groups SDS (d-e) showed better cell removal and preserved microfibers of the connective tissue, including endomysium and perimysium. Yellow arrow: perimysium. Red arrow: endomysium. Asterisk (*): remnant muscle fibers. Scale bar: 30 µm.

cells from tissues, they have been associated with other reagents, such as SDS.

Sodium dodecyl sulfate (SDS) is an ionic detergent responsible for protein denaturation and causes cell membrane solubilization. It has been described as an essential reagent for decellularization of innumerous tissues and organs, such as aorta (Ott et al. 2008), kidney (Burgkart et al. 2014) and lung (O'Neill et al. 2013). The incubation time in SDS may vary depending upon the organ, tissue type, donor's age, method of incubation, among other factors (Crapo et al. 2011).

Triton X-100, a nonionic surfactant, was used as it removes the remnant SDS (Gilpin & Yang 2017), leads to tissue disruption and is less aggressive than SDS, preserving protein-protein bonds (Crapo et al. 2011).

However, chemical agents may not completely remove cellular remnants, such as dsDNA, mitochondria, mitochondrial DNA and cell membrane phospholipids, which may remain attached to the matrix and compromise the remodeling process. The immune rejection of matrices containing such residual elements may vary depending upon the source of ECM, the type of tissue in which it will be implanted and the host's immune function (Crapo et al. 2011). For instance, to avoid some of these complications, it is recommended that dsDNA be kept at levels below 50 ng of DNA/mg of ECM dry weight (Keane et al. 2012).

With respect to the temperature, freezing tissues lead to formation of intracellular ice crystals, which causes ruptures of cell membrane, and allow greater penetration of the

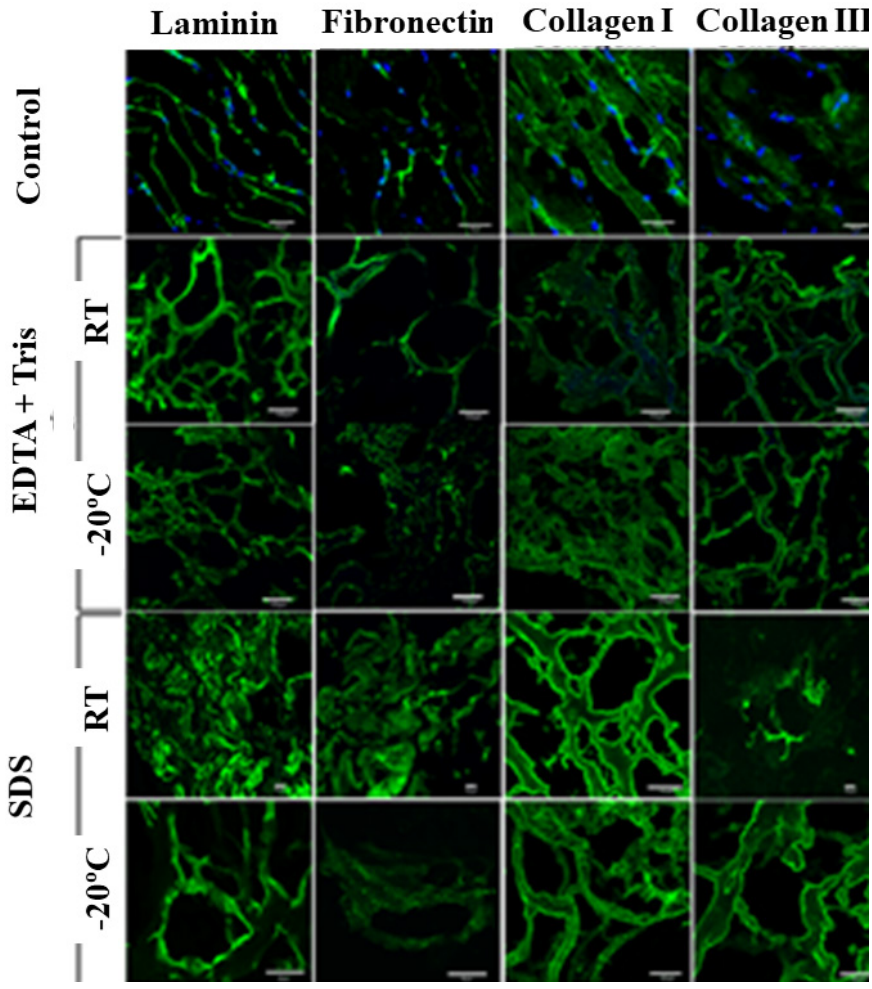

Figure 5. Preservation of cell adhesion (laminin, fibronectin) and structural proteins (collagen types I and III) in the extracellular matrix (ECM) of control and decellularized skeletal muscles of rats analyzed by immunofluorescence. Scale bars= 40 and 100 µm.

chemical reagents within the tissue, favoring cell removal, regardless of which solution was used first (Gilbert et al. 2006). Freeze-thaw procedures may contribute to minor disruptions of the ECM ultrastructure (Crapo et al. 2011).

In this study, application of 1% SDS in the beginning of the process, regardless of the temperature, favored decellularization and led to earlier macroscopic changes of the matrix (translucency, decrease in size and altered consistency in handling). It also favored elimination of cell remnant and dsDNA, as compared to its utilization after EDTA+Tris incubation. This demonstrated that the sequence in which the chemical reagents are used affects the resulting features of the ECM.

Increased tissue processing leads to decrease of ECM mechanical resistance and physical structure, provided by the 3D arrangement of its proteins. The structural features and biochemical composition of the ECM will modulate the microenvironment and influence several cellular activities (Zhang et al. 2016). Therefore, establishing a protocol that minimizes the damages caused to the ECM is crucial (Crapo et al. 2011).

Collagen is one of the main proteins of the muscle ECM and several types have been identified: I, III, IV, V, VI, XI, XII, XIV, XV and XVIII. Among these, collagen types I and III predominate in the adult muscle, particularly within the epimysium, perimysium and endomysium (Gillies & Lieber 2011, Wolf et al. 2012). Other

molecules, such as proteoglycans (decorin and biglican), glycosaminoglycans (dermatan) and glycoproteins (laminin and fibronectin), are part of the skeletal muscles' ECM and their maintenance, in conjunction with growth factors, could support and/or induce tissue remodeling (Gillies & Lieber 2011, Wolf et al. 2012). Collagen types I and III, laminin and fibronectin were preserved after decellularization processes analyzed in this study.

Therefore, our data suggested that protocol SDS -20°C had the best results and should be applied to decellularize murine skeletal muscles. These results represent an important step for the validation of a protocol, considering that murine ECM can be applied as allografts or xenografts in translational investigations related to VML treatments.

Acknowledgments

The authors would like to thank Dra. Ana Cláudia Carreira, researcher at Nucel (Universidade de São Paulo, Brazil), for spectrophotometer reading; Dr. Moysés Miranda, professor at Universidade do Pará (Brazil), for statistical analysis and Dr. Edson Liberti, professor at Institute of Biological Science (Universidade de São Paulo, Brazil) for helping with histological preparations. We are also thankful to Grant sponsors: Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP and Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq; Grant numbers: 14/50844-3 (FAPESP) and 467476/2014-4 (CNPq).

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How to cite

MIRANDA CMFC, LEONEL LCPC, CAÑADA RR, MARIA DA, MIGLINO MA, DEL SOL M & LOBO SE. 2021. Effects of chemical and physical methods on decellularization of murine skeletal muscles. An Acad Bras Cienc 93: e20190942. DOI 10.1590/0001-3765202120190942.

Manuscript received on August 15, 2019;
accepted for publication on October 07, 2019

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