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Biomaterials 25 (2004) 2679–2686
Biomaterials
www.elsevier.com/locate/biomaterials

Process development of an acellular dermal matrix (ADM) for biomedical applications

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Received 20 January 2003; accepted 5 September 2003

Abstract

The object of this study was to compare the extent of decellularization at each critical step of processing porcine skin to produce an acellular dermal matrix (ADM) for biomedical applications. The results demonstrated that the removal of epidermis using treatment with 0.25% trypsin for 18 h and 0.1% sodium dodecyl sulfate (SDS) for 12 h at room temperature was beneficial for the subsequent treatment to remove cells in the dermal structure. Lengthy incubation in 0.25% trypsin (12 h) and then 560 units/l Dispase (12 h) at 25°C of small pieces of porcine skin from which the epidermis had been removed efficiently removed cells and cellular components from the skin. Histological examinations revealed that the epidermis, dermal fibroblasts, and epidermal appendages were completely removed by these treatments, and the basic dermal architecture of collagen bundles was that of a loose meshwork. Examinations by TEM showed that the characteristics of collagen fibers in the ADM were retained after complete removal of cells present under optimal conditions defined in this study. SDS-PAGE and size-exclusion HPLC revealed that collagen fibers in the ADM were mostly type I and showed two typical component peaks identified as oligomers and monomers, respectively.

Keywords: Acellular dermal matrix; Trypsin; Sodium dodecyl sulfate; Dispase

1. Introduction

Currently, due to excellent achievements in a variety of different biomedical applications, the performance of acellular dermal matrices, which are derived from full-thickness skin treated to remove cells and cellular components but which retain the native dermal structure, has drawn the attention of researchers in many fields [1–3]. Many recent studies have presented several methods for producing an acellular dermal matrix (ADM) from porcine skin [4] and the submucosal layer of the small intestine [5]. Walter et al. commented that these methods using treatment with trypsin, freeze-thawing, and prolonged incubations with enzymes produced ADMs which were too highly antigenic when implanted into recipients, where they induced immune reactions resulting in poor graft survival [6]. Two more effective and controlled extraction methods have been reported for producing ADMs which exhibit very low antigenicity and excellent stability, while retaining the native dermal structure. One method uses hypertonic NaCl followed by sodium dodecyl sulfate (SDS) and freeze-drying [1,7], while the other uses sequential treatments with Dispase followed by Triton-100 [8,9]. Walter et al. compared these two methods and concluded that both methods of ADM preparation resulted in extensive extraction of both cellular and extracellular components of the skin but retained the basic dermal architecture. Lee et al. also published another alternative method to produce a truly cell-free porcine dermal matrix [10]. The optimal aseptic method for preparing a cell-free dermal matrix with a looser collagen structure required the treatment of porcine skin of 0.03 in thickness with 0.25% trypsin at 4°C for 24 h followed sequentially by treatments with 0.1% Triton X-100 for 8 h at room temperature, Dispase (560 units/l) at 4°C for 24 h, and a final extensive washing with R.O. water.

Recently, scaffolds derived from a xenogenic extracellular matrix (ECM) have been shown to be effective in the repair and reconstruction of several body tissues including the lower urinary tract, dura mater, esophagus, musculotendinous structures, and blood vessels [11]. The characteristic of these scaffolds recognized as important for their effectiveness is their ability to induce

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doi:10.1016/j.biomaterials.2003.09.070

a host cellular response that supports constructive remodeling rather than default scar tissue formation. In a study comparing purified collagen, naturally occurring ECM scaffolds, and synthetic scaffold materials for in vitro endothelial cell attachment [12], it was found that ECM possessed the ability to recruit circulating marrow-derived progenitor cells and attract mature endothelial cells from selected organs such as the heart and liver to promote successful vascularization of engineered tissue structures. These studies reveal that extracellular components in a cell-free or ADM are critical for success in biomedical applications as scaffolds. The object of this study, therefore, was to compare the extent of decellularization at each critical step in the processing of porcine skin into an ADM for biomedical applications.

2. Experimental methods

2.1. Materials

2.1.1. Acellular dermal matrix

Fresh porcine skin was obtained from a local slaughterhouse. After a complete cleaning, excision of the subdermal fat tissue, and removal of hair, the resulting skin was kept at -20°C until use. The skin was divided into four groups for comparison, and the optimal conditions for processing porcine skin into an ADM were defined in the final results.

Group A was processed as follows to compare the influence of treatment conditions of the trypsin solution on the extent of decellularization. Porcine skin was cut in pieces which were $0.5 \times 0.5 \times 0.3\,\text{cm}^3$. They were further divided into four subgroups (A1–A4), and five pieces of this size of porcine skin were allocated to each subgroup. Porcine skin in the four subgroups was soaked in a 0.25% trypsin solution at 4°C for 12 h (A1), at 4°C for 24 h (A2), at 25°C for 12 h (A3), and at 25°C for 24 h (A4), respectively. This was followed by treatment with a 0.1% SDS solution at room temperature for 6 h, then by 560 units/l of a Dispase solution at 4°C for 12 h, and finally in a 0.1% SDS solution at room temperature for 6 h before being washed twice with PBS buffer for 15 min each. Samples were preserved in PBS buffer. Gentamicin at 10 μg/ml was added to all solutions to prevent bacterial growth.

Porcine skin in the four subgroups (B1–B4) of group B was processed in similar respective conditions to that of group A except that both treatments with the 0.1% SDS solution were extended from 6 to 12 h. This was designed to compare the influence of washing time with 0.1% SDS solution on the extent of decellularization.

Group C was processed as follows to compare the influence of treatment conditions of both trypsin and Dispase on the extent of decellularization after removing the epidermis of porcine skin. Porcine skin was cut into pieces which were $10 \times 7 \times 0.3\,\text{cm}^3$. The epidermis of the skin was removed after treating with a 0.25% trypsin solution at 25°C for 18 h, and then the dermal part was cut into pieces which were $0.5 \times 0.5 \times 0.3\,\text{cm}^3$. These were divided into two subgroups (C1–C2) with 10 pieces of skin in each subgroup. This skin was treated with a 0.25% trypsin solution followed by shaking at 25°C for 12 h for one subgroup and at 25°C for 24 h for the other. Porcine skin in both subgroups was then washed with a 0.1% SDS solution at room temperature for 12 h, followed by 560 units/l of a Dispase solution at 4°C for 12 h (C11 and C21) or at 25°C for 12 h (C12 and C22), respectively. Sequentially, porcine skin in the four subgroups was washed with 0.1% SDS at room temperature for 12 h and then washed with PBS buffer twice for 15 min each. Samples were preserved in PBS buffer. Gentamicin at 10 μg/ml was added to all solutions to prevent bacterial growth.

Porcine skin in group D was processed in similar respective conditions as those of subgroup C2 except that treatment with the Dispase solution and SDS solution in the final step was shortened from 12 to 6 h. This was designed to compare the influence of washing time of the Dispase solution and SDS solution on the extent of decellularization.

2.1.2. Histological examinations

ADM samples were first dehydrated with an increasing series of alcohol concentrations and then embedded in paraffin. Paraffin-embedded ADMs were sectioned at a thickness of 5 μm. After removing the paraffin, samples were stained with hematoxylin and eosin. After sealing, samples were examined by light microscopy at a magnification of 200× to inspect fibroblast cells (stained by hematoxylin to a bluish-purple color) and collagen fibers (stained by eosin to a pink color).

2.1.3. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examinations

ADM samples after treatment with various conditions or those without treatment were freeze-dried for SEM and TEM examinations. For SEM examinations, samples were loaded onto aluminum studs and coated with gold for 3 min at 8 mA under a pressure of 0.1 Torr. Collagen morphologies were examined under a scanning electron microscope (Hitachi model S-2400, Department of Pathology, Taipei Medical University). Samples were scanned, and the micrographs were recorded. Comparisons were made of morphological changes to collagen fibers before and after treatment under various conditions. For TEM examinations, samples were scanned using a transmission electron microscope (Hitachi model

H-600). Characteristics of the sequences of black and white bands of collagen fibers of samples treated with various conditions were compared using the TEM examination.

2.1.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of collagen types and their α-chains was performed on SDS-polyacrylamide slab gels, using 5%

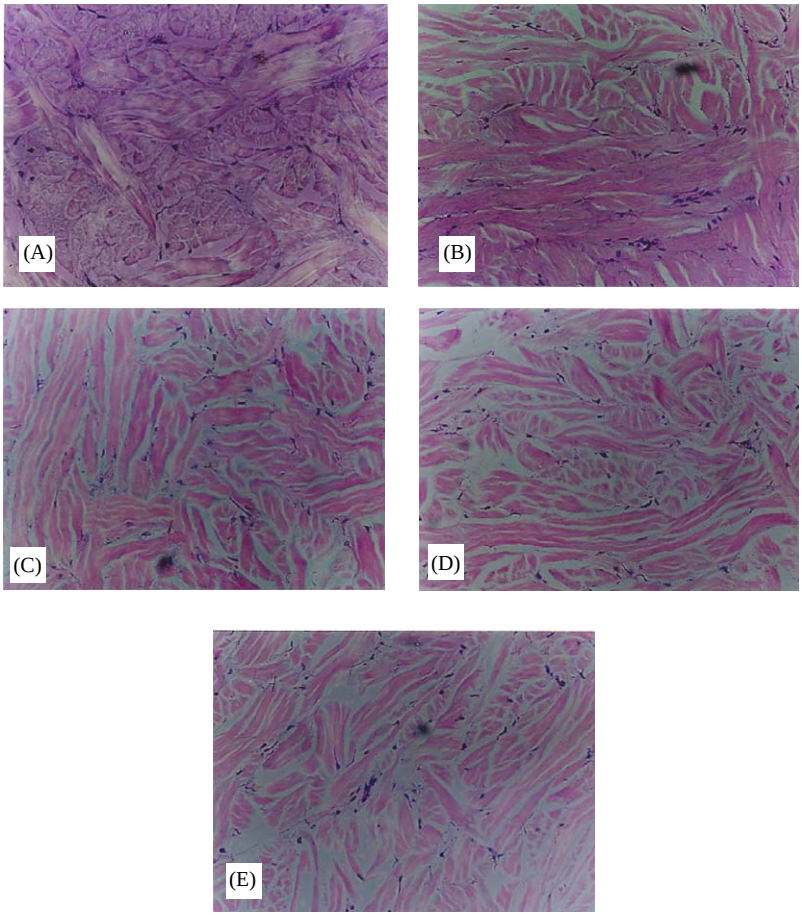

Fig. 1. Histological photographs of ADM samples from group A. (A) Control, (B) A1, (C) A2, (D) A3 and (E) A4.

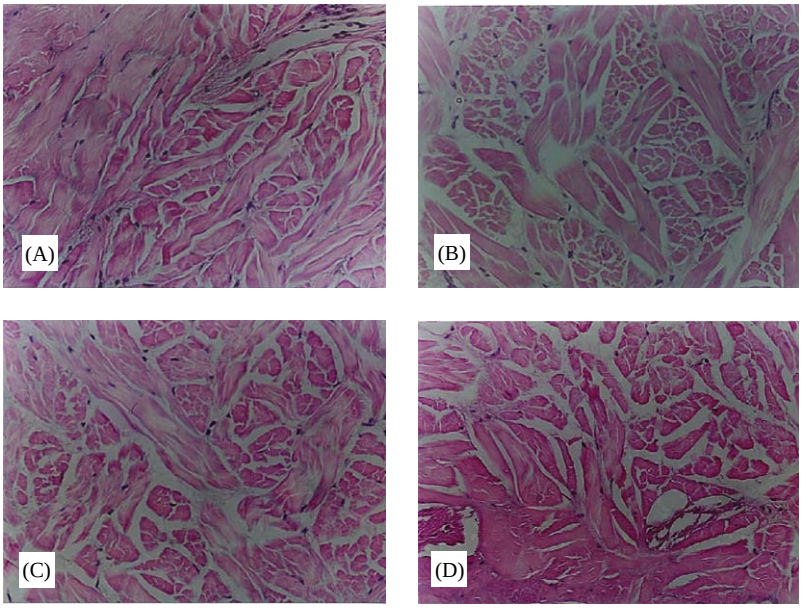

Fig. 2. Histological photographs of ADM samples from group B. (A) B1, (B) B2, (C) B3 and (D) B4.

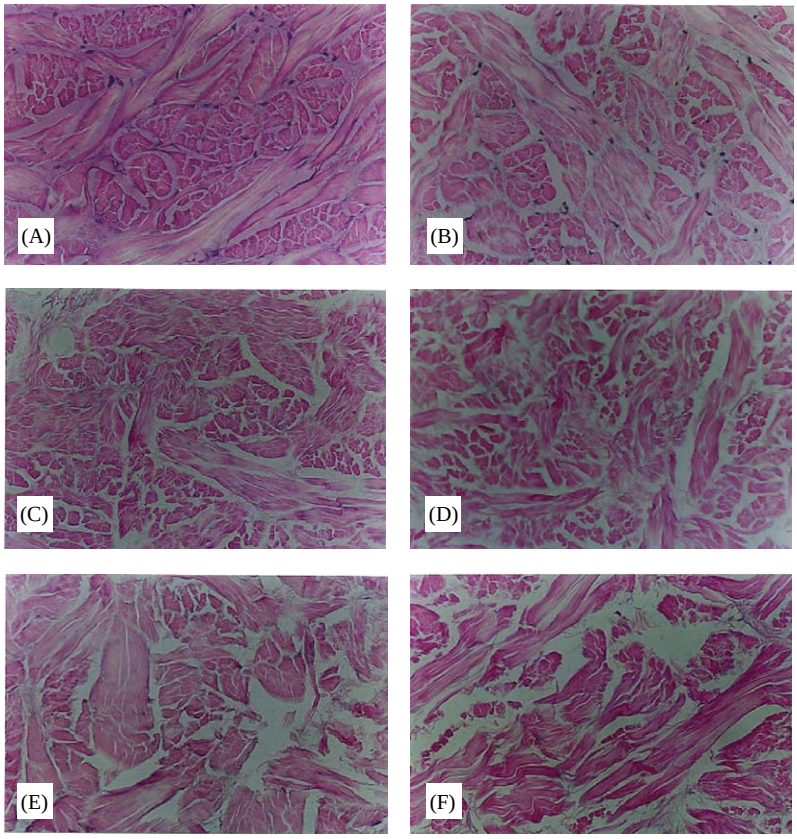

Fig. 3. Histological photographs of ADM samples from group C. (A) C1, (B) C2, (C) C11, (D) C12, (E) C21 and (F) C22.

(w/v) polyacrylamide for the separating gel and 6% (w/v) polyacrylamide for the stacking gel. ADM samples, purified samples of ADM prepared by the isoelectric point method [6], and a standard of a collagen sample (type I) were prepared in 1.5 M Tris-HCl buffer (pH 6.8) containing 10% SDS, 11.14% 2-mercaptoethanol, 40% glycerol, and 0.02% bromophenol blue at a concentration of 0.5 mg/ml and heated to 95°C for 5 min. Then 20 μl of each sample was loaded and electrophoresed at 85–100 V on vertical slab gels until the bromophenol blue had moved out of the gel. Polyacrylamide gels were stained for 2 h in 0.1% Coomassie blue R-250 in methanol/acetic acid/water 5:2:5 (v/v/v) and destained in 15% methanol/7.5% acetic acid.

2.1.5. HPLC size exclusion

Prior to HPLC analysis, ADM samples containing collagen were diluted to 0.6 mg/ml with an HCl solution (pH 2.0) and filtered through a 0.45-μm membrane filter. The size-exclusion column was a Nucleogel aqua-OH 50-8 with dimensions 7.5 × 300 mm and a 13-μm particle diameter. The mobile phase was 5 mM acetic acid with the addition of 0.25 M sodium chloride, and the flow rate was set at 0.2 ml/min. The injection volume was 20 μl. The UV absorbance was monitored at 220 nm.

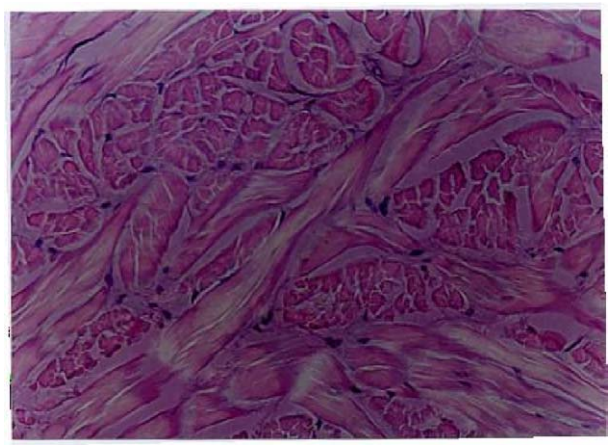

Fig. 4. Histological photographs of ADM samples from group D.

2.1.6. Biocompatibility test

The biocompatibility of injectable ADM was confirmed by evaluation using an in vitro cytotoxicity test with fibroblasts (3T3) as a qualitative indicator based on a morphological examination of cell damage and growth rates when in direct contact with the materials.

3. Results and discussion

Histological examinations of ADM samples from group A are shown in Fig. 1. A histological photograph of porcine skin with no treatment as the control is shown in Fig. 1A. It shows a dense structure of collagen fiber (a gray color) with the presence of many fibroblast cells (dark granules) among them. The histological photographs of the dermal matrix for samples of groups A1–A4 are in Fig. 1B–E, respectively. Apparently, no epidermis is shown, and the appendages and basic dermal architecture of the collagen were in a looser

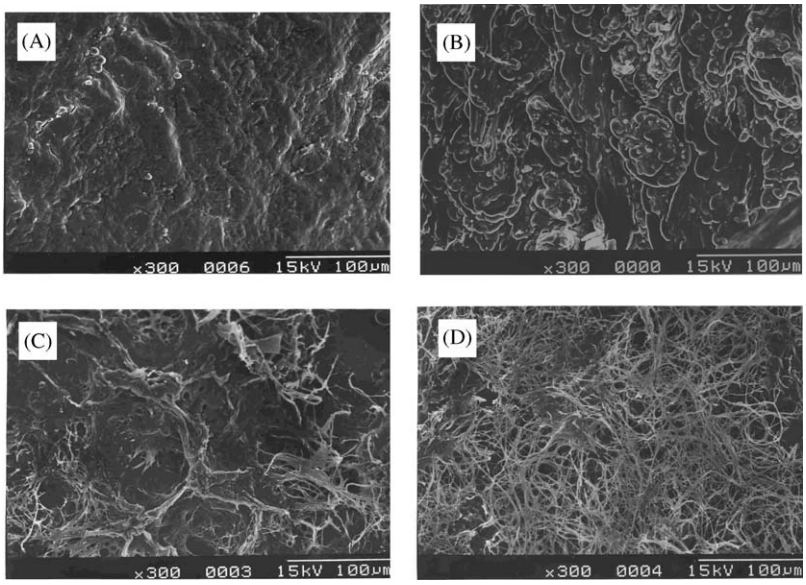

Fig. 5. SEM photographs of ADM samples.

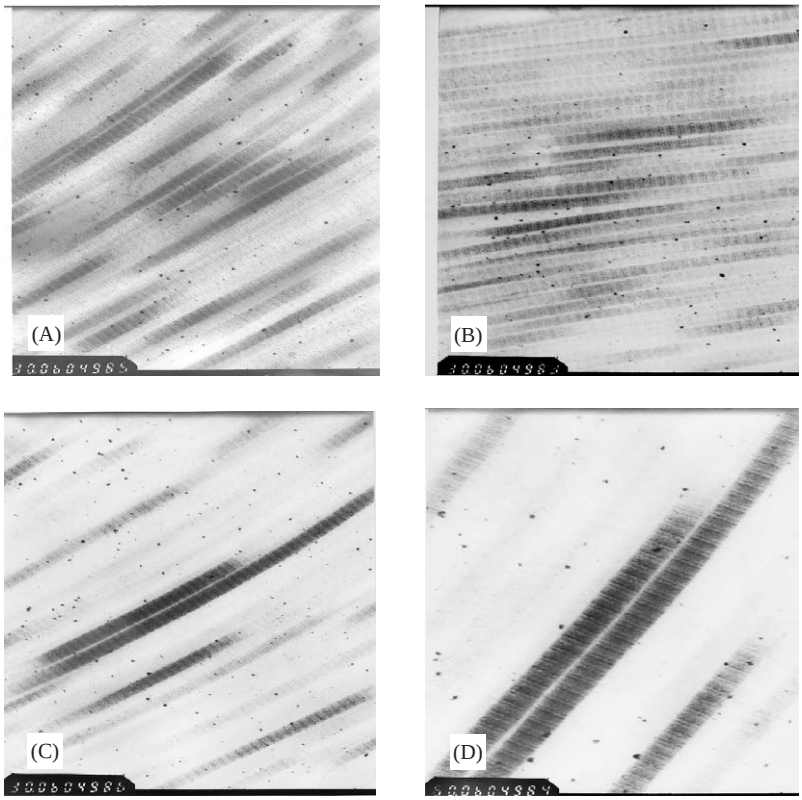

Fig. 6. TEM photographs of ADM samples.

meshwork with the presence of a fewer number of fibroblast cells among them than that with no treatment. However, cells were not completely removed in these four samples. In a comparison of treatment A4 (trypsin at 25°C for 24 h) with treatments A1–A3, a longer time of treatment at a higher temperature with trypsin led to more-complete removal of cells and a looser meshwork of the collagen fiber.

Since the above conditions were not able to completely remove cells from the skin structure, extension of the treatment time with 0.1% SDS from 6 to 12 h in both steps of the procedure was first tested at the same conditions for trypsin and Dispase treatments. Histological photographs of the results for groups B1–B4 are shown in Fig. 2. Obviously, cell removal under these four conditions was much greater than before, but still incomplete. This confirmed that extending the treatment time with the SDS solution was able to improve the efficiency of cell removal. Further, the dermal matrix obtained by the treatment conditions of B4 appeared to have a fewer number of cells present than that for the remaining treatment conditions (B1–B3).

Since the epidermis of porcine skin used in groups A and B was retained during treatments, it was thought that it would be less efficient for enzymes to remove cells present in the dermis. With treatment conditions of group C, removal of the epidermis was preceded by treatment with trypsin and the SDS solution. Results demonstrated in Fig. 3A and B are photographs for ADM samples of groups C1 and C2 after de-epidermis. Both still show the incomplete removal of cells in the dermal matrix. This indicates that treatment with Dispase and SDS in the subsequent steps is necessary. Fig. 3C–F comprises photographs of ADM samples of treatments C11, C12, C21, and C22, respectively. Complete removal of cells originally present in the dermal matrix can be observed in all four samples. This reveals that removal of the epidermis before treatment is necessary to produce a truly cell-free dermal matrix.

Fig. 4 further compares the influence of shortening treatment time with Dispase and SDS in the final step of the process on the efficiency of removal of cells originally present in the dermal matrix. Obviously, cell removal with a shortened treatment time with Dispase and SDS in the final step of the process was incomplete. To achieve the complete removal of cells originally present in the dermal matrix, the process cannot be simplified by shortening the treatment time. Finally in this work, it was found that de-epidermis using treatment with 0.25% Trypsin at 25°C for 18 h was beneficial for efficiently removing those cells present in the dermal matrix. When followed by incubation with 0.25% Trypsin at 25°C for 12 h, washing with 0.1% SDS at room temperature for 12 h, treatment with 560 units/l Dispase at room temperature for 12 h, and a final washing with 0.1% SDS at room temperature for

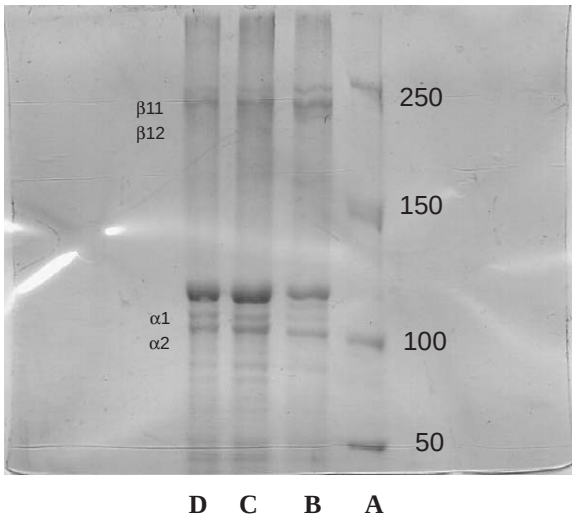

Fig. 7. SDS-PAGE electrophoresis patterns. (A) Marker, (B) standard, (C) ADM sample and (D) ADM sample further purified by pH precipitation.

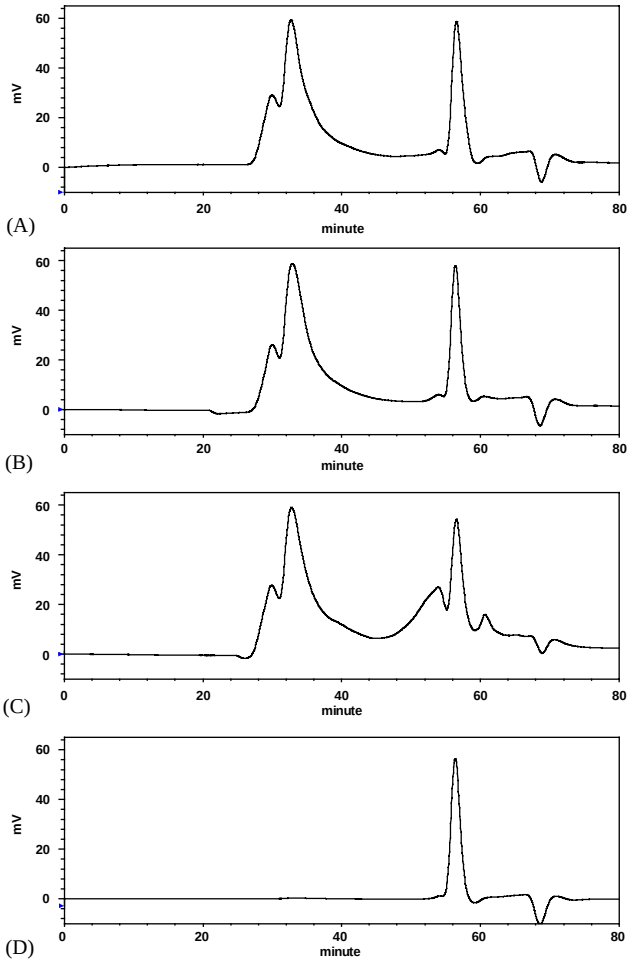

Fig. 8. Size-exclusion HPLC patterns. (A) Standard, (B) ADM sample, (C) ADM sample further purified by pH precipitation and (D) solvent peak.

12 h, the removal of cells and cellular components was efficient and complete. Ultimately, the process to produce ADMs was achievable at room temperature with a yield of about 55%.

Histological comparisons using SEM examination of ADM samples at each major step following the processing conditions defined above are shown in Fig. 5. The structure of the dermal matrix in the original sample with no treatment is shown to be dense and integral (Fig. 5A). After treatment with trypsin for 18 h to remove the epidermis, the dermal structure as shown in Fig. 5B is a loosened meshwork. Further washing with 0.1% SDS and incubation with 0.25% trypsin for 12 h produced an even looser meshwork (Fig. 5C). The SEM photograph for ADM produced with the process conditions defined above is shown in Fig. 5D. Although the sample underwent a long period of processing with trypsin, SDS, and Dispase, the structural pattern of collagen fiber still remained. It seems to reveal that modification of collagen fibers or loss of other extracellular components might be minimal.

Fig. 6 shows the TEM photographs for ADM samples for each major step following the processing conditions defined above. Fig. 6A is the structural pattern of collagen fiber in the dermal matrix of the original ADM sample. Apparently, a sequential arrangement of black and white bands at a bandwidth of 64 nm is the typical pattern of collagen fibers under TEM examination. A

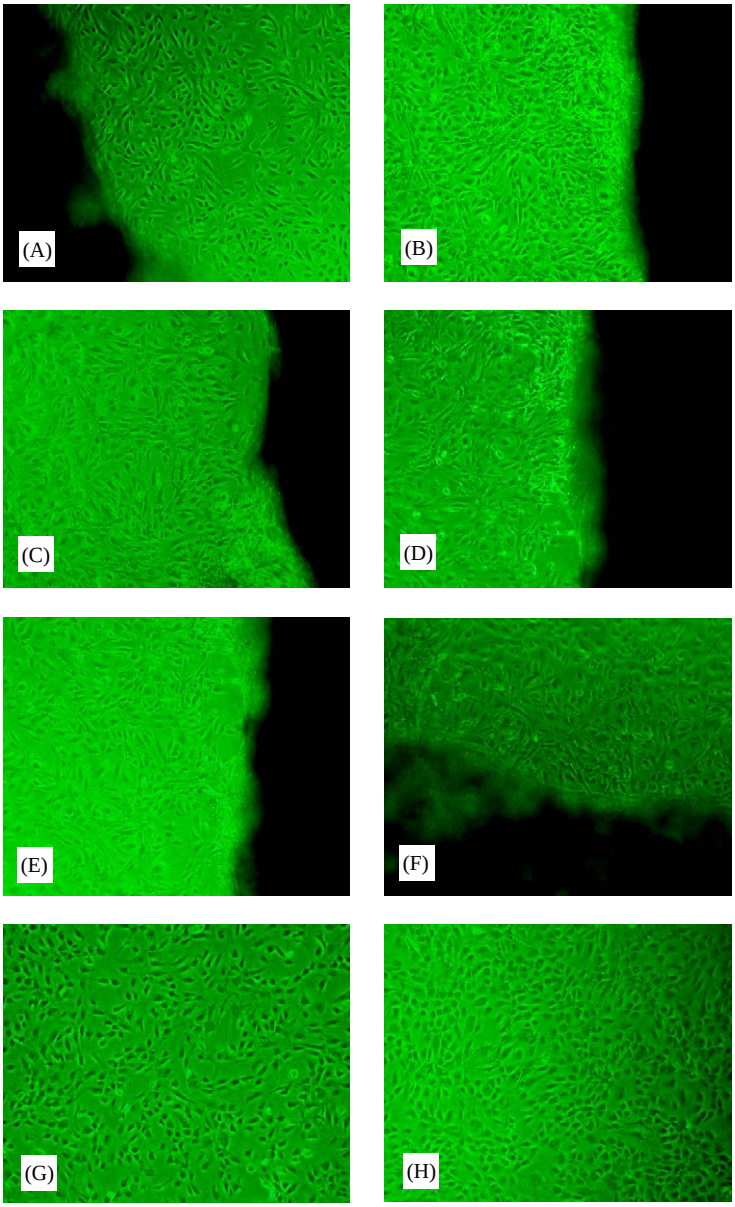

Fig. 9. Biocompatibility tests of ADM and collagen samples co-cultured with fibroblasts for 24 and 48 h. (A) ADM, 24 h; (B) ADM, 48 h; (C) ADM further purified by pH precipitation, 24 h; (D) ADM further purified by pH precipitation, 48 h; (E) collagen isolated by pH precipitation, 24 h; (F) collagen isolated by pH precipitation, 48 h; (G) control, 24 h and (H) control, 48 h.

similar structural pattern of collagen fibers for ADM samples obtained at each major step of the processing conditions can be observed in Fig. 6B–D in sequence with that of no treatment. This indicates that characteristics of collagen fiber in ADM samples obtained at each major step of the processing conditions was preserved or altered to a small extent.

Fig. 7 shows the SDS-PAGE electrophoresis patterns of the standard (B) and two collagen samples (ADM obtained from treatment C2 (C) and collagen further purified by pH precipitation from ADM obtained from treatment C2 (D)). The collagen standard and the two ADM samples display the same bands, two α bands ($\alpha 1$ and $\alpha 2$, MW = 95,000) and two β bands ($\beta 11$ and $\beta 12$, MW = 19,000). These results are consistent with the fact that the known helical structure of type I collagen is composed of three polypeptide chains of $\alpha 1$, $\alpha 1$, and $\alpha 2$, and the ADM samples contain type I collagen in great abundance.

Compared to the standard (A) using the size-exclusion HPLC method, both collagen in the ADM obtained from treatment C2 (B) and that in the ADM further purified by pH precipitation of ADM obtained from treatment C2 (C) showed two typical component peaks eluting serially from the column, which were identified as oligomers and monomers (Fig. 8). Collagen oligomers represent intermolecular crosslinking of native collagen molecules. After processing of the skin, some of the crosslinks of collagen still remain. Hence, the ADM preparations generally contain a mixture of monomers, dimers, and higher oligomers.

The biocompatibility tests were carried out for ADMs obtained from treatment C2 by co-culture with fibroblasts (3T3) for 24 and 48 h (Fig. 9). For all materials, microscopic observations show that fibroblasts adhered as rapidly to all materials as they did to the control. No significant morphologic changes were observed in those cells in contact with ADM obtained from treatment C2 for all studied time periods.

4. Conclusions

Optimum process conditions were defined with the ability to completely remove all cellular components and maintain the ECM structure of porcine dermis. Examinations by SEM and TEM and analysis by SDS-PAGE and HPLC size-exclusion revealed that the ADM obtained with optimal processing conditions retained most of the original type I collagen. In the in vitro biocompatibility test, there were no significant morphological changes observed for those fibroblast cells (3T3) in contact with the ADM obtained from these optimal processing conditions. For safety issues in biomedical applications, this material should be further subjected to in vivo biocompatibility and biodegradability tests.

Acknowledgements

Financial support from the National Science Council of the ROC is highly appreciated (NSC 91-2320-B038-035).

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