Original Article

An Effective Method for Decellularization of Human Foreskin: Implications for Skin Regeneration in Small Wounds

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Abstract

Objective: Acellular matrices of different allogeneic or xenogeneic origins are widely used as structural scaffolds in regenerative medicine. The main goal of this research was to optimize a method for decellularization of foreskin for skin regeneration in small wounds.

Materials and Methods: In this experimental study, the dermal layers of foreskin were divided into two sections and subjected to two different decellularization methods: the sodium dodecyl sulfate method (SDS-M), and our optimized foreskin decellularization method (OFD-M). A combination of non-ionic detergents and SDS were used to decellularize the foreskin in OFD-M. The histological, morphological, and biomechanical properties of both methods were compared. In addition, human umbilical cord mesenchymal stem cells (hucMSCs) were isolated, and the biocompatibility and recellularization of both scaffolds by hucMSC were subsequently determined.

Results: We observed that OFD-M is an appropriate approach for successful removal of cellular components from the foreskin tissue, without physical disturbance to the acellular matrix. In comparison to SDS-M, this new bioscaffold possesses a fine network containing a high amount of collagen fibers and glycosaminoglycans (GAG) (P≤0.03), is biocompatible and harmless for hucMSC (viability 91.7%), and exhibits a relatively high tensile strength.

Conclusion: We found that the extracellular matrix (ECM) structural integrity, the main ECM components, and the mechanical properties of the foreskin are well maintained after applying the OFD-M decellularization technique, indicating that the resulting scaffold would be a suitable platform for culturing MSC for skin grafting in small wounds.

Keywords: Decellularized Scaffolds, Foreskin, Mesenchymal Stem Cell

Introduction

For decades, skin grafts have been used to restore wound healing defects following trauma, vascular diseases, and cancer (1). Skin grafts can reduce the complexity of treatment and minimize the risk of infection and hospitalization, to achieve satisfactory repair results in patients with chronic or recurrent ulcers (2). However, the availability of adequate healthy skin, remains as an overwhelming obstacle. In addition, scar formation and deformity of the donor's skin is a problem that must be considered. To address these challenges, scientists and surgeons work collaboratively to develop appropriate bioengineered and synthetic alternatives that promote the healing of the wounded skin (1). Moreover, it has been shown that when scaffolds are located within the defect, they protect against dehydration, contaminants, and microorganisms (3).

Acellular dermal matrix (ADM) is one of the most widely used grafts in skin transplantation today. ADM can be derived from various allogeneic or xenogeneic sources, such as human cadavers and animal dermises (4). Decellularized extracellular matrix (ECM) from the target tissue is the ideal scaffold for tissue engineering. Decellularization technique, in which most cell components are removed by physical and/ or chemical procedures, enables researchers to obtain cell-free, natural ECM that is primarily made up of collagen and glycosaminoglycans (GAGs). ECM retention, especially the maintenance of structural integrity of the collagen mesh, is very important for the effectiveness of recellularization (5). Collagen provides tensile strength and promotes cell migration and tissue development by controlling cellular adhesion. In fact, since collagen has high electrostatic properties, it draws water in the interstitial spaces and causing it to inflate (6, 7). Thus ,it is important to take advantage of such characteristics to achieve a scaffold with ideal physicochemical properties for effective tissue regeneration.

A well-known technique in regenerative medicine

involves the use of a tissue scaffold in combination with stem cells. Because of their trophic and paracrine functions, mesenchymal stem cells (MSCs) can improve cutaneous regeneration. Specifically, they can improve wound healing by enhancing keratinocyte and fibroblast migration following transplantation (8). MSCs secrete several cytokines and growth factors [e.g., vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), stromal cell derived factor 1 (SDF-1), and angiopoietin-1], which can act as chemo-attractants to induce endogenous cell recruitment to the injured tissue (9). The human umbilical cord is considered to be an important and non-invasive source of MSCs (10). While these cells may have a valuable impact on healing, the harsh environment of the wound site limits the grafting, retention, and transplanted cells' survival rate. Thus, the delivery of cells into the wound site through scaffold-based cell applications remains a significant challenge in regenerative medicine (11).

The multifaceted nature of human foreskin, which is a highly vascularized and densely innervated bilayer tissue, is comparable to that of few other parts of the human anatomy. Foreskin circumcision has ancient roots, but its modern version dates back to the early 19th century (12). Foreskin circumcision is performed on most male children in Iran, providing an abundant supply of waste tissue that can be used as a skin graft without damaging the donor. Consequently, in the present study, we compare two methods of decellularization, with the goal of optimizing a technique for creating a foreskin acellular matrix (FAM).

Materials and Methods

Foreskin acellular matrix preparation

In this experimental study, the foreskin samples used in this study were from boys aged one month to four years, and were obtained from Imam-Ali Clinic, Shahre-Kord, with parental informed consent. Specimens were transferred to the laboratory in phosphate-buffered saline (PBS, BIO-IDEA, Iran) containing 10% gentamicin and stored for 6 hours at 4°C. The outer adipose layer was then removed using a scalpel after washing the specimen in PBS. The isolated dermal layers were divided into two parts and subjected to two decellularization methods: the optimized foreskin decellularization method (OFD-M), and the sodium dodecyl sulfate method (SDS-M, Sigma St. Louis, MO), which are both described below.

The SDS-M method was previously reported elsewhere (13). Briefly, samples were placed in SDS 1% in deionized water, and fresh SDS/H_2O media were substituted every 6 hours for three days until the sample color was completely transparent. Finally, specimens were placed in PBS for 2

hours to remove nuclei, cell residues, and detergents from the tissue.

For the OFD-M, the samples were placed in 5% SDS and incubated for 6 hours, then washed for 1 hour in distilled water. In the next step, 0.05% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA, Life Technologies, Carlsbad, CA), were added to the samples. The specimens were incubated for 6 hours, followed by a wash with Hank's balanced salt solution (HBSS, BIO-IDEA, Iran). The tissues were digested by Triton-X100 (1%) for 1 hour (Bio Basic Inc. cas # 9002-93-1). All the above steps were performed on a shaker (IKA-WERKE Hs501, 90 rmp), and the solutions were replaced every 2 hours. Finally, the tissue was submerged in the HBSS solution at 4°C for 48 hours to remove residual SDS (Fig. S1, See Supplementary Online Information at www. celljournal.org) (14).

Both groups of FAM samples were rinsed several times with distilled water and subsequently stored in 75% alcohol at -20°C. Prior to testing, samples were subjected to UV radiation for 20 m.

Colorimetric detection of sodium dodecyl sulfate

The amount of SDS remaining in the scaffolds was measured during the experiments for each method, using a colorimetric assay with methylene blue. First, the standard curve was obtained by measuring the optical density (OD) (Fig.S2A, See Supplementary Online Information at www.celljournal.org). Next, to determine the amount of residual SDS in the tissues, 1 g of FAMs was weighed and prepared according to the method explained by Alizadeh et al. (15). Finally, each sample's OD was measured at 650 nm (microplate reader, STAT FAX 2100), and a standard curve was used to determine the amount of SDS in each sample.

Histological analysis

To assess the decellularization procedure's efficiency regarding ECM integrity and the removal of cell components, the samples were fixed in 10% formalin solution and then embedded in paraffin. Then three 5 to 6 μm thick serial sections were prepared and mounted on slides for further analysis (microtome Leitz1512, Ramsey, USA). The slides were stained with hematoxylin and eosin (H&E) as follows: the samples were first immersed in hematoxylin and acid-alcohol for 6 minutes and 30 seconds, respectively. After washing with distilled water, the sections were submerged in eosin for 1 minute. Finally, serially diluted alcohol (70°, 90°, and 100°) were used for dehydration. The number of residual cells was then observed in three microscopic fields of each stained slide using a 10x objective lens. to analyze the collagen and elastic fibers the sections were also stained with Masson's trichrome following a standard protocol

(16). Briefly, the slides were immersed in Bouin's fluid overnight at room temperature, then rinsed with water and placed in Weigert's haematoxylin solution for 5 minutes, then immersed in Biebrich scarlet-acid fuchsin for 15 minutes. After that, they were rinsed in water and placed in phosphomolybdic/phosphotungstic acid for 15 minutes, then aniline blue stain for 10 minutes, and finally rinsed in water. The slides were finally placed in 1% acetic acid for 5 minutes and dehydrated by rinsing with 100% ethanol.

To assess the efficacy of cell removal and the number of remaining nuclei in decellularized scaffolds in each method, samples were stained with the DNA staining dye Hoechst 33258 (Sigma-Aldrich Corp., MI, USA). After preparation of paraffin-embedded sections, deparaffinization was performed, and the samples were stained by 1:5000 diluted Hoechst in PBS for 30 seconds in a dark room. Finally, the slides were washed with PBS and then visualized using a fluorescence microscope (Nikon-TS-100F, Tokyo, Japan). The collagen content was analyzed using ImageJ software (NIH, Bethesda, MD) as previously reported (17).

DNA quantification

The amount of remaining DNA in the decellularized samples was measured by Geno PlusTM Mini extraction kit (GG2001, Viogene, Taipei, Taiwan). Specifically, 30 mg of each dried FAM and native tissue samples were homogenized in 180 μL of lysis buffer, and after adding 20 μL of proteinase K, the rest of the protocol was carried out in accordance with the manufacturer's instructions (GG2001). Finally, DNA was dissolved in trace HCl (10 mM, pH=9), and the quality and quantity of DNA was evaluated by nanodrop 2000 (Thermo Scientific, USA).

Morphological analysis by scanning electron microscopy

To determine the pore sizes and ensure that the ECM structure has remained intact, the samples were analyzed by scanning electron microscopy (SEM). The samples were fixed in 2.5% glutaraldehyde fixative solution (Sigma-Aldrich, USA) for 90 minutes at room temperature, then washed with PBS to remove the fixative solution. Dehydration was carried out through rinsing the samples with serially diluted ethanol (30, 50, 70, 80, 90, and 100%); 10 minutes for each concentration (18). Finally, the samples were dried, fixed on SEM stubs, and coated with gold (Desk Sputter Coater-DSR1). The samples were then subjected to SEM (Philips XL 30, North Billerica, MA) examination, and multiple images were taken using fluorescence microscope.

Glycosaminoglycans quantification

The GAG content is an indicator of the degree of structural modification in decellularized tissue (18).

The standard curve was drawn using the chondroitin 6-sulfate kit (KGAG96, Kiazist, Iran) according to the manufacturer's instructions (Fig.S2B, See Supplementary Online Information at www.celljournal.org). Next, 20 mg of each sample (dried FAMs and native) individually was digested through incubation with 400 μL papain at 65°C for 16 hours, then centrifuged at 8000g for 15 minutes. After that, the supernatants were transferred into 1.5 mL eppendorf tubes and 50 μL of protein precipitant was added to each tube and centrifuged at 8000g for an additional 15 minutes. Finally, 200 μL of GAG reagent was added to 30 μL of each sample or the standard solution, in triplicates (96 well plate). Finally, the OD of the test was obtained at 510-560 nm using a microplate reader (Stat Fax 2100, Awareness Technology, Palm City, FL).

Collagenase assay

One of the most reliable and cost-effective methods for the measurement of collagen content is biochemical calculation of hydroxyproline (19). The amount of hydroxyproline in the decellularized samples was determined by employing the Kiazist Kit (KHPA96, Kiazist, Tehran, Iran) according to the manufacturer's instructions. First, the standard curve was obtained according to the kit instructions (Fig.S2C, See Supplementary Online Information at www.celljournal. org). Next, 20 mg of native and dried FAM samples were homogenized in 100 μL H_2O plus 100 μL of 12M HCL and incubated for 4 hours at 120C. Oxidation solution and activated charcoal were then added to the samples, and the mixture was centrifuged at 12000g for 15 minutes. The supernatants were harvested, and the OD of the test was obtained at 540-560 nm using the microplate reader in triplicates.

Biomechanical properties: tensile strength

To determine the mechanical properties of the scaffolds, FAMs, and native foreskins were tested by a tensile-test device (TANSAM. STM-1). Briefly, samples of size 2×5 cm were prepared and clamped to the sample holders, then stretched at a speed of 10 mm/minutes. until tearing. The stiffness (N/m), elastic modulus, and maximum force (N) were determined from the resulting stress-strain curve.

Isolation of mesenchymal stem cells from human umbilical cord

The protocol was approved by the Ethical Research Committee of Kurdistan University of Medical Sciences (KUMS), according to the criteria set by the declaration of Helsinki and numbered as #IR.MUK.REC.1397.220. Informed consent was obtained from all the participating women (for the umbilical cord). In the next step, the human umbilical cord mesenchymal stem cells (hucMSCs) were isolated from the entire umbilical cord

using the modulated explant/enzyme method (MEEM) and characterized as previously described (20). The expression of CD29, CD90, CD105, CD34, and CD45 on the cell surface of hucMSCs was analyzed by flow cytometry. Furthermore, hucMSCs were subjected to differentiation into adipogenic, osteogenic, and chondrogenic cell phenotypes (Fig.S3, See Supplementary Online Information at www.celljournal.org). Finally, the isolated cells were cultured in a T75 culture flasks (SPL, Pocheon-si, South Korea) containing Dulbecco's modified Eagle's medium-low glucose (DMEM, Gibco, Thermo Fisher Science, US) supplemented with 13% fetal bovine serum (FBS, Gibco, USA) and 100 IU/mL-100 mg/mL penicillin-streptomycin (Gibco, USA). The flasks were placed in a humidified incubator with 5% CO_2 at 37˚C. Every 2 days, the medium was replaced with fresh medium until the cells were confluent, then they were detached using 0.25% trypsin-0.04% EDTA (Gibco, USA) for cell passages.

Cell /Contact cytotoxicity assay

The biocompatibility/cytotoxicity of FAMs was evaluated through the co-incubation of scaffolds with hucMSCs. Specifically, 2×10^5 cells/well of hucMSCs were seeded in a six-well plate and were allowed to grow until reaching confluency. Then, native tissue and both groups of FAMs (3 mm^2) were placed inside each well. Tissues and medium were subsequently removed from the wells, at time intervals of 48 hours and 72 hours, respectively to evaluate the cytotoxicity of FAMs. Next, 1 ml of FBS-free medium containing 50 μL of resazurin (Kiazist, Iran) was added to each well and the samples were incubated for 4 hours at 37˚C. Finally, the OD was measured at 520-570 nm in a microplate reader (State Fax 2100), and the viability percentage of each of the three groups of hucMSCs was analyzed.

Recellularization of FAMs with hucMSCs

The hucMSCs were seeded in a six-well plate (2×10^5 cells/well) in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin (Pen/Strep) and subsequently incubated at 37°C and 5% CO_2. The optimized foreskin decellularization FAM (OFD-FAM) samples were washed in PBS, cut into 10 mm pieces, and placed on hucMSCs for 24 hours. The resulting samples were monitored for 8 days, and recellularization was investigated by H&E and Hoechst staining. Furthermore, the morphology and the attachment of the hucMSCs onto the surfaces of FAMs were observed by SEM.

Statistical methods

Each experiment was performed three times, and the mean values of the results were evaluated. All data were analyzed using SPSS statistical software (18.0 version, SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Tukey's post-hoc test were performed to compare datasets. Mean differences were considered significant at P<0.05. All numerical data analyses were performed using GraphPad Prism 8.0 (GraphPad, CA, USA).

Results

Macroscopic observations

Macroscopic evaluation of the intact human foreskin specimens showed a pink appearance with a fatty layer before the decellularization process (Fig.S1A, See Supplementary Online Information at www.celljournal.org). The tissues were white after decellularization via the OFD-M, implying that the epidermoid layers and cells were probably removed, without any physical tissue disturbance or effects on the elasticity (Fig.S1B, See Supplementary Online Information at www.celljournal.org). However, FAMs obtained by SDS-M showed a shrinking appearance with a rough surface and a rigid structure. In contrast, FAMs obtained by OFD-M were smooth and tensile. (Fig.S1C, See Supplementary Online Information at www.celljournal.org), indicating that they might have more collagens and fewer cells compared to the FAMs obtained by SDS-M.

Histological staining and electron microscopic analysis

H&E staining of the two FAM groups revealed a complete lack of an epidermal layer and residual cells in the group of FAMs obtained by the OFD-M and a partial removal of cells by the SDS-M, compared to the intact tissue. It was also found that the ECM is well preserved in FAMs obtained via the OFD-M (Fig.1A). Masson's trichrome staining, which appears blue in the presence of collagen, indicated that collagen is conserved in the OFD-FAM but not in FAMs obtained via the SDS-M (Fig.1B). ImageJ analysis further confirmed well-maintained collagen content in the OFD-FAM group in comparison to the SDS-FAM group (native tissue 65.1%, OFD-M 74.5%, and SDS-M 69.4%).

Furthermore, Hoechst staining confirmed the complete removal of the cells without residual DNA in the OFD-FMS group, whereas this was not the case in the group obtained by the SDS-M (Fig.1C). We further investigated the degree of cell removal, the pore size, and the matrix fibers' structure in the intact tissue and the two FAM groups using a scanning electron microscope. As illustrated by Figure 2, the OFD-FAMs displayed a network of collagen fibers with high structural integrity and appropriate pore size with no deformation or disruption of the tissue and no residual cells. We see in Figure 3 that this was not the case for FAMs obtained by the SDS-M: the matrix, in this case, was highly irregular, and there were residual cells present.
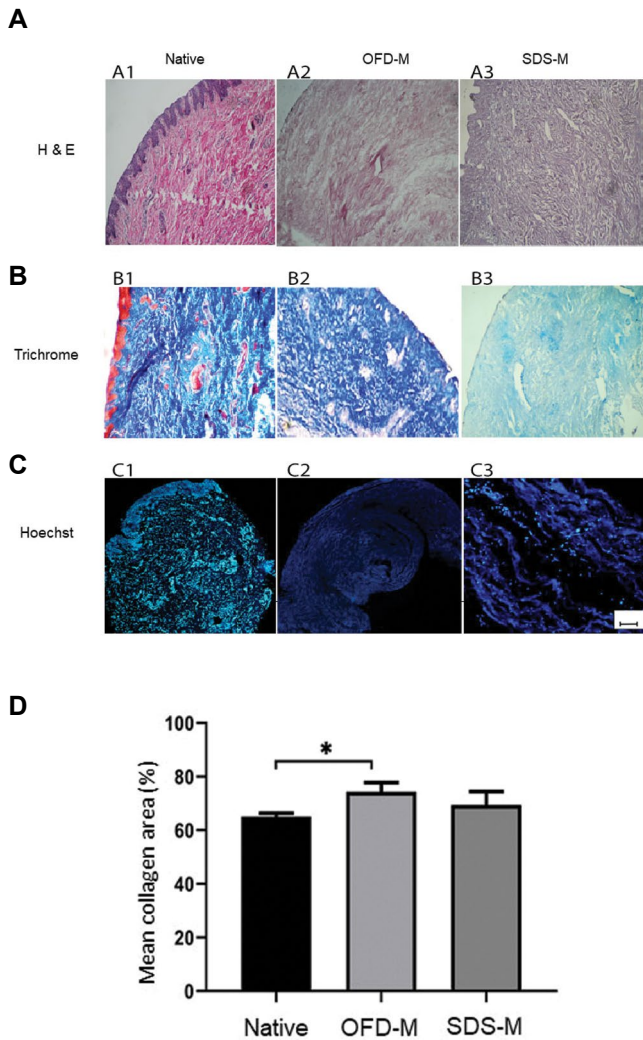
A

B

C

D

Fig.1: Histological staining of FAMs shows the effectiveness of the OFD-M in comparison to the SDS-M. **A1.** H&E staining showed that both cells and the ECM are intact in native samples. **A2.** The OFD-FAM samples possessed a preserved ECM and lacked any residual cells. **A3.** Decellularized foreskin obtained by the SDS-M partially preserved the ECM and contained residual cells. **B1.** Masson Trichrome staining was used to identify changes in collagen intensity (blue staining) of the matrix in the native samples in comparison to the FAM obtained by the **B2.** OFD-M and the **B3.** SDS-M. **C1.** Hoechst staining of the native samples, where the fluorescent color indicates the location of cell nuclei. **C2.** The FAM prepared by the OFD-M did not contain cell nuclei and residual DNA, and **C3.** FAM obtained by the SDS-M contained fluorescent spots which shows residual DNA (scale bar: 100 μm). **D.** Shows the collagen content in the OFD-FAM compared to the SDS-FAM. The data from 4 independent experiments were semi-quantified by ImageJ (*; P≤0.05). FAM; Foreskin acellular matrix, OFD-M; optimized foreskin decellularization method, SDS-M; Sodium dodecyl sulfate method, H&E; Hematoxylin and Eosin, and ECM; Extracellular matrix.

The OFD-FAMs do not show cytotoxic activity against hucMSCs

A resazurin assay was used to evaluate the toxicity of FAMs for hucMSCs. This assay indicated that the time-dependent cell viability percentage of the FAMs obtained byt the SDS-M was significantly lower than the cell viability of FAMs obtained via the OFD-M, especially at 72 hours. Precisely, the viability percentages of the hucMSCs co-cultured with FAMs obtained by the OFD-M were 91.7% and 83.1% at 48 hours and 72 hours, respectively, whereas they were only 83.5% and 66.7% at

48 hours and 72 hours, respectively, in the hucMSCs co-cultured with FAMs obtained via the SDS-M. In addition, there was a statistically significant difference between both of the two FAM groups and the native tissue group (P≤0.001, Fig.3).
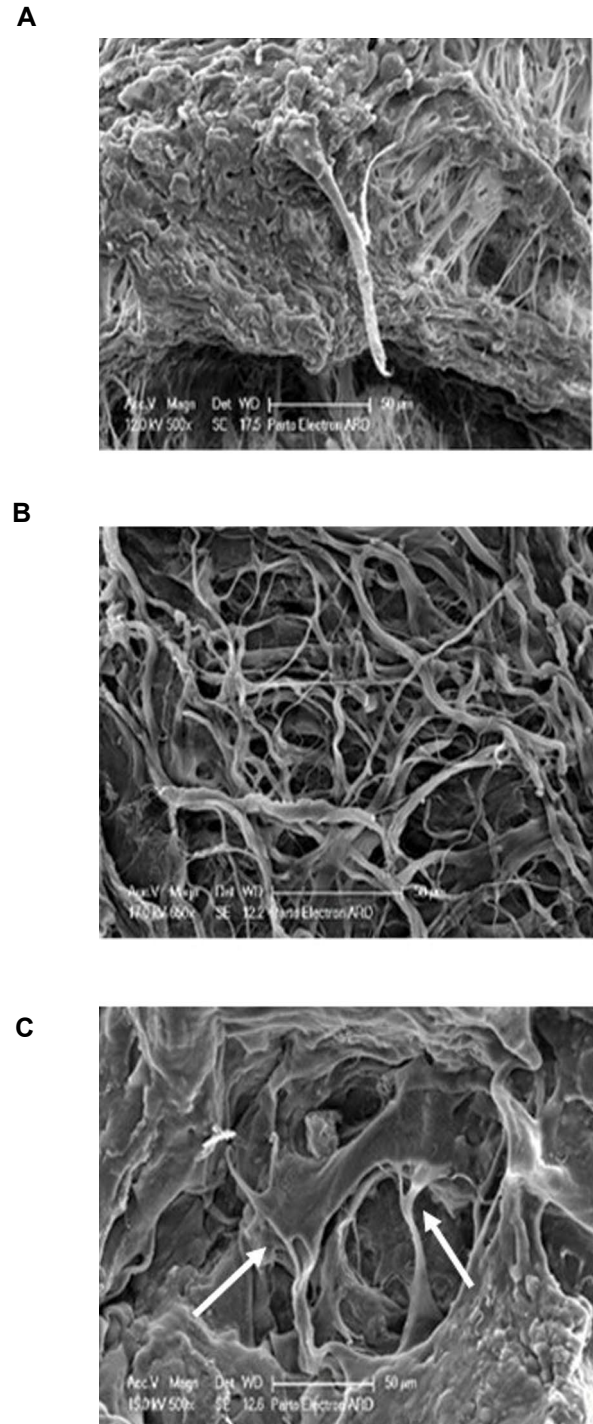
A

B

C

Fig.2: Comparison of the construction of natural and decellularized scaffolds by SEM analysis. **A.** Shows cellular ultrastructure in native tissues. **B.** FAMs obtained via the OFD-M did not contain any cells, and they exhibited an intact matrix and collagen structure. **C.** FAMs obtained via the SDS-M showed an irregular matrix structure. The presence of some cells is shown by arrows (500x, 650x). A representative of at least 3 independent experiments is shown (scale bar show 100 μm in all photos). SEM; Scanning electron microscope, FAM; Foreskin acellular matrix, OFD-M; Optimized foreskin decellularization method, and SDS-M; Sodium dodecyl sulfate method.
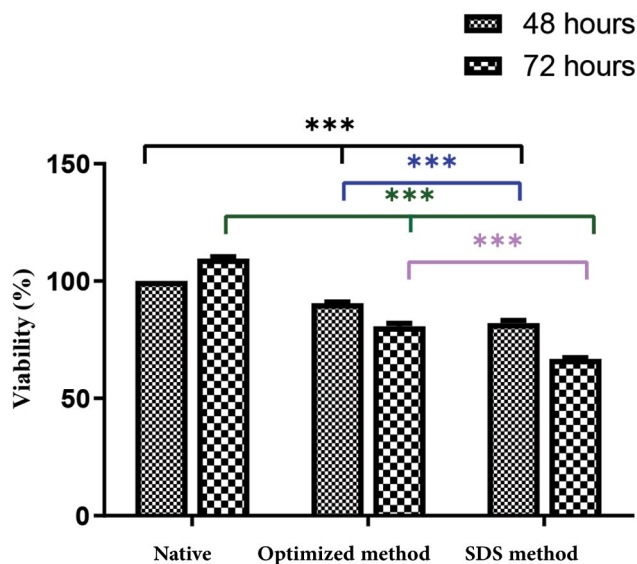
Fig.3: The OFD-M is biocompatible for hucMSCs. The time-dependent cell viability percentage in the FAMs obtained by the SDS-M was significantly lower than the FAMs obtained by the OFD-M (***; P≤0.001). OFD-FAMs showed similar behavior in terms of cell viability when compared to the native group. Pooled data are exhibited as the mean (± standard deviation, SD) of at least four independent experiments, each performed in triplicates. FAM; Foreskin acellular matrix, OFD-M; Optimized foreskin decellularization method, SDS-M; Sodium dodecyl sulfate method, and hucMSCs; Human umbilical cord mesenchymal stem cells.

Analysis of DNA ,SDS ,GAG ,and collagen content

In addition to histology outcomes, the DNA extraction experiments indicated that both the OFD-FAM group and the SDS-FAM group had less DNA than the native tissue. In particular, the DNA content was measured to be 1367 ng/μL in the native tissue, 338 ng/μL in SDS-FAMs, and 86 ng/μL in OFD-FAMs (Fig.4A). These results demonstrated that the OFD-FAMs had the least amount of residual DNA, which indicated that the decellularization processes was most effective in the OFD-M. Furthermore, the colorimetric assay revealed that the amount of residual SDS in the samples obtained via the SDS-M was approximately three folds higher than the OFD-FAMs (Fig.4B).

The remaining collagen and GAGs content were also quantified in both FAM groups. The collagenase assay indicated that the OFD-M approach resulted in the highest amount of collagen content per dry weight of tissue, in comparison to the SDS-M and the native tissue (Fig.4C). The GAG assay indicated that both methods decreased the GAG content in comparison to the native samples, however, the FAMs derived via the OFD-M retained significantly more ECM and GAGs than those perfused with the SDS-M (Fig.4D). These results indicate that the OFD-M is more appropriate for decellularization of foreskin since this method retained the highest amount of major ECM proteins.
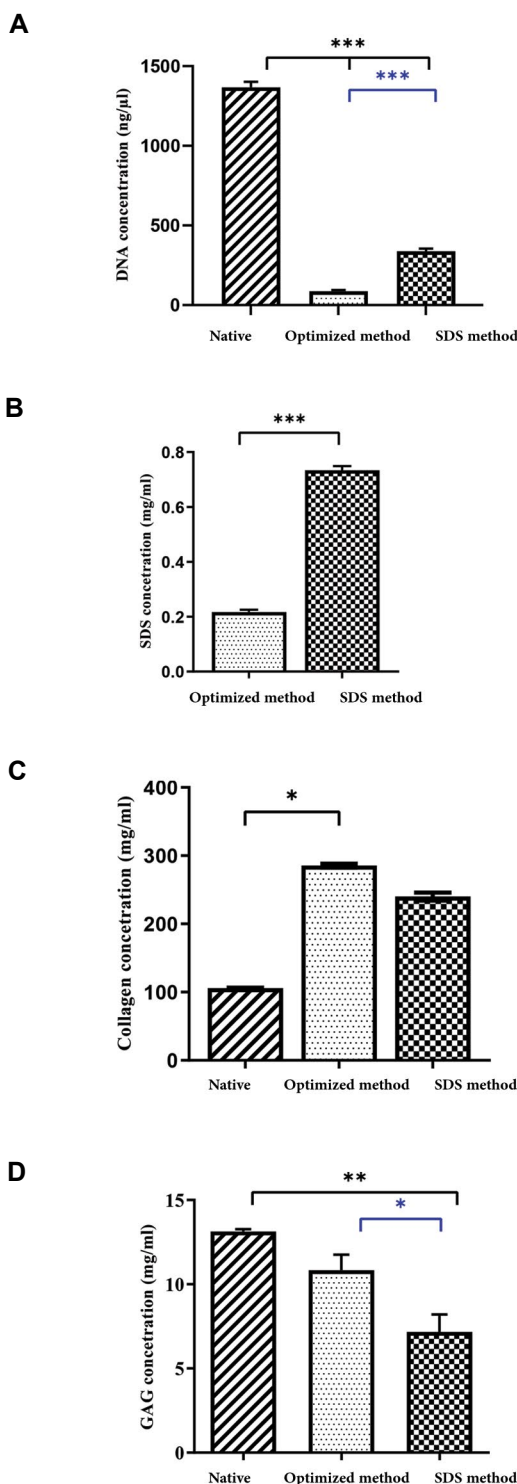

Fig.4: Comparison of the DNA, SDS, hydroxyproline and GAGs content of native tissue and FAM scaffolds. **A.** The DNA content was significantly reduced in the acellular scaffolds in comparison to the native samples. **B.** The content of residual SDS was significantly lower in the OFD-FAM group in comparison to the SDS-FAM group. **C.** A comparison of collagen concentration in the native tissue with the decellularized tissues showed that the FAMs contain a higher concentration of collagen, especially FAMs derived via the OFD-M. **D.** The content of GAGs in the native group compared to the two FAM groups showed that GAGs content was preserved during decellularization. The native foreskin was included as a control. Data with *P≤0.03, **P≤0.002, and ***P≤0.003 were statistically different according to one-way ANOVA and post hoc testing (Tukey's procedure). Pooled data are exhibited as the mean (± standard deviation, SD) of at least four independent experiments, each performed in triplicates. SDS; Sodium dodecyl sulfate method, GAGs; Glycosaminoglycans, FAM; Foreskin acellular matrix, and OFD-M; Optimized foreskin decellularization method.

Mechanical properties of OFD-FAM

In addition to biological efficiency, it is essential for bioscaffolds to have suitable mechanical properties. To address this point, we measured the tensile strength of the samples using a tensile-test device. As shown in Table 1, our data suggested that the native tissue had the highest tensile strength (4.33 ± 0.19 MPa), followed by FAMs derived by the OFD-M (3.66 ± 0.15 MPa), and finally FAMs obtained via the SDS-M (3.21 ± 0.01 MPa). The differences observed between these groups were significant (P=0.002, Fig.S4, See Supplementary Online Information at www.celljournal.org).

Moreover, the experiments indicated that in terms of the stress-strain elongation properties, the OFD-FAMs was similar to the native group (Table 1). Furthermore, the yield strain was shown to be significantly increased in the OFD-FAMs (P=0.01), indicating that the tissue durability had decreased, while at the same time, the elasticity had increased, in comparison to the native tissue.

OFD-FAM recellularization with hucMSCs

It is worth noting that in the recellularization experiments, we did not use seeding or any other technique to deliver cells to the tissues. Rather, we simply placed the OFD-FAMs on top of the cultured hucMSCs for eight days. Interestingly, we observed that hucMSCs were significantly more distributed on the FAMs obtained via the OFD-M compared to the FAMs obtained via the SDS-M. In addition, SEM images demonstrated the adherence of hucMSCs to the FAM surface (Fig.5).

Table 1: Mechanical properties of FAMs obtained by the two methods

	Modulus (Mpa)	Yield stress (Mpa)	Elastic strain	Ultimate tensile strength (Mpa)	Yield strain
Native	11.78 ± 0.68	4.06 ± 0.15	0.34 ± 0.01	4.33 ± 0.19	0.35 ± 0.02
OFD-M	9.66 0.29	3.7 ± 0.2	0.36 ± 0.02	3.66 ± 0.15	0.41 ± 0.02
	P value*: 0.002	P value*: NS#	P value*: NS	P value*: 0.002	P value*: 0.01
SDS-M	9.01 ± 0.01	3.2 ± 0.1	0.34 ± 0.01	3.21 ± 0.01	0.34 ± 0.01
	P value**: 0.0006	P value**: 0.001	P value**: NS	P value**: 0.0002	P value**: NS

FAM; Foreskin acellular matrix, OFD-M; Optimized foreskin decellularization method, SDS-M; Sodium dodecyl sulfate method, Native vs. OFD*; Native vs. SDS**, NS#; Not significant.
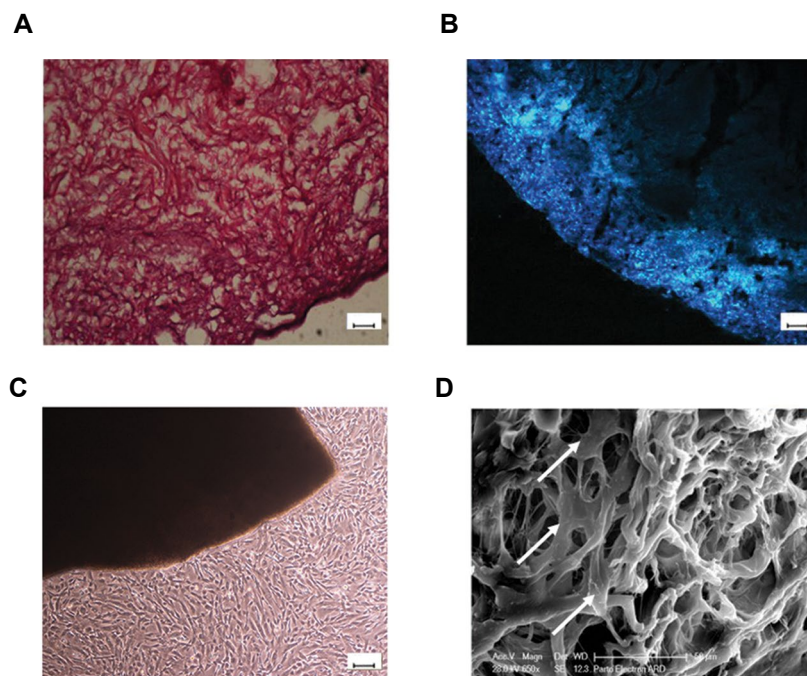

Fig.5: Recellularization of OFD-FAMs on day 8. **A.** H&E staining showed the number of migrated hucMSCs to the acellular scaffold (scale bar: 50 μm). **B.** The presence of cells was also confirmed using nuclear staining by Hoechst (scale bar: 50 μm). **C.** Growth and adherence of hucMSCs to FAMs (scale bar: 100 μm). **D.** SEM images of recellularized FAM on day 8 (650x) (cells are shown by arrows). A representative of at least 3 independent experiments is shown (scale bar: 50 μm).

Discussion

Natural biological scaffolds are commonly used in tissue engineering applications since they are biocompatible and mimic the native cellular microenvironment (20).

However, developing an optimal procedure for removing as much cell debris as possible from the tissue without compromising the structural and mechanical integrity of the ECM remains a challenge. Depending on the process of decellularization, the scaffold will have different properties. Therefore, all of these considerations should be carefully evaluated for designing decellularized scaffolds that are appropriate for tissue-specific stem cell applications (3). In the current study, an effective method for decellularization of the human foreskin was developed so that the resulting bioscaffold could be used as a potential therapy source in skin grafts for small wounds.

Continuous innovations in regenerative medicine have led to the development of various commercially available biological and synthetic substitutes for the reconstruction of skin defects caused by burns, chronic ulcers, and other types of damage (21). In some countries, including Iran, circumcision is a common practice, and the foreskin is considered a wasted tissue (22). The importance of repurposing this wasted tissue as a biological scaffold is compounded by the fact that it possesses components that are anatomically and structurally similar to skin (12). Furthermore, thicker skin grafts contain undesired hair follicles, eventually leading to hair growth in the graft (23). In contrast, the foreskin does not have any hair follicles, and so it has the potential to substitute for small injuries as a skin scaffold while alleviating this problem.

Here we compared two decellularization techniques: the so-called SDS-M and an OFD-M developed in this work to obtain a better scaffold. Other methods of skin decellularization have been reported in the literature (12, 24), and each protocol aims to remove the cells while maintaining the ECM components and the three-dimensional structure of the ECM (25). SDS is a popular detergent for tissue decellularization, which can solubilize both nuclear and cytoplasmic membranes efficiently. It can be considered a standard treatment for complete cell removal and approximately 90% of host DNA in several tissues (26). Treatment with SDS can disrupt protein-protein interactions and lead to protein denaturation, so choosing an optimal concentration of SDS and a suitable exposure time is crucial. In the present study, we developed an OFD-M for decellularization, which used 5% SDS over a limited period of time (6 hours) with Triton X100 and EDTA. This method was found to result in less disruption to the ECM and also better biological activity and cell removal in comparison to the SDS-M, which uses just 1% SDS for about 72 hours.

The FAM samples were evaluated using histology, residual DNA, GAGs, collagen content, cell biocompatibility, and tensile properties. Histological analysis and Hoechst staining showed that the cells were more completely removed in the OFD-FAM group compared to the SDS-FAM group. The findings were supported by H&E results, which revealed a trend that was similar to Hoechst staining. Furthermore, DNA extraction experiments showed that the DNA content was significantly reduced in the OFD-FAM group, compared to the native foreskin tissue.

The extent of disruption to the ECM was also evaluated with the aid of GAG and collagen assays. GAGs play a crucial role in survival, proliferation, differentiation, adhesion, and migration of the cells, so any decrease in GAG content will affect the tissue structure (18). The results of a recent study on decellularization of porcine livers showed that the amount of remaining collagen and GAGs in scaffolds varies, depending on the decellularization method. In particular, scaffolds perfused with Triton-SDS and sodium deoxycholate-SDS were found to retain more ECM and its associated compounds, including total collagen and GAGs, than those perfused with SDS only (27). Our results on human foreskin scaffolds support the findings of those studies. Moreover, collagen content was also quantified in this study using a hydroxyproline assay, to ensure that the decellularization process did not decrease the collagen content (28). As verified by other studies, the increased amount of collagen found in scaffold samples could be attributed to a decrease in sample mass due to the decellularization process (29).

Importantly, our findings indicated that GAG and collagen content in OFD-FAMs were more conserved than in the SDS-FAMs. These results were supported by Masson's trichrome staining (12), which showed that the preserved collagen network in the ECM of the OFD-FAMs was more structurally stable than that in the SDS-FAMs. These findings were substantiated by SEM analysis as well, which revealed that the three-dimensional structures and suitable porosity were well preserved only in the FAMs obtained by the OFD-method. The mechanical resistance of the scaffolds provided useful information regarding its structural integrity under an applied force. The results of the tensile test experiments indicated that during the decellularization process, the scaffold's mechanical strength was preserved.

Conclusion

In this study we have optimized the decellularization process for foreskin and have extensively and effectively removed the cells, while preserving GAG, collagen contents, and the ECM structure. Knowing that MSCs play a crucial role in wound healing, by utilizing these cells in our study, we have introduced a new approach to regenerative medicine. Importantly, hucMSCs were found to be well distributed on the OFD-FAM scaffolds after eight days of co-culture, and the cell viability of the co-culture remained high over the time of the experiment. The results of this study showed that the acellular scaffold obtained from human foreskin has appropriate mechanical properties and structural integrity for use in regenerative medicine, and in particular, for the

reconstruction of human foreskin. Importantly, one of the benefits of using FAM is that it is widely available and is considered a waste tissue after circumcision. In the current study, we found that the structural integrity of the ECM, the major ECM components such as collagen and GAGs, and the mechanical properties of the foreskin are well maintained after the decellularization process, opening up the possibility that FAMs may be well suited for several applications in regenerative medicine, including the treatment of small chronic wounds such as diabetic foot ulcers.

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Authors' Contributions

S.R.; Carried out the experiments and wrote the first draft of the manuscript. M.B.D.; Conceived and planned the experiments and directed the project. A.J.; Supervised the project and performed the first round of editing on the manuscript. M.P.; Performed the final editing, rewriting of the manuscript and data analysis. All authors read and approved the final manuscript.

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