Evaluation of Two Purion Processed Amniotic Tissue Allografts: Bioactivity with Targeted Structural Distinctions

Sarah Moreno, Lisa Godwin, Shauna Campbell, Michelle Massee, Thomas J. Koob PhD, and John R. Harper PhD

SAWC, November 2023

INTRODUCTION

Amniotic membrane allografts continue to garner recognition for the clinical benefits in treating acute and chronic wounds. As the underlying mechanism of action is better understood, these products are being used in increasingly diverse applications. Developing novel processing techniques to provide the most appropriate form factors for these procedures is necessary for clinical success, but not at the expense of matrix composition, protein content and biological activity. This study evaluated the physical and biological properties of two PURION processed amniotic membrane products: a lyophilized human amnion, intermediate layer and chorion membrane (LHACM*) and a dehydrated human amnion, chorion membrane (DHACM**).

MATERIALS AND METHODS

Histological Evaluation: Hematoxylin and Eosin (H&E) staining was performed on paraffin embedded sections of DHACM and LHACM. Immunofluorescence analysis was used to visualize type I and type IV collagens.

Thickness Measurements: A digital caliper was used to measure thickness of each DHACM and LHACM graft. Three measurements were distributed across each graft. N=15 grafts.

Barrier Properties: Grafts were hydrated in phosphate buffered saline (PBS) and placed in the dialysis chamber. A standard molecular weight marker or PBS was added to the cavity on each side of the dialysis chamber. The dialysis chamber was incubated at 4°C, for 3 days, allowing the passage the protein of the molecular weight marker with a diffusion dependent on the permeability of the barrier. Permeable proteins were evaluated using SDS-PAGE. N=3 grafts.

Extract Preparation: Human amniotic tissue (amnion, intermediate, and chorion layers) was processed using a proprietary and patent-pending cleansing process followed by lyophilization and terminal sterilization‡. Soluble factors from LHACM and DHACM were extracted in assay-appropriate basal media at 4°C for 24 hours.

Bioactivity: Proliferation: Human dermal fibroblasts (HDFs) were treated with basal media supplemented with DHACM or LHACM extract at final concentrations of 10 and 1 mg/mL. Following a 72 hour incubation at 37˚C, cellular proliferation was determined by CyQuant Assay. Migration: HDFs were plated at confluence on ImageLock plates (Sartorius) and incubated overnight at 37°C. Monolayers were scratched using the WoundMaker (Sartorius) and treatments applied at final concentrations of 10 and 1 mg/mL DHACM or LHACM extract. Basal (DMEM with 0% FBS) and complete (DMEM with 10% FBS) media served as controls. Cellular migration was determined by live cell imaging for 120 hours with automated image processing to determine % Wound Confluence at each time point (S3 IncuCyte, Sartorius).

Metabolism: HDFs were plated at 15,000 cells per well of a 96-well plate for 24 hours in DMEM containing 10% FBS. After 24 hour incubation to allow for cell adhesion, the cells were serum starved for 24 hours in basal DMEM. The cells were treated with extract at 10 and 1 mg/mL concentrations. After 48 hour treatment, L-Lactate in the media was assessed using the Lactate-Glo assay (Promega). Data was normalized to the Cell Titer-Glo assay (Promega).

***In vivo* mouse model:** Female and male NU/J athymic nude mice were implanted with a 1 cm x 1 cm piece of DHACM or LHACM into a surgical pocket. Mice were euthanized at 1, 2, and 4 weeks post implantation. The implant sites were harvested *en bloc* with >10 mm tissue margins to include epidermis, dermis, muscle, and other surrounding soft tissues. Samples were fixed in 10% neutral buffered formalin for at least 12-24 hours, then transferred into 70% ethanol. Samples were paraffin-embedded and sections stained for Hematoxylin and Eosin (H&E).

Immunofluorescence: Immunofluorescence was performed on formalin-fixed paraffin-embedded sections. Briefly, sections were deparaffinized, subjected to antigen retrieval followed by blocking in Serum-Free Protein Block (Agilent Dako) for 1 hour at room temperature. Incubation with primary antibody against human-specific type IV collagen, mouse-specific type I collagen, and CD31 in Antibody Diluent (Agilent Dako) was carried out overnight at 4°C. For visualization, cells were incubated with Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 and Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 (Thermo Fisher) and DAPI (Vector Laboratories) to identify the nuclei. Images were acquired on a Leica microscope fitted with 10x and 40x objectives, using Leica Application Suite Advance Fluorescence software and the THUNDER Imager (Leica Microsystems).

RESULTS

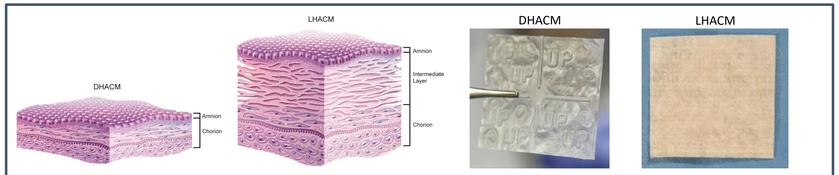

Figure 1. Left: Schematic illustration of DHACM and LHACM allografts highlighting the layers in each amniotic membrane allograft. Right: Top view of DHACM and LHACM allografts.

RESULTS

LHACM is thicker than DHACM due to inclusion of the intermediate layer and lyophilization

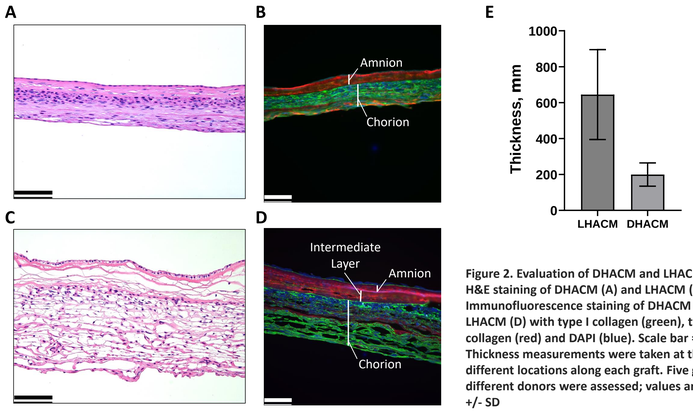

Figure 2. Evaluation of DHACM and LHACM allografts. H&E staining of DHACM (A) and LHACM (C). Immunofluorescence staining of DHACM (B) and LHACM (D) with type I collagen (green), type IV collagen (red) and DAPI (blue). Scale bar = 100 µm. (E) Thickness measurements were taken at three different locations along each graft. Five grafts from 3 different donors were assessed; values are the means +/- SD

DHACM and LHACM function as an effective barrier

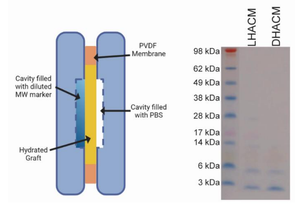

Figure 3. Barrier properties of LHACM and DHACM. (Left) Experimental apparatus diagram depicting the setup of the dialysis chamber. (B) Image of SDS-PAGE analysis of permeable proteins that moved into the chamber with PBS after 3 days of dialysis. Data shows that proteins >14 kDa molecular weight did not diffuse through the membrane.

DHACM and LHACM promote cellular proliferation, migration and metabolism of HDFs in vitro

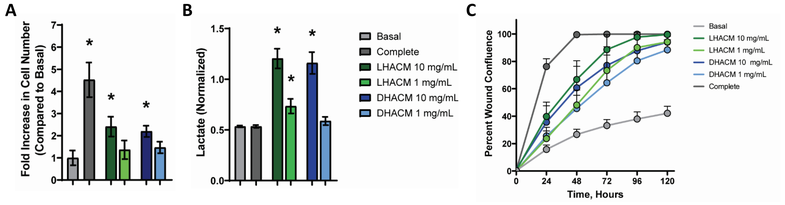

Figure 4. *In vitro* bioactivity in HDFs. (A) Proliferative response to LHACM and DHACM when HDFs were incubated with extract for 72 hours. Data normalized to the basal group as average fold change ± the standard deviation. (B) Metabolic response to LHACM and DHACM. Lactate measurements normalized to cell viability. (C) Migration of HDFs in a 2D scratch assay. Live cell label-free imaging of HDF over 5 days under exposure of LHACM or DHACM extracts or media controls. Data presented as % coverage of the initial scratch area at each time point ± standard error. *p<0.05 relative to the basal group.

RESULTS

Implantation of DHACM or LHACM results in reorganization, neocollagen formation associated with infiltrating host fibroblasts, and recruitment of endothelial cells and neovascularization

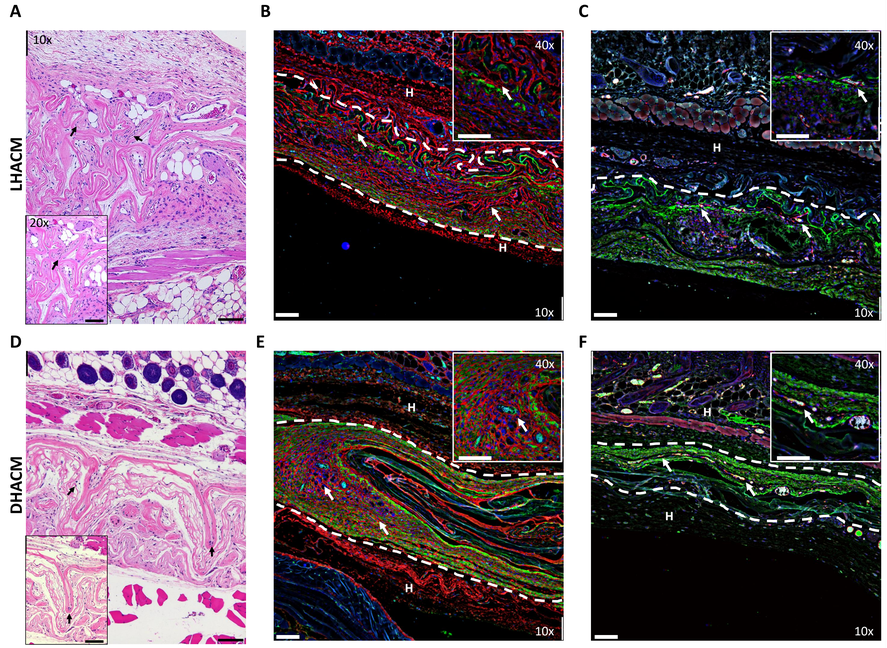

Figure 5. Host cell infiltration in response to DHACM or LHACM post *in vivo* implantation. The cellular response to DHACM (A) and LHACM (D) was assessed after 4 weeks of subcutaneous implantation. Reorganization and neocollagen formation associated with infiltrating host fibroblast cells (arrows). Immunofluorescence of cellular infiltration and associated neocollagen formation (arrows); DHACM (B) and LHACM (E): human type IV collagen (green); mouse type I collagen (red); cell nuclei (blue). Recruitment of endothelial cells and neovascularization within the DHACM (C) and LHACM (F) implant (arrows). Immunofluorescence of endothelial cells: human type IV collagen (green); CD31 (red); cell nuclei (blue). H: host tissue; Scale bar = 100 µm

CONCLUSION

The physical and biological properties of an amniotic membrane allograft are determined by the preservation processes employed during manufacture and the presence or absence of constituent anatomical layers. PURION processed LHACM and DHACM differ in thickness and handling properties but possess similar *in vitro* and *in vivo* activities highlighting the versatility of this processing method. LHACM offers an allograft with the biological properties synonymous with the PURION‡ process, but with alternative structural and handling properties relative to DHACM.

ACKNOWLEDGEMENTS

In vivo study was conducted at Global Center for Medical Innovations (Atlanta, GA). Histology of the *in vivo* study was conducted by StageBio (Fredrick, MD).

** AMNIOFIX®, MIMEDX Group Inc. Marietta, GA;*AMNIOEFFECT™, MIMEDX Group Inc. Marietta, GA; ‡PURION® Process, MIMEDX Group, Inc., Marietta GA

All authors are employees of MIMEDX Group, Inc.