***In Vivo* Comparison of Three Human Acellular Dermal Matrices for Breast Reconstruction**

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Abstract. *Background/Aim:* Acellular dermal matrices (ADMs) have become popular in implant-based breast reconstruction. The aim of this study was to compare three commonly used ADM products *in vivo* in an animal model. *Materials and Methods:* The nucleic acid content (residual double-stranded DNA) and the levels of the remaining growth factors after decellularization were measured for each ADM. Cytocompatibility with ADMs was documented using NIH 3T3 mouse fibroblast cells. *In vivo*, the implanted ADMs were histologically evaluated at 1, 2, 3, and 6 months (n=5) using male 8-week-old Sprague-Dawley rats. *Results:* Fibroblasts grew in the SureDerm HD and DermACELL with no cytotoxicity. In a rat model, SureDerm HD and DermACELL incorporated more readily into the surrounding host tissue, as measured by rapid cell influx and collagen deposition, and showed more delayed tissue remodeling with decreased matrix metalloproteinases levels compared to AlloDerm. *Conclusion:* SureDerm HD and DermACELL can be used as biological materials for breast reconstruction.

Acellular dermal matrix (ADM) is a tissue graft obtained from cadaveric skin, which is widely used in tissue reconstruction, particularly burn injuries, abdominal wall repair, dural repair,

tympanic membrane replacement and breast reconstruction (1-8). Decellularization technique has been employed for the preparation of ADM, followed by terminal sterilization to preserve the biochemical and structural components of the extracellular skin matrix. ADMs are increasingly used for skin graft in tissue engineering since they contain complex proteins, signaling cascades, and biomolecules for restoration of damaged tissue. The presence of collagen and elastin maintain favorable tensile strength and elasticity, while proteoglycans and laminin induce angiogenesis and connective tissue binding, respectively. Besides, they provide a biocompatible environment and promote reepithelization, neovascularization, and fibroblast infiltration without inducing immune response at implant site (1, 9-11). Furthermore, ADM degradation results in the release of growth factors and biochemical signals similar to the native tissue, maintaining cellular behavior in terms of attachment as well as growth. Therefore, ADM implantation into the human body influences host remodeling responses such as rapid ingrowth of blood vessels, cell migration, proliferation, differentiation and support for regenerative healing and prevent scar tissue formation after implantation (12-15).

The application of ADMs as breast implants was reported in 2005 by Breuing and Warren using AlloDerm sling (LifeCell Corporation, Branchburg, NJ, USA) to directly implant 10 patients undergoing bilateral mastectomy (16). By single stage alloplastic reconstruction, they showed that their technique “avoids or shortens the tissue expansion/implant reconstructive process, avoids mastectomy flap contraction during the latency period of expansion, and provides an additional option for immediate, single stage breast implant reconstruction”. Furthermore, this approach has significantly influenced surgery and become standard of care in breast reconstruction after mastectomy. The benefits of using AMD as a sling are useful for immediate prosthetic breast reconstruction and can provide a tissue expander or a larger implant pocket with extremely low

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complication rate and long-term safety, while being time- and cost-effective. In addition, the use of ADMs not only helps to reconstruct pleasing breasts with natural soft tissue appearance but also facilitate speedy completion of the reconstruction (16-19). Maintaining three-dimensional ultra-structure and conserving the complex composition of ADMs during decellularization is challenging at current time. It is well recognized that all decellularization techniques disrupt the architecture of tissue to some degree and affect surface structure and composition (20). In addition, insufficient removal of cellular and nuclear components is frequently observed during decellularization. As a result, several types of major complications have occurred including immunogenic reaction, fibrotic capsular formation, inflammation, and scar tissue formation (21-23). Therefore, it is crucial to choose the most applicable implants among the available biological scaffolds and consider the factors influencing host response during the clinical application.

The current work was focused on comparing the performance of three commercial human ADMs, namely AlloDerm, SureDerm HD and DermACELL. Their biochemical composition as well as their effect on *in vitro* cell viability were evaluated. Moreover, these commercial ADMs were implanted *in vivo* in rat models of breast reconstruction to determine host tissue response. Furthermore, quantitative histomorphologic metrics were established to recognize the inflammatory and tissue remodeling responses *in vivo*.

Materials and Methods

Materials. Three samples of each of the following ADMs were used in each experimental test; 1) AlloDerm from LifeCell Corp (Branchburg, NJ, USA); 2) SureDerm HD from Hans Biomed Corporation (Seoul, Republic of Korea); 3) DermACELL from LifeNet Health (Virginia Beach, VA, USA). Paraformaldehyde (4%) was obtained from KCFC (Ansan, Republic of Korea). Hematoxylin and eosin (H&E), xylene, dehydration agent (ethanol), and HCl acid were supplied by Duksan Pure Chemicals (Ansan) and paraffin was from Merck (Branchburg, NJ, USA). Modified Mayer's H&E Y solution was from Richard-Allan Scientific (Kalamazoo, MI, USA) and ammonia solution was from JunsieChemical (Tokyo, Japan). In order to determine collagen synthesis after ADM implantation by Masson's trichrome (MT) staining, aniline blue solutions, Biebrich scarlet-acid fuchsin, phosphotungstic acid and phosphomolybdic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA) and acetic acid (1%) was supplied by Duksan Pure Chemicals (Ansan). For Immunofluorescent Staining, antigen-retrieval solution was acquired from Dako (Glostrup, Denmark). The primary antibodies anti-hTGF- β 1 (ab92486, Abcam, Cambridge, MA, USA), anti-VEGF (ab46154, Abcam) and anti-bFGF- β 1 (MAB233, R&D systems, Minneapolis, MN, USA) were used in this study.

Decellularization assessment. The level of decellularization was determined for AlloDerm, SureDerm HD, and DermACELL based on the established criteria such as 4',6 diamidino-2-phenylindole (DAPI) stain and H&E staining, to determine the presence of DNA

and intact cells and nuclei, respectively. Further, the size of existing DNA fragments was determined by gel electrophoresis methods as well as by quantifying double stranded DNA (dsDNA) by the Quant-IT PicoGreen assay (Invitrogen, Carlsbad, CA, USA).

Biochemical analysis. Concentrations of vascular endothelial growth factor (hVEGF), transforming growth factor- β 1 (hTGF- β 1), and basic fibroblast growth factor (bFGF) were determined using human VEGF immunoassay, human TGF- β 1 immunoassay and human FGF basic Immunoassay (all R&D Systems) as described at the manufacturer's protocol. All examinations were performed in duplicate.

***In vitro* cytocompatibility analysis.** The mouse fibroblast cell line NIH 3T3 was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% of penicillin-streptomycin and maintained in an incubator at 37°C in 5% CO₂ until 80%-90% confluence. All ADMs used for cell seeding were cut uniformly 2.5 cm² and subsequently placed in 6 well plates, hydrated according to description on the manufacturer's protocol and incubated in the media at 37°C, 5% CO₂ for 15 min. Then, the media were discarded and the cells were seeded at a density of 10⁶ cells per 1.5 cm². After 7 days of culture, the cells attached to the grafts were fixed with 10% formalin for 24 h and embedded in paraffin blocks to prepare slices for H&E staining. The cell phenotype and cell infiltration into the ADM substrate were analyzed.

***In vivo* experiment.** Eight-week-old Sprague Dawley rats were used for the *in vivo* experiment. The animals weighing 250-300 g were kept to 12/12 h light/dark cycle and maintained in pathogen-free conditions with free access to food and drinking water. The animal experiments were approved by the Institutional Animal Care and Use Committee of Bundang Seoul National University Hospital (approval number: BA-1802-241-014-08) and all procedures were conducted based on NIH guidelines for the Care and Use of Laboratory Animals. Twenty animals for each group were implanted with the AlloDerm, SureDerm HD, or DermACELL separately. In order to implant ADM, the rats were anesthetized using inhalation of isoflurane (Hana Pharm, Seoul, Republic of Korea). After shaving the dorsal region of the three groups of rats, 70% alcohol and betadine were applied to the surgical site. #15 scalpel blades were used for implantation of ADM into the subpanniculus pocket and the incision site (2-3 cm long) was sutured (Nylon 4/0, Ethicon, New Brunswick, NJ, USA). A light dressing was applied after disinfection of the surgical site with 70% alcohol and betadine. The implanted rats were observed for up to 6 months. We monitored inflammation and wound healing progress in detail. Moreover, randomly chosen five animals from each AlloDerm, SureDerm HD, and DermACELL groups were biopsied at 1, 2, 3, and 6 months. The biopsy area was tissue near the implanted dorsal region including epidermis, dermis, and posterior as well as anterior capsules.

Quantification of collagen deposition by histological assessment. For paraffin embedding, 10% paraformaldehyde was used for fixation of the biopsied tissue for 24 h before embedding in paraffin. The paraffin blocks were sliced into 4- μ m slices. According to type and period of implantation, 5 slices from each of the 5 randomly selected rats per each group were stained by H&E to determine infiltration of cells and perform statistical analysis. Collagen fiber production and collagen intensity were evaluated by Masson's trichrome stain and quantified by using ImageJ software.

Figure 1. Decellularization assessment of acellular dermal matrices (ADMs). (A) The remaining double stranded DNA (dsDNA) in ADMs was analyzed by 1% agarose gel electrophoresis and staining with ethidium bromide; Lines 1-3 present three independent samples of AlloDerm; Lines 4-6 present three independent samples of SureDerm HD; Lines 7-9 present three independent samples of DermACELL. (B) PicoGreen assay to measure dsDNA concentration. (C) H&E and DAPI staining to probe for intact cells and remnant nucleic acid, respectively.

Western blot analysis. The western blot analysis was conducted for MMP-2, MMP-3 and MMP-9 expression. The implants collected at 1-, 2-, 3-, and 6-months following implantation were lysed in PRO-PREPTM Protein Extraction Solution (C/T) (iNtRON, Seongnam, Republic of Korea) according to the manufacturer's protocol. A standard BCA method was used to determine protein concentration and subsequently, the protein samples were prepared for western blot. The same amount of protein was loaded on 12% SDS-PAGE gel, and then the proteins were transferred onto the blotting membrane. Membrane blocking was performed using 5% of skim milk in the TBST buffer. Mouse anti-MMP2 antibody (1:1000; Abcam; Cat# ab86607), mouse anti-MMP9 antibody (1:1000; Abcam; Cat# ab38898), mouse anti-MMP3 antibody (1:1000; Abcam; Cat# ab52915), mouse anti-TIMP1 antibody (1:1000; Abcam; Cat# ab61224), rabbit anti-TGF beta 1 antibody (1:1000; Abcam; Cat# ab92486), mouse anti- α -SMA antibody (1:1000; Abcam; Cat# ab7817) were used as primary antibodies and incubated with the membranes overnight at 40C. Subsequently, goat anti mouse IgG H&L (HPP) (1:5000; Abcam; Cat# ab6789) secondary antibody was used. The level of protein expression was measured by an image analyzer (Las-3000, Photo Fuji, Tokyo, Japan). The images were acquired using Image Lab Touch (Bio-Rad, Berkeley, CA, USA).

Statistical analysis. The mean values and $\pm$ SEM obtained by ImageJ are shown in all figures. Prism statistical software (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. The statistical significance was determined using an unpaired *t*-test. Statistical significance was set at $p < 0.05$.

Results

Decellularization evaluation of ADMs. The efficacy of the process of decellularization for AlloDerm, SureDerm HD, and DermACELL was evaluated by DNA fragment analysis using agarose gel electrophoresis (Figure 1A). AlloDerm contained large DNA fragments ($>1,000$ bp), however SureDerm HD and DermACELL were not found to contain large DNA fragments ($>1,000$ bp) (Figure 1A). The DNA content in AlloDerm was 24.94 ± 2.17 ng/mg, whereas SureDerm HD and DermACELL contained 0.93 ± 0.25 ng/mg and 0.03 ± 0.01 ng/mg, respectively (Figure 1B). Furthermore, H&E staining showed a lack of hematoxylin-stained nuclei in SureDerm HD and DermACELL indicating efficient cell removal, while AlloDerm showed dense darkly stained cell nuclei (Figure 1C). DAPI staining revealed complete removal of intact nuclei and remaining nuclear DNA in decellularized SureDerm HD and DermACELL as compared to AlloDerm, which showed heavy nuclear staining throughout the tissue (Figure 1C). These results suggest that SureDerm HD and DermACELL are appropriate for use in breast reconstruction.

Biochemical properties. A consideration when utilizing this biomaterial is whether it retains growth factors after the decellularization process, which support the activity of recipient

Figure 2. Biochemical properties. ELISA was used to quantify the growth factors retained in the acellular dermal matrices. (A) VEGF, (B) hTGF- β 1, and (C) bFGF. The data are shown as the mean value $\pm$ SEM of three independent experiments.

cells, leading to better incorporation and recovery. The biochemical properties of AlloDerm, SureDerm HD, and DermACELL were analyzed by examining the levels of VEGF, TGF- β 1, and bFGF using established ELISA methods. SureDerm HD and DermACELL contained more VEGF, TGF- β 1 and bFGF than AlloDerm (Figure 2A-C). VEGF content in SureDerm HD and DermACELL was found to be about 2.0-fold and 2.2-fold higher compared with AlloDerm, respectively (Figure 2A). The protein levels of TGF- β 1 in SureDerm HD and DermACELL increased about 1.3-fold and 2.0-fold compared to AlloDerm, respectively (Figure 2B). In addition, SureDerm HD and DermACELL contained significantly increased levels of bFGF, approximately 2.0-fold and 1.9-fold, compared to AlloDerm, respectively (Figure 2C). Overall, the levels of the remaining growth factors after decellularization were higher in SureDerm HD and DermACELL and, therefore, may support recovery after implantation more efficiently than AlloDerm.

Cell adhesion and infiltration on ADMs. To confirm the capability of ADMs to support cell growth after implantation into body, we quantified cell confluence and cell infiltration using NIH 3T3 mouse fibroblasts (Figure 3A). ADMs were seeded with fibroblasts for up to 7 days. Fibroblasts cultured on AlloDerm were unevenly distributed on the surface of the dermal matrix showing no increase in cell number (Figure 3B). Fibroblasts were adherent and spread on the surface of SureDerm HD and DermACELL at day 7 (Figure 3B). A confluent fibroblastic monolayer lining SureDerm HD surface was noticed (Figure 3B), however few cells were observed inside the SureDerm HD at this time (Figure 3B). A higher percentage of cells was observed not only on the surface of DermACELL but also inside the ADM compared to AlloDerm and SureDerm HD (Figure 3B). The surface of DermACELL was more readily infiltrated by fibroblasts than that of the other two ADMs. These results indicated that SureDerm HD and DermACELL were more cytocompatible than AlloDerm for cell adhesion and infiltration (Figure 3B).

Histologic analysis of implanted ADMs. In vivo evaluation of each ADM material for bilateral breast reconstruction was performed in a rat model (Figure 4). And the result of host remodeling response was confirmed through histomorphologic observation as a function of time and location of the explants. After implantation of AlloDerm, SureDerm HD, or DermACELL, all ADMs were well accepted and were not harmful to living tissue. Moreover, no infection and no graft rejection were observed at different time points after implantation of these materials (Figure 5A). After implantation of the porous architecture of AlloDerm, SureDerm HD, and DermACELL, vascular and connective tissue was formed and developed within ADMs over time (Figure 5A). SureDerm HD and DermACELL exhibited mild inflammation with immune cell infiltration and extracellular matrix (ECM) deposition compared to the AlloDerm (Figure 5B). The infiltration level of immune cells in DermACELL ($26.20\% \pm 9.94$) was lower than that in SureDerm HD ($54.80\% \pm 10.65$) and AlloDerm ($81.40\% \pm 7.67$) at 1 month after implantation (Figure 5B). After 2 months of implantation, the infiltration level of immune cells significantly increased in AlloDerm ($140.8\% \pm 4$) compared to the first month. In contrast, it was decreased in both of SureDerm HD ($38.00\% \pm 6.55$) and DermACELL ($24.20\% \pm 5.67$) compared to the first month. Moreover, fibroblasts were observed in all ADMs at 1 month after implantation and the infiltration levels of fibroblasts in all ADMs were gradually increased over time. Specially, the infiltration level of fibroblasts in SureDerm HD and DermACELL revealed a 2 fold and 3-fold increase at 1 month compared to the AlloDerm (Figure 5C). However, the infiltration of fibroblasts in all ADMs was decreased after 6 months of implantation (Figure 5C).

Evaluation of collagen content of implanted ADMs. The content and distribution pattern of collagen, an important structural component of ADMs, were evaluated by MT staining. Quantification of collagen density showed that the amounts of collagen in AlloDerm, SureDerm HD, and DermACELL were

Figure 3. Cytocompatibility with fibroblasts. (A) Schematic representation of cell adhesion and infiltration into the acellular dermal matrices (ADMs) *in vitro*. (B) H&E staining of ADMs cultured with cells. NIH 3T3 fibroblasts were seeded on the surface of ADMs and cultured for 7 days. Invasion of fibroblasts on the dermal surface is shown by arrows. The experiment was performed in triplicate.

Figure 4. Schematic diagram of the *in vivo* experiment.

significantly increased and their porous architecture was replaced by new collagen over time (Figure 6A). After decellularization, small amounts of collagen remain on ADMs. However, numerous collagen fibers appeared over time and the porous architecture of the ADM was fully restored by new collagen fibers after 6 months of implantation (Figure 6A).

Collagen deposition within the implanted SureDerm HD and DermACELL was significantly increased approximately 64.8% and 55.2% at 1 month, 40.9% and 54.5% at 2 months, 7.6% and 47.1% at 3 months, and 38% and 35.8% at 6 months compared to AlloDerm after implantation, respectively ($p < 0.05$, Figure 6B).

Figure 5. Tissue suitability assessment. (A) Host tissue ingrowth and cellular infiltration of implanted acellular dermal matrices (ADMs) over time were analyzed by H&E staining ($\times 200$, scale bar = 50 μ m). (B) Infiltration of immune cells (C) Infiltration of fibroblasts. The data is shown as the mean value $\pm$ SEM of five independent experiments. $p < 0.05$; significantly different from the AlloDerm.

Effect of ADMs on the expression of MMPs. MMPs are a family of enzymes that include at least 28 members, most of which degrade ECM. Analysis of MMPs by western blotting showed differential expression of these proteins at different time points. The expression and activities of MMP-2 were increased during the acute phase of wound and decreased during the recovery stage. As depicted in Figure 7A, clear bands were detected for MMP-2, thereby indicating that this MMP is expressed in ADMs. The differences in MMP-2 among all groups were not statistically significant at 3 months after implantation ($p > 0.05$). The MMP-2 levels in AlloDerm were the highest and that in SureDerm HD were the lowest among the three groups at 6 months after implantation (Figure

7B). The differences in MMP-3 among all groups were not statistically significant at 6 months after implantation ($p > 0.05$) (Figure 7C). The MMP-9 levels in AlloDerm were the highest and those of SureDerm HD and DermACELL were lower at 6 months as shown in Figure 7A and D. These results suggest that expression of proteins involved in ECM remodelling in SureDerm HD is delayed, compared AlloDerm and DermACELL during wound healing process.

Discussion

In this study, we investigated the histological and biochemical characteristics of the novel human derived SureDerm HD,

Figure 6. Histological analysis of collagen deposition after acellular dermal matrix implantation was analyzed by Masson's trichrome stain. The data is shown as the mean value $\pm$ SEM of five independent experiments. $p < 0.05$; significantly different from the AlloDerm.

which is produced through a unique process, and compared them with those of two commercial ADM products.

The process of development and manufacturing of human ADMs consists of decellularization, cross-linking, preservation, and sterility assurance (24, 25). Decellularization performed through chemical, physical, biological, or combinatorial methods plays the most important role in all phases. The products of each method are distinctive. The elimination of all immunogenic factors from donor tissue and antigenic materials such as cells, cell debris and chromosome fragments while retaining the ECM biochemistry and structure are the goals of decellularization process. The ECM, which retains dermal collagen architecture, has an important role (26). Moreover, the inflammatory response is caused by the existence of residual DNA in biological scaffold materials (27). H&E staining, DAPI staining and evaluation of DNA samples on agarose gel

electrophoresis indicated that the human derived SureDerm HD was completely decellularized compared with AlloDerm and DermACELL. In addition, essential growth factors promoting hyaluronan production, through an increase in the mRNA levels of hyaluronic acid synthase 2, depend on bFGF (28). In the present study, we found that SureDerm HD contains significant levels of TGF- β 1, VEGF, and bFGF. TGF- β 1 and VEGF stimulate cell proliferation whereas bFGF is an effective inducer of hyaluronic acid production. Furthermore, the tensile and elasticity of AMD are contributed by collagen and elastin fibers, which are the main components of this material. Therefore, it can be considered that SureDerm HD has a good biological behavior and immunological safety during the implantation process.

When breast is reconstructed by implanting ADMs, it is recolonized by fibroblasts, myofibroblasts and cells of the

Figure 7. Remodeling related protein expression in implanted acellular dermal matrices was evaluated by western blot. The data represent the mean value $\pm$ SEM of four independent experiments. $p < 0.05$; significantly different from the AlloDerm.

immune system namely lymphocytes, macrophages, granulocytes, and mast cells (12-29). The fibrosis and neovascularization processes establish capsule and capillaries as well as lymphomagenesis. Therefore, AMDs biocompatible with host tissue, activate biologic responses as adhesion and proliferation of host cells without cytotoxicity (30). Based on our results of increased infiltration of fibroblasts, cell adhesion, and morphological stability, we highly recommend SureDerm HD as a good product for employing in breast reconstruction.

The phases of wound healing consist of haemostasis, inflammation, proliferation, deposition and remodeling of ECM that affect tissue integration after AMD implantation (11, 31-33). In our study, histological analysis showed increased deposition of collagen fibers over time. Especially, the intensity of collagen at sixth months was approximately double compared with that at 1 month after implantation. This result suggests rapid cellular infiltration of the implant. The loosely organized collagen of ADMs was replaced by newly synthesized collagen at 6 months of implantation.

Additionally, elastin fiber deposition was gradually increased only in DermACELL during the observed time, whereas it was decreased over time in both SureDerm HD and AlloDerm. These results may be due to the method of decellularization.

MMPs are enzymes belonging to the family of metalloendopeptidases that contribute to all phases of the wound healing process by regulating immune cell influx, facilitating migration of fibroblasts, keratinocytes and scar tissue reconstruction (34, 35). In addition, they play a critical role in the remodeling of ECM components. Normally, throughout the process of wound healing, the levels of MMPs increase in an acute wound and decrease as wound healing occurs. MMP-2 (gelatinase A, 72-kDa gelatinase) can degrade several matrix components including collagen types I, IV, V, VII, XI, and laminin (36). MMP-3 has the ability to hydrolyse collagen types II, III, IV, IX and laminin, while MMP9 (gelatinase B, 72 kDa) is able to degrade collagen types III, IV, V, XIV and elastin (37, 38). In our study, AlloDerm showed dramatically increased levels of

MMP-2, MMP-3, and MMP-9 at 6 months after implantation compared with those of SureDerm HD and DermACELL ($p < 0.05$). These results suggest that SureDerm HD could provide long-term structural integrity, and result in prolonged remodeling.

In summary, our study focused on the three ADMs for breast reconstructive application and evaluated their performance through *in vitro* and *in vivo* studies. Among them, SureDerm HD showed long term structural integrity and provided a more favorable environment for cell infiltration and ECM deposition. Besides, protein expression results demonstrated SureDerm HD had a significant remodeling effect on the wound site during the healing stage. based on our data, SureDerm HD could be a potential implant in clinical applications for breast reconstruction.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization, S.Y.N. and C.Y.H.; methodology, P.N.C.; validation, P.N.C. and X.R.Z.; investigation, P.N.C., D.N., O.F., and X.R.Z.; data curation, P.N.C. and S.Y.N.; writing—original draft preparation, P.N.C. and S.Y.N.; writing—review and editing, P.N.C., O.F., and S.Y.N.; supervision, S.Y.N. and C.Y.H.; project administration, C.Y.H. All Authors have read and agreed to the published version of the manuscript.

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