Research Article

Preparation of human decellularized peripheral nerve allograft using amphoteric detergent and nuclease

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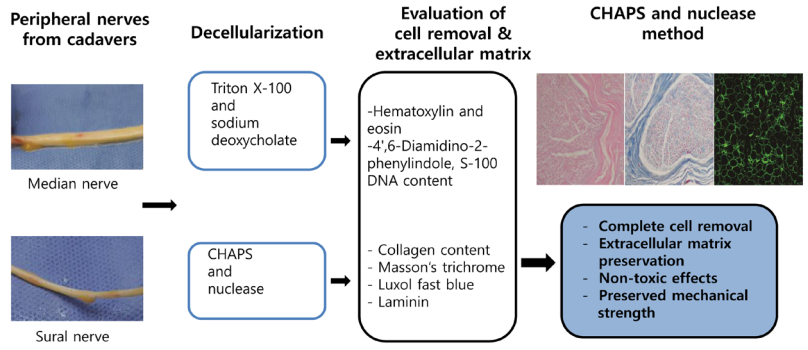
Joo-Yul Bae[1, #], Suk Young Park[2, #], Young Ho Shin[2], Shin Woo Choi[1], Jae Kwang Kim[2, *]

Graphical Abstract *Comparison of two decellularization methods in the context of human peripheral nerves from cadavers*



Abstract

Animal studies have shown that amphoteric detergent and nuclease (DNase I and ribonuclease A) is the most reliable decellularization method of the peripheral nerve. However, the optimal combination of chemical reagents for decellularization of human nerve allograft needs further investigation. To find the optimal protocol to remove the immunogenic cellular components of the nerve tissue and preserve the basal lamina and extracellular matrix and whether the optimal protocol can be applied to larger-diameter human peripheral nerves, in this study, we decellularized the median and sural nerves from the cadavers with two different methods: nonionic and anionic detergents (Triton X-100 and sodium deoxycholate) and amphoteric detergent and nuclease (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), deoxyribonuclease I, and ribonuclease A). All cellular components were successfully removed from the median and sural nerves by amphoteric detergent and nuclease. Not all cellular components were removed from the median nerve by nonionic and anionic detergent. Both median and sural nerves treated with amphoteric detergent and nuclease maintained a completely intact extracellular matrix. Treatment with nonionic and anionic detergent decreased collagen content in both median and sural nerves, while the amphoteric detergent and nuclease treatment did not reduce collagen content. In addition, a contact cytotoxicity assay revealed that the nerves decellularized by amphoteric detergent and nuclease was biocompatible. Strength failure testing demonstrated that the biomechanical properties of nerves decellularized with amphoteric detergent and nuclease were comparable to those of fresh controls. Decellularization with amphoteric detergent and nuclease better remove cellular components and better preserve extracellular matrix than decellularization with nonionic and anionic detergents, even in large-diameter human peripheral nerves. In Korea, cadaveric studies are not yet legally subject to Institutional Review Board review.

Key Words: median nerve; sural nerve; nuclease; detergent; human decellularized nerve graft

Chinese Library Classification No. R459.9; R364; R622

Introduction

Damage to the peripheral nerve is a common injury pattern and is reported in up to 3% of all trauma patients (Rasulić et al., 2015; Zhu et al., 2017). It is a significant clinical challenge to overcome for microsurgeons (Sameem et al., 2011). If possible, primary tension-free end-to-end neurorrhaphy is the treatment of choice (Jesuraj et al., 2014). However, it is often difficult to repair the nerve primarily due to nerve defects or resection of the lesion. In this situation, autogenous nerve grafts are used alternatively (Beris et al., 2019). However, there is a limitation to the use of autogenous nerve grafts due to donor site morbidity, lack of sufficient tissue, or size mismatch between donor and recipient (Beris et al., 2019).

Recently, acellular nerve grafts have been studied to overcome these drawbacks (Beris et al., 2019; Rbia et al., 2019). To enable allogenic nerve grafting, decellularization methods should eliminate graft antigenicity by complete removal of cellular components and maintain the integrity of the basal lamina and extracellular matrix (ECM). Currently available decellularization techniques can be classified into

[1]Department of Orthopedic Surgery, University of Ulsan College of Medicine, Gangneung Asan Hospital, Gangneung-si, Korea; [2]Department of Orthopedic Surgery, University of Ulsan College of Medicine, Asan Medical Center, Seoul, Korea

*Correspondence to: Jae Kwang Kim, MD, PhD, orth4535@gmail.com.

https://orcid.org/0000-0001-5104-4634 (Jae Kwang Kim)

#These authors contributed equally to this work.

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physical methods (Zalewski and Gulati, 1982; Evans et al., 1998), chemical detergents (Sondell et al., 1998; Hudson et al., 2004), biological agents (Sridharan et al., 2015), and miscellaneous methods (Szynkaruk et al., 2013; Ishida et al., 2014). These may be used alone or in combination of two or more (Sondell et al., 1998; Hudson et al., 2004; Kim et al., 2016). Among them, chemical agents, particularly detergent-based decellularization, are the most commonly used (Kim et al., 2016). Recently, many researchers have reported the usefulness of a decellularization method using a combination of nuclease and detergent (Wang et al., 2014; Mangold et al., 2015; Sridharan et al., 2015).

Although several methods have been reported, the most effective procedure for eliminating cellular components and maintaining the integrity of the basal lamina as well as the ECM using decellularization has not been established (Wang et al., 2014; Mangold et al., 2015; Sridharan et al., 2015; Gilpin and Yang, 2017; Simsa et al., 2018; Shin et al., 2019). Simsa et al. (2008) reported the results of a study comparing the decellularization of blood vessels with five different mixtures of detergent and nuclease. Furthermore, Shin et al. (2019) compared various detergents with nuclease-based decellularization methods on nerve fragments. They systematically compared the effects of six different mixtures of detergents with nuclease methods in animals and found that a combination of amphoteric detergent (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, CHAPS) and nucleases (deoxyribonuclease I and ribonuclease A) was the most reliable chemical agent for decellularization.

Based on the research of Shin et al. (2019), we attempted to apply reliable decellularization methods to human peripheral nerve tissue. Compared to other methods, amphoteric detergent and nucleases significantly eliminated the immunogenic cellular components of the tissue, thereby preserving the basal lamina and ECM. Among the six types of decellularization methods, no statistical differences were observed between the other methods except the amphoteric detergent and nucleases. Therefore, we selected only two methods, amphoteric detergent and nucleases (CHAPS, deoxyribonuclease I and ribonuclease A) and nonionic and anionic detergent (Triton X-100 and sodium deoxycholate) for the decellularization of the human peripheral nerve. The combination of Triton X-100 and sodium deoxycholate is a well-known decellularization method; therefore, it was selected among the five for comparative analysis (Sondell et al., 1998; He et al., 2015; Zhu et al., 2017).

It is known that decellularization is difficult in thick and less vascularized tissues (Syed et al., 2014; Koenig et al., 2019), and each agent has a different ability to remove cells depending on the tissue size (Petersen et al., 2010; Crapo et al., 2011; Du et al., 2011). Therefore, relatively large-diameter median nerves and small-diameter sural nerves were prepared. The purpose of this study was to compare the results of decellularization by applying two well-established decellularization methods on the human peripheral nerve. In addition, we evaluated the decellularization status in both the median and sural nerves to investigate whether this protocol could be applied to large-diameter human peripheral nerves.

Materials and Methods

Nerve harvest from cadaver

Median nerves and sural nerves were obtained from two cadavers at the Korea Public Tissue Bank. Both the body donation agreement and the human body research agreement were obtained from the National Public Tissue Bank. The isolation of the median nerves from both arms and the sural nerves from both legs was performed at the Korea Public Tissue Bank. We obtained two median nerves (20 cm long) and two sural nerves (50 cm long) from a male cadaver (47 years old) and two median nerves (40 cm long) and two sural nerves (30 cm long) from a female cadaver (23 years old). The median and sural nerves were 3.0–3.8 mm and 1.2–1.5 mm in diameter, respectively (**Figure 1**). The median and sural nerves were cut into 2.0–2.3 cm segments. The 24 segments of the median nerves and 32 segments of the sural nerves were placed separately in a 15-mL tube containing PBS. We divided them into six groups: (A) fresh median nerve, (B) median nerve decellularized by nonionic and anionic detergent (Triton X-100 and sodium deoxycholate), (C) median nerve decellularized by amphoteric detergent and nuclease (CHAPS, deoxyribonuclease I and ribonuclease A), (D) fresh sural nerve, (E) sural nerve decellularized by nonionic and anionic detergent (Triton X-100 and sodium deoxycholate), and (F) sural nerve decellularized by detergent amphoteric and nuclease (CHAPS, deoxyribonuclease I and ribonuclease A).

All nerve fragments were stored at 4°C in phosphate buffered saline (PBS), and decellularization was initiated within 12 hours of nerve harvest. All reagents not indicated by the manufacturer were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used in the experiment. Unless otherwise noted, buffer treatment was performed at room temperature.

All consents for approval of research were managed by the Korea Public Tissue Bank. And in general, research using cadaver is not subject to IRB. Since our research was not conducted on living persons, although we submitted the protocol to the Institutional Review Board (IRB) committee, the review was not conducted. Please note that in Korea, cadaveric studies are not yet legally subject to IRB review.

Decellularization process

Nerve fragments mixed with sterilized distilled water (DW) were continuously shaken for 7 hours in a 15-mL centrifuge tube (100 r/min, room temperature: 20°C). Based on the results of the previous study, two decellularization methods were applied to the median and sural nerves (Shin et al., 2019). Steps involving detergent were performed at room temperature with the exception of nuclease treatment. Nonionic, anionic, amphoteric detergent, and 1 M sodium chloride solution were prepared with 10 kIU/mL aprotinin (Roche Diagnostics GmbH, Mannheim, Germany).

(1) Nonionic and anionic detergent: The nerve fragments were treated with 3% Triton X-100 for 24 hours and transferred to 1 M sodium chloride solution for 15 hours. Subsequently, these fragments were treated with 4% sodium deoxycholate for 24 hours, and then washed for 72 hours by changing PBS every 12 hours to remove residual reagents.

(2) Amphoteric detergent and nuclease detergent: The nerve fragments were treated with 100 mM CHAPS in PBS buffer (pH 7.5, CHAPS) for 1 day. The nerve fragments were then transferred to a 1 M sodium chloride solution for 15 hours. After rinsing, the nerve fragments were placed in Dnase I and RNase A (10 μg/mL deoxyribonuclease I and 200 μg/mL ribonuclease A in 10 mM Tris-HCl at pH 7.5) at 37°C for 24 hours, and then the residual reagents were washed out for 72 hours by changing the PBS solution every 12 hours.

The samples were stored at 4°C in PBS after decellularization. Histological analysis and DNA quantification were performed immediately after decellularization. Collagen content quantification, biomechanical strength evaluation, and cytotoxicity assays were performed within 3 days following decellularization.

Histological analysis

Five nerve fragments per group were selected. These were transferred to a fixative solution (10% neutral buffered formalin, NBF), dehydrated, and embedded in paraffin. Three pairs of 4-μm thick nerve sections were cut five times at even

intervals of 2–2.3 cm of the nerve fragment, as shown in **Figure 1D**. Fifteen sections were obtained from a single nerve fragment. Five sections were used for hematoxylin and eosin (H&E) staining, five sections for 4,6-diamidino-2-phenylindole (DAPI) staining, and five sections for laminin staining (**Figure 1D**). After H&E staining, the blue hematoxylin-stained nuclei of cells were counted in the area (200 μm^2) for the 25 nerve sections using 100× magnification under a light microscope (Olympus, Tokyo, Japan), and the cellSens software (Olympus) was used for image capture using an Olympus DP73 digital camera (Olympus).

Immunofluorescence staining against laminin was performed for the 25 nerve sections within each group. After deparaffinization and rehydration, the nerve sections were immersed in heat-mediated antigen for 40 minutes. Following this, the nerve sections were rinsed with permeabilization buffer (0.2% Triton-X 100 in phosphate-buffered saline) for 5 minutes. For the blocking step, normal goat serum (10% in PBS with 0.1% sodium azide) was used for 90 minutes. The rabbit polyclonal anti-laminin primary antibody (Cat# ab11575, 1:500, Abcam, Cambridge, UK) was applied to the sections in blocking solution and placed in a 4°C refrigerator overnight. The goat anti-rabbit IgG-Alexa Fluor 488 secondary antibody (Cat# ab150081, 1:500, Abcam) was incubated at 20°C for 90 minutes. The primary antibody was replaced with blocking buffer as a negative control. Slides were cover-slipped with mounting medium (HIGHDEF IHC fluoromount, Enzo Life Sciences, Farmingdale, NY, USA). The degree of staining was observed using confocal microscopy (LSM980, Carl Zeiss, Jena, Germany), and images were captured using ZEN software (Carl Zeiss).

Basal lamina preservation of the decellularized nerve fragments was evaluated by calculating the percentage area relative to the fresh nerve using 200× magnification images, which was quantified by a scoring system (**Additional Table 1**) (Kim et al., 2016).

DNA staining for nuclei of nerve sections was performed with DAPI (1 μg/mL in PBS). The blue colored DAPI-positive cells were evaluated in the area (200 μm^2) using non-overlapped images at 100× magnification under a confocal microscope.

Additional histological evaluation was carried out for S-100, Masson's trichrome, and Luxol Fast Blue staining. Thirty sections per group were prepared (10 sections for S-100, 10 sections for Masson's trichrome, and 10 sections for Luxol Fast Blue staining).

S-100 immunohistochemistry (IHC) was used to compare the degree of clearance of Schwann cells in the nerve fragments. The sections were deparaffinized with EZPrep (Roche Diagnostics, Indianapolis, IN, USA), rehydrated with serial dilutions of ethanol (95%, 70%, and 50%) and DW. The sections were stained using an immunohistochemical staining device (Benchmark XT Ventana Medical Systems, Tuscon, AZ, USA). After rinsing the slides with reaction buffer (Tris buffer, pH 7.6), sections were transferred to an antigen retrieval buffer (Tris-Borate-EDTA buffer, pH 8.4). The sections were then blocked using Ultraview peroxidase inhibitor (Roche Diagnostics, Indianoapolis, IN, USA) for hydroperoxidase blocking, and immersed in rabbit polyclonal anti-S-100 primary antibody (Cat# ab868, 1:100, Abcam) diluted in Dako REAL antibody diluent (S2022, Agilent Technologies, Santa Clara, CA, USA) for 40 minutes at 37°C, followed by the introduction of DISCOVERY UltraMap anti-rabbit HRP (Cat# 760-4315, 1:1000, Roche Diagnostics) as the secondary antibody for 30 minutes at 37°C. Antigen-antibody reactions were visualized using the Ventana UltraView Universal DAB Detection Kit (Ventana Medical Systems, Roche). After rinsing the slides with reaction buffer and Ultraview copper, counterstaining was performed with hematoxylin and bluing reagent on a Ventana Benchmark XT instrument for 4 minutes each at 37°C. An isotype of the primary antibody served as the negative control. Slides were mounted with mounting medium (HIGHDEF IHC fluoromount, Enzo Life Sciences, Farmingdale, NY, USA) and evaluated at 100× magnification under a light microscope.

Nerve collagen, cytoplasm, and nuclei were stained using Masson's trichrome (MT) staining method. After deparaffinization and rehydration, the sections were re-fixed in Bouin's solution for 1 hour at 56°C to improve staining quality. The sections were rinsed with running tap water for 10 minutes, followed by staining with Weigert's iron hematoxylin for another 10 minutes and then finally washed in DW. The sections were stained in Biebrich scarlet-acid fuchsin solution for 15 minutes, rinsed in DW, and then differentiated in phosphomolybdic-phosphotungstic acid solution for 10 minutes. Sections were transferred directly to aniline blue solution, stained for 5 minutes, rinsed in DW, and differentiated in 1% acetic acid solution for 3 minutes. After washing in DW, the sections were dehydrated and mounted with Permount medium (Thermo Fisher Scientific, Waltham, MA, USA). The MT staining slides were observed at 100× magnification under a light microscope.

Myelin sheath and neurons were stained with Luxol Fast Blue (LFB)-Cresyl violet. After deparaffinization, sections were rinsed in 95% ethyl alcohol and incubated in Luxol Fast Blue (0.1% in ethyl alcohol and glacial acetic acid, Cat# 26056-10, Electron Microscopy Sciences, Hatfield, PA, USA) for 24 hours at 60°C. After washing with 95% ethyl alcohol and DW, the samples were placed in differentiation buffer (0.05% lithium carbonate solution) and stained with cresyl violet acetate (0.1% in DW, Polysciences Inc., Warrington, PA, USA) for 5 minutes at room temperature. The stained sections were dehydrated and mounted with permount medium (Thermo Fisher Scientific) and then evaluated at 100× magnification using light microscopy. For histological analysis, two independent and blinded observers counted sections of staining. If the results of the two observers did not match, a consensus was reached through discussion.

DNA quantification

An extraction kit (CME0111, COSMOgenetech, Seoul, Korea) was used to isolate and quantify DNA. Five lyophilized nerve fragments of 10 mg dry weight obtained from each group were digested for 4 hours at 56°C in a tissue lysis buffer containing proteinase K. DNA quantification was determined using a NanoDrop micro spectrophotometer (model 1000, Thermo Fisher Scientific).

Collagen quantification

An assay kit of hydroxyproline (MAK008, Sigma-Aldrich) was used to quantify collagen. Seven nerves lyophilized to 10 mg dry weight from each group were treated with HCl (12 N hydrochloric acid) for hydrolysis for 3 hours at 120°C. Each sample and standard well were oxidized using sodium N-chloro-p-toluenesulfonamide and p-dimethylaminobenzaldehyde. Absorbance was measured at 560 nm using a multi-detection microplate reader (SpectraMax 190, Molecular Device, San Jose, CA, USA). The concentration of hydroxyproline was determined by plotting a standard curve. The total collagen content was calculated using a hydroxyproline to collagen ratio of 1:7.69 (Ignat'eva et al., 2007).

Contact cytotoxicity

MRC-5 human fibroblast cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin. Cyanoacrylate glue served as a positive control and type I collagen was used as a negative control.

Four nerve fragments, each 5 mm long, were selected from the fresh sural nerve and sural nerve decellularized with amphoteric detergent and nuclease, respectively. Samples were attached to the bottom of the plates (6-well culture dish) with gel (rat tail collagen type I, #C3867) for 30 minutes on a clean bench. After washing with PBS, human fibroblasts (1.5×10^5 cells/well) were incubated with nerve fragments at 37°C in a 5% CO_2 cell incubator for 48 hours.

After incubation of the cytotoxic test, culture medium was washed and incubated with fixative solution [4% paraformaldehyde in PBS (Biosesang, Gyeonggi, Korea)] for 1 hour at 4°C. Attached cells were stained with Giemsa solution for 1 hour at room temperature. Each well was then rinsed with PBS five times and dried in a hood. Cell morphology and density were examined at 40× magnification under a Nikon eclipse TS100 phase contrast microscope (Nikon, Tokyo, Japan).

Testing of biomechanical tensile properties

From the male cadaver, three 2.3 cm long nerve fragments were excised from fresh sural nerves and placed in PBS (pH 7.4) and sural nerves were decellularized using the amphoteric detergent and nuclease method, respectively. Nerve fragments were rinsed in PBS and mounted under zero strain in a material testing machine (MCT-1150 model, A&D company, Tokyo, Japan), and the initial length was recorded. The samples were stretched at a rate of 10 mm/min to the point of tensile failure. All tests were conducted at room temperature, and normal saline was applied to keep the sample moist during the procedure. Ultimate load, stress, and strain were measured and compared with the load-displacement curve.

Statistical analysis

The results are expressed as the mean and standard deviation for all variables. Differences between groups were measured using the Student's *t*-test in GraphPad Prism software (Version 5.01, GraphPad, San Diego, CA, USA). Differences were considered statistically significant at *P* values of 0.05.

Results

Cell removal of nerves using two different methods

The remaining cellular components were identified by H&E staining (**Figure 2**). Using nonionic and anionic detergent, the cellular components were well removed from the sural nerve (**Figure 2E**), whereas the cellular components remained in the median nerve (**Figure 2B**). Groups C and F using amphoteric detergent and nuclease showed almost complete removal of cells. The cellular components visible after DAPI staining are shown in **Figure 3**. The quantified values of H&E and DAPI staining, with cell removal, showed similar trends in each group. In the DAPI analysis, groups C and F using amphoteric detergent and nucleases and group E using nonionic and anionic detergent showed almost complete removal of cells (**Figure 3**). Group B showed significantly higher cellular components than the other median nerve groups. The DNA contents of groups C and F were significantly lower than in the other groups treated with nonionic and anionic detergent method (**Figure 4A**).

In order to compare the degree of removal of Schwann cells, immunohistochemical staining was performed using the S-100 antibody. Results from decellularization with nonionic and anionic detergent showed that a considerable number of Schwann cells remained in the median nerve and partially stained cells in the sural nerve. On the other hand, Schwann cells were removed completely from both the median and sural nerves using the amphoteric detergent and nuclease decellularization method (**Figure 4B–G**).

Extracellular matrix integrity

The mean scores for basal lamina using laminin immunohistochemical staining are shown in **Figure 5**. The structural integrity of median and sural nerves decellularized using amphoteric detergent and nuclease was better preserved than that of median and sural nerves decellularized using nonionic and anionic detergent (**Figure 5**). The collagen content of groups B and E was significantly lower than that of groups A and D (**Figure 6**). The collagen content in the median and sural nerves decellularized by the amphoteric detergent and nuclease was slightly, but not significantly, lower than that in the fresh median and sural nerves (**Figure 6A**).

MT staining was performed to visualize the distribution of collagen in the median and sural nerves. Blue collagen, black nuclei, and red cytoplasm were observed in the fresh median and sural nerve. Result of MT staining showed that a larger amount of blue collagen was preserved in the groups C and F, in which amphoteric detergent and nuclease were used, than that in the groups B and E, in which the nonionic and anionic detergents were used (**Figure 6B–G**). The distribution of the myelin sheath and neurons was compared with Luxol Fast blue-cresyl violet staining. In the fresh median and sural nerve, myelin stained blue green and neurons stained violet were observed. Significantly reduced myelin was observed in the groups C and F (**Figure 6H–M**).

Contact cytotoxicity

Analysis of contact cytotoxicity revealed that inhibition of cell proliferation or lysis was not observed in either fresh or decellularized sural nerve fragments (**Figure 7**).

Maintenance of the mechanical strength

There was no significant difference in the ultimate load, stress, and strain between the fresh and decellularized sural nerves (**Table 1**).

Table 1 | **Biomechanical evaluation of fresh and decellularized sural nerves from cadavers**

Group	Ultimate load (N)	Ultimate stress (MPa)	Ultimate strain (%)
Fresh sural nerve	10.26±1.021	2.637±0.315	0.471±0.036
Decellularized sural nerve	14.27±3.166	2.903±0.647	0.523±0.060

There was no statistically significant difference in the decellularized nerve group using the amphoteric detergent-nuclease method when compared to the fresh nerve group. All data are expressed as the mean ± SD (*n* = 3/group).

Discussion

Decellularization had been attracting attention as an alternative to autografts and synthetic replacements in the past two decades (Zilic et al., 2016; Boriani et al., 2017). Decellularization is the formation of a neural scaffold, which removes cellular components while preserving the ultrastructure and ECM components (Zilic et al., 2016; Philips et al., 2018). The basal lamina and ECM play important roles in the development of peripheral nerves and in the repair of injured peripheral nerves (Spivey et al., 2012). The basal lamina provides a pathway for axonal elongation as well as maintenance and maturation of regenerating axons (Ide et al., 1983). The ECM not only influences cell morphology, phenotype, and function but also promotes cell-cell interactions (Zilic et al., 2016).

In this study, we compared amphoteric detergents and nucleases with nonionic and anionic detergents, using a modification of the Sondell method based on a previous study (Shin et al., 2019). Most of the decellularization methods using chemical detergents are based on Sondell and Hudson's methods (Sondell et al., 1998; Hudson et al., 2004). These two methods with some modifications were used in clinical studies (He et al., 2015; Zhu et al., 2017). The decellularization method devised by Sondell is a combination of an ionic

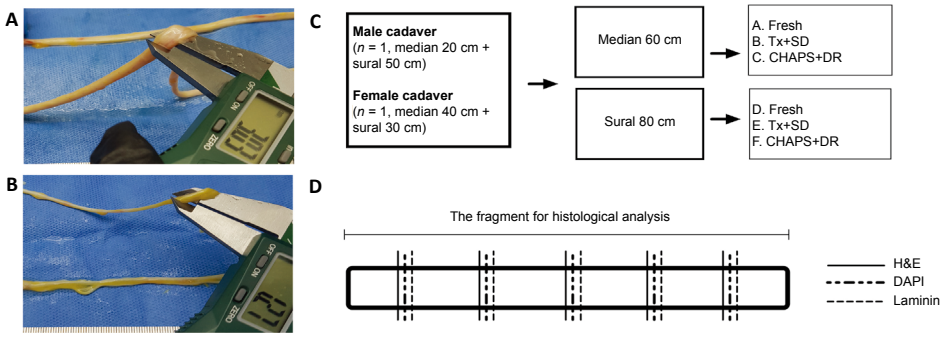


Figure 1 | Preparation of the median and sural nerves and of nerve fragments for staining.
(A) Harvested median nerve. (B) Harvested sural nerve. (C) Schematic diagram of grouping. The number of segments per group is as follows: n = 8 segments each for groups A–D and n = 12 segments each for groups E and F. (D) Section diagram of a single nerve showing histological analysis. CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; DAPI: 4′,6-diamidino-2-phenylindole; DR: Dnase + Rnase; H&E: hematoxylin and eosin; SD: sodium deoxycholate; Tx: triton X-100.

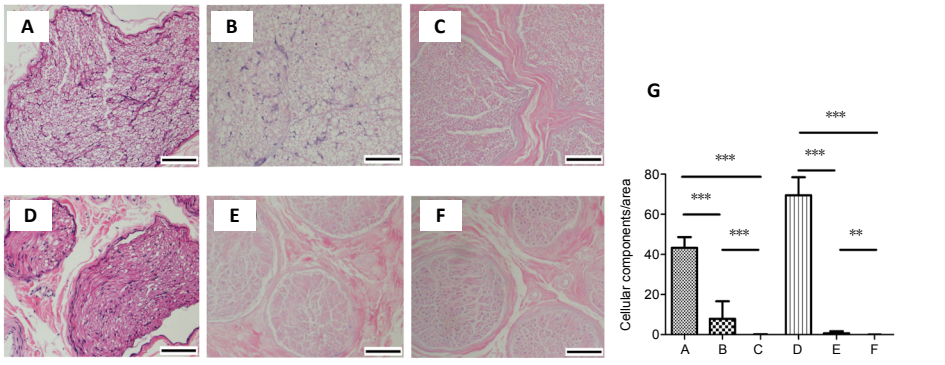


Figure 2 | Hematoxylin-eosin staining results of median and sural nerves decellularized by two different methods.
(A–F) Both non-ionic and anionic detergents showed satisfactory cell removal in the median (Group B) and sural (Group E) nerves. The amphoteric detergent and nuclease showed almost complete cell removal in both the median (Group C) and sural (Group F) nerves. Groups A and D refer to fresh median and sural nerves, respectively. Original magnification, 100×; scale bars: 100 μm. (G) Quantification of cellular components. Data are presented as the mean ± SD, n = 5. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's t-test). H&E: Hematoxylin and eosin; SD: standard deviation.

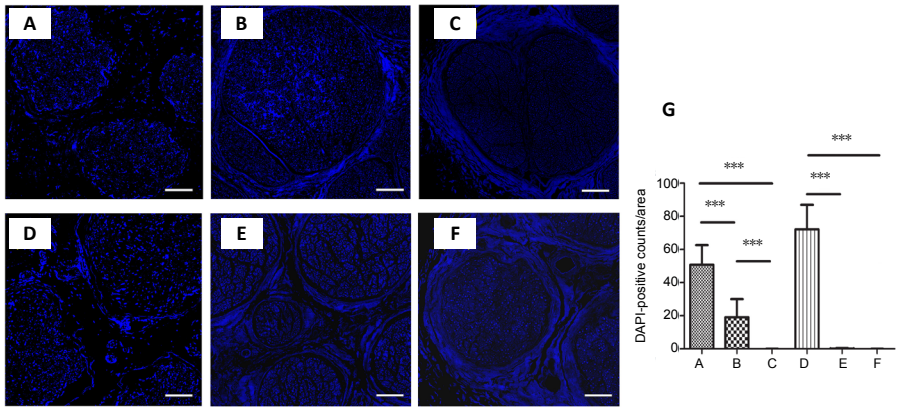


Figure 3 | Comparison of DAPI staining of median and sural nerves between two decellularization methods.
(A–F) Treatment with amphoteric detergent and nuclease showed almost complete cell removal in both the median (Group C) and sural (Group F) nerves. Additionally, in the context of the use of nonionic and anionic detergents, the sural nerve showed almost complete cell removal (Group E), whereas a significantly higher number of cells remained in the median nerve (Group B). Blue color indicates remnant cellular components. Groups A and D refer to fresh median and sural nerves, respectively. Original magnification, 100×; scale bars: 100 μm. (G) DAPI-positive counts. Data are presented as the mean ± SD, n = 5. $^{***}P < 0.001$ (Student's t-test). DAPI: 4′,6-Diamidino-2-phenylindole.

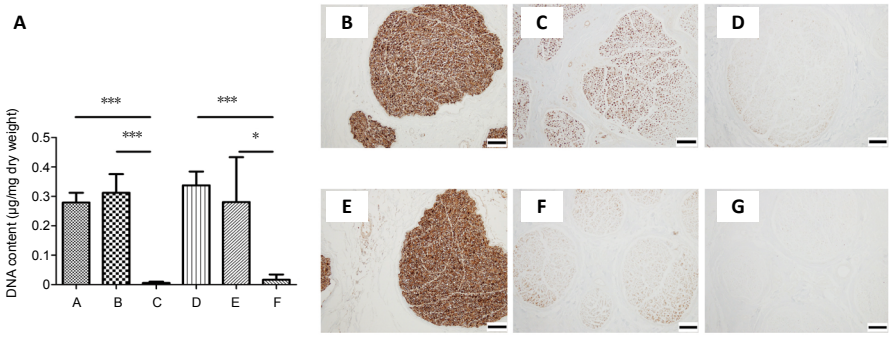
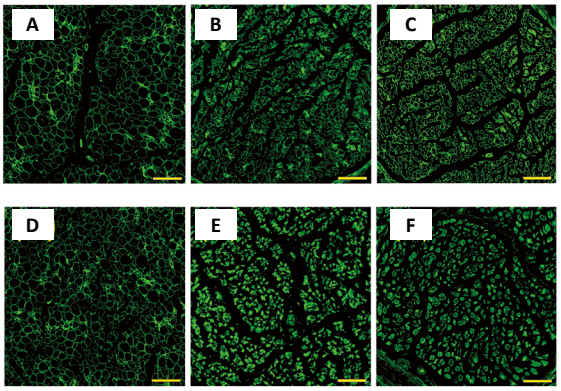


Figure 4 | DNA content and S-100 immunohistochemistry of median and sural nerves decellularized by two different methods.
(A) Comparison of the DNA content of sural and median nerve between two decellularization methods. Median and sural nerves decellularized using amphoteric detergent and nuclease showed significantly lower DNA contents (Group C and F) than median and sural nerves decellularized using nonionic and anionic detergent (Groups B and E). Data are presented as the mean ± SD, n = 5. $^{*}P < 0.05$, $^{***}P < 0.001$ (Student's t-test). (B–G) S-100 immunohistochemistry of the sural and median nerves decellularized by two different methods. Schwann cells were removed almost completely in the median and sural nerves treated with amphoteric detergent and nuclease. Original magnification, 100×, scale bars: 100 μm.



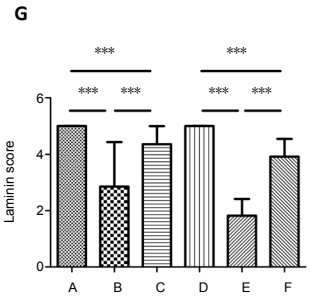


Figure 5 | Immunostaining against laminin of median nerves and sural nerves decellularized by two different methods.
(A–F) Groups A–C: Median nerve; Groups D–F: sural nerve; Groups A and D: fresh nerve tissues; Groups B and E: nonionic and anionic detergent treatment; Groups C and F; amphoteric detergent and nuclease treatment. The median nerve (Group C) and sural nerve (Group F) treated with amphoteric detergent and nuclease showed better preservation of the structural integrity than those treated with nonionic and anionic detergents (Groups B and E). Original magnification, 200×. Scale bars: 50 µm. (G) Quantification of laminin expression. Data are presented as the mean ± SD, $n = 5$. ***$P < 0.0001$ (Student's t-test).

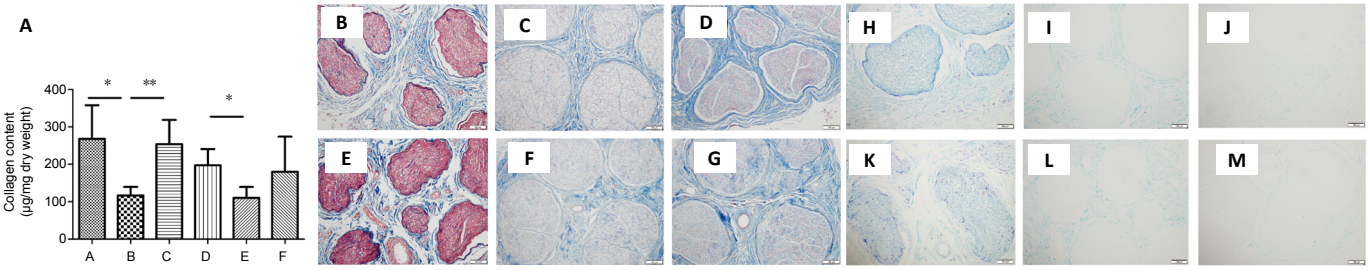


Figure 6 | Comparison of collagen content on median and sural nerves decellularized using two different methods after collagen myelin staining.
(A) Comparison of collagen content of sural and median nerves (hydroxyproline assay). Groups A-C: median nerve; Groups D-F: sural nerve; Groups A and D: Fresh nerve tissue; Groups B and E: nonionic and anionic detergent; Groups C and F; amphoteric detergent and nuclease. Data are expressed as the mean ± SD, $n = 7$. *$P < 0.05$, **$P < 0.01$ (Student's t-test). (B–G) Masson's trichrome staining. Median (D) and sural (G) nerves treated with amphoteric detergent and nuclease show stronger blue staining (collagen) than those decellularized with nonionic (C) and anionic detergents (F). (H–M) Luxol fast blue-cresyl violet staining. The median and sural nerves treated with amphoteric detergent and nuclease (J, M) showed significantly reduced myelin content (blue-green color) than those decellularized with nonionic and anionic detergents (I, L). Original magnification, 100×. Scale bars: 100 µm.

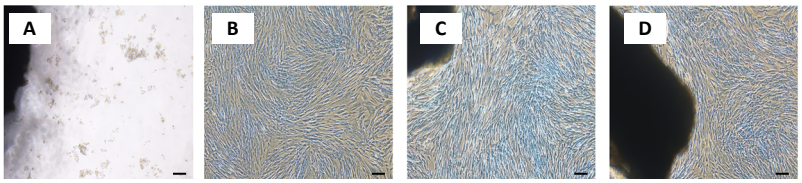


Figure 7 | Contact cytotoxicity assay of sural nerve decellularized with amphoteric detergent and nuclease.
(A) Cyanoacrylate glue. (B) Collagen gel. (C) Fresh surface nerve segment. (D) Sural nerve segment decellularized by amphoteric detergent and nuclease. Original magnification, 40×. Scale bars: 1 mm. The images show that the decellularized nerves (D) are harmless to normal human fibroblasts.

detergent (sodium deoxycholate) and a nonionic detergent (Triton X-100). Hudson's method consists of an ionic detergent (Triton X-200) and two amphoteric detergents (SB-10 and SB-16), although Triton X-200 has been discontinued and is currently unavailable for experiments (Philips et al., 2018).

In the present study, amphoteric detergent and nuclease showed better cell removal than the nonionic detergent and anionic detergent method. Amphoteric detergent and nuclease showed almost complete removal of cells in both the median and sural nerves, while nerve tissue processed using nonionic and anionic detergent method showed remaining cells in the median nerve. Thus, it appears that amphoteric detergent and nuclease more effectively remove cells in larger-diameter nerves. In addition, amphoteric detergent and nuclease have the advantage of maintaining ECM and basal lamina over nonionic and anionic detergent methods. Previous *in vitro* studies on decellularization have mainly used tissues of animals, especially small animals, and no studies have been conducted on human peripheral nerves. This study has important implications in that it was performed using human peripheral nerves directly obtained from cadavers.

Recently, some authors reported clinical results of peripheral nerve reconstruction using decellularized nerve grafts (He et al., 2015; Zhu et al., 2017). He et al. (2015) used a graft processed with a modified protocol based on Sondell's method and reported that a human acellular nerve graft is safe and effective. However, most gap lengths were below 3 cm. Similarly, Zhu et al. (2017) also used allografts decellularized by Sondell's method, and significant functional recovery was observed in groups with gap lengths below 3 cm. These results do not seem to be different from previous animal studies that showed poor outcomes in allografts with the diameter of a defect of 14 mm or longer (Whitlock et al., 2009; Moore et al., 2011).

Nucleases are enzymes and are another effective option for decellularization. These agents catalyze the hydrolysis of DNA and RNA chains and aid in the complete removal of cells. Although nucleases alone have limited cell removal abilities, they demonstrate better decellularization in various tissues when coupled with detergents (Wang et al., 2014; Mangold et al., 2015; Sridharan et al., 2015; Kasbekar et al., 2018; Vafaee et al., 2018). Various decellularized tissues, such as the conjunctiva, cardiac valve, and amniotic membrane, were developed with a combination of detergent and nuclease (Perea-Gil et al., 2015; Kasbekar et al., 2018; Vafaee et al., 2018). Additionally, Mangold et al. (2015) reported that nuclease treatment is not only required to remove nucleic acid in all cases, but also to obtain a cleaner scaffold for vascular tissue engineering using human umbilical vein. Sridharan et al. (2015) reported the usefulness of a combination of amphoteric detergent and nuclease in peripheral nerves. However, the study was conducted with rat sciatic nerves, and a 50% reduction in collagen content was observed in nerve tissues treated with a combination of amphoteric detergent and nuclease. In the present study, we decellularized

peripheral nerves with amphoteric detergent and nuclease. Results showed cell components were reduced, the integrity of ECM was preserved, and there was no significant difference in collagen content between human peripheral cells decellularized with amphoteric detergent and nuclease and fresh peripheral nerves.

The present study, however, has some limitations. First, we did not compare amphoteric detergent and nuclease with other methods except the detergent-based method. Cold preservation is one of the most studied decellularization methods (Zalewski and Gulati, 1982; Ide et al., 1983; He et al., 2015), but prolonged processing time and poor mechanical properties of friable acellular grafts limit the clinical application of cold preservation techniques and make it available for research only (Crapo et al., 2011). Freezing and repeated freeze–thaw cycles have also been studied extensively, although they produce irreversibly damaged residual cells, resulting in an immune response (Ide et al., 1983; Evans et al., 1998; Szynkaruk et al., 2013). Second, this study was performed *in vitro*. Despite several previous studies on decellularization showing benefits with their devised methods (Kim et al, 2016; Zilic et al., 2016), *in vivo* studies have not yet demonstrated whether the increase in regenerative capacity improves functional nerve regeneration and recovery after transplantation (Boriani et al., 2017; Shin et al., 2019). Third, this study was conducted with nerves from a small number of cadavers.

In summary, we studied a novel method of processing decellularized human nerve grafts using amphoteric detergent and nuclease and showed superior results compared to the established detergent-based method in terms of cell removal and ECM integrity in human nerve tissues. Furthermore, this study revealed that the amphoteric detergent and nuclease method could be effectively used even in large-diameter human peripheral nerves.

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Institutional review board statement: *Donated cadavers were used in this study. All consents for approval of research were managed by the Korea Public Tissue Bank. And in general, research using cadaver is not subject to the Institutional Review Board (IRB). Since our research was not conducted on living persons, although we submitted the protocol to the IRB, the review was not conducted. Please note that in Korea, cadaveric studies are not yet legally subject to IRB review.*
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Additional file:
Additional Table 1: *Scoring system for basal lamina integrity using laminin immunohistochemical staining.*

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Additional Table 1 Scoring system for basal lamina integrity using laminin immunohistochemical staining

Score	Immunostained nerve fiber area for laminin
5	Comparable to fresh nerve fiber
4	≥ 75% of fresh nerve fiber
3	≥ 50% but < 75% of fresh nerve fiber
2	≥ 25% but < 50% of fresh nerve fiber
1	< 25% of fresh nerve fiber