Characterization of Human Amniotic Membranes for Clinical Wound and Wrap Applications

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Introduction

Human amniotic membrane (HAM) has been used since the early 1900's as an aid in tissue replacement. Over the decades it has been applied as a wound covering for integumentary acute and chronic wounds, burns, ophthalmic damage, surgical wraps such as tendons and spine and dermatological applications.

The human placenta is a multi-layered organ comprised of maternal (decidua) fetal (chorion and amnion) tissue. The amnion, innermost membrane that surrounds the fetus, is a thin, avascular membrane consisting of an epithelium thick basement membrane, compact avascular stroma, and a fibroblast layer. An intermediate spongy layer is loosely connected to the chorion (Figure 1). The amnion is readily separated from the chorion by blunt dissection, then washed with PBS to remove blood and debris, dehydrated, packaged, and terminally irradiated.

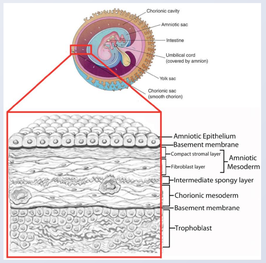


Figure 1: Amnion structure. McDonald, 2011

Many amniotic membrane products are regulated by the Food and Drug Administration (FDA) as a Medical Device or a Human Cells, Tissues and Tissue Based Product (HCT/P), based on several factors, including processing and claims for use. For an HCT/P, amniotic tissue is minimally manipulated, preserving the basic functions of the tissue *in utero*, e.g., covering and structural barrier. It retains the native cells and architecture, has low immunogenicity, is an effective wound covering, provides a structural component with the presence of a basement membrane and is a rich source of growth factors, cytokines and antimicrobial peptides (AMPs).

Presented here is a research use, non-claims-based characterization of human dehydrated amniotic membrane (dHAM) to better understand how this tissue, normally medically discarded may facilitate healing in a multitude of applications. We start with DualGraft™†, a dual layered amnion layered with stromal layer inside and epithelial side facing out for the initial studies, assessing biocompatibility, antimicrobial activity, then quantifying the porosity and mechanical properties of cesarean section amnion.

† DualGraft™ is a product of Axolotl Biologix, Inc.

Biocompatibility

The biocompatibility of dHAM used as a wound covering or structural barrier in clinical applications is important to ensure the treatment fosters the healing process by integrating into the wound bed, leading to improved healing outcomes. This was tested by a cell viability assay and confocal evaluation of DualGraft™ cellularized with primary adult human dermal fibroblasts (hDF).

Cell Viability: Fibroblasts were seeded with DMEM + 10% FBS in a 96 well plate in the presence or absence of 4 mm discs of Axolotl DualGraft™ and cultured for 48 hours. CellTiter Glo reagent (Promega, Madison, WI), a luciferase-based ATP detection system was added to lyse the cells. ATP levels were quantified by luminescence, Figure 2A.

Confocal: Fibroblasts were cultured on dHAM for 48 hours, incubated with CellTracker Green CMFDA, which is converted to a fluorescent analog by viable cell esterases, then visualized on a confocal microscope, Figure 2B.

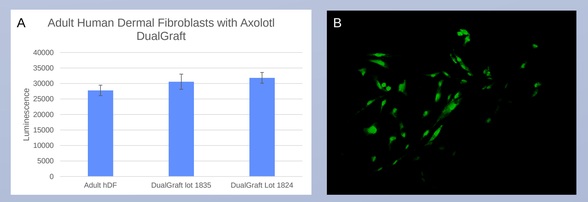


Figure 2: A) Viability of adult fibroblasts cultured with dHAM for 48 hours. Adult hDF column represents cells cultured without dHAM. B) Fluorescent cells are adult human dermal fibroblasts growing on dHAM following 48 hours culture.

Antimicrobial Properties

A Kirby-Bauer antimicrobial disk diffusion assay was used to assess the antimicrobial activity using three species known to cause nosocomial infections (*Candida albicans*, *Escherichia coli*, *Staphylococcus epidermidis*) with and without organism-specific antibiotic-, antimycotic-treated disks. Two antibiotics, one control, one dHAM and one conditioned media treated disk were applied to a lawn of each microbe, except for the *C. albicans* where only one antifungal was added, n = 8 per organism.

For all plates, the microorganisms demonstrated growth inhibition with the positive controls but did not show growth inhibition (0 mm) on the dHAM, conditioned media and the negative control disks, Table 1.

Average Inhibitory Zone (n=8)						
Organism	**Tetracycline**	**Chloramphenicol**	**Gentamycin**	**Fluconazole**	**Dual Graft**	**Control**
E. Coli	26	27	---	---	0	0
S. Epidermis	18	---	26	---	0	0
C. Albicans	---	---	---	30	0	0

Table 1: Zone of inhibition for each treatment group per organism in mm. (---, not tested)

Porosity

The ability for cells to grow on, in or through a biocompatible membrane is partly related to its porosity. Tests were performed to determine the size and type of pores present in DualGraft, including through pores, open pores, blind pores and inter-material voids.

Capillary Porosimetry: The presence of through pores, continuous channels which traverse the membrane from one side to another were measured by Porometer 3G. The samples were analyzed wet, then following dehydration, analyzed dry, Figure 3.

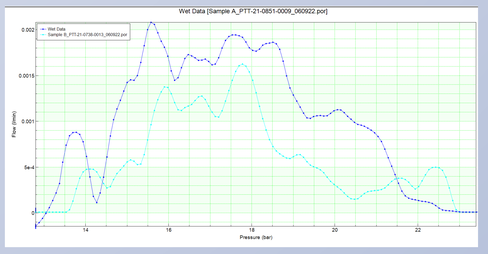


Figure 3: Flow rate (L/min) versus pressure (bar), demonstrating the absence of through pores.

Mercury Intrusion Porosimetry: The size of inter-material voids and intra-material blind and open pores was quantified by mercury intrusion, measuring the pressure required to intrude pores with liquid mercury, quantified by contact angle, Figure 4.

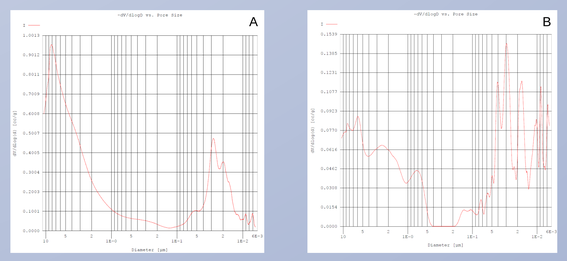
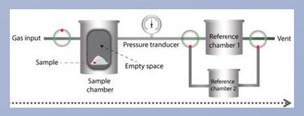


Figure 4: Pore size range in two lots of dHAM, quantified by mercury intrusion porosimetry. The largest pores are on the left of the x-axis. A) Donor 0851, pore diameter range 10.6 to 0.006 µm with mean of 0.079 µm. B) Donor 0738, pore diameter range 10.7 to 0.006 µm, with mean of 0.035 µm.

True Density: Gas pycnometry uses Boyle's Law to quantify the volume of gas displaced by a sample and is used to calculate the volume or true density of a solid.

Density varied 19% across donors at 1.4903 and 1.2226 g/cm^3 with % CVs of 0.45% and 0.19%.



Mechanical Testing

Understanding the mechanical properties of a membrane enhances the ability to compliance match the target tissue and reduce or eliminate compressive, shear or fracture failures. DualGraft was tested dry or hydrated with PBS, pH 7.4, in conditions that mimic physiological processes. Thickness varies from ≈ 50 µm dry to ≈ 67 µm hydrated. All tests were performed with a HR-2 rheometer (TA Instruments) at room or body temperature.

Tensile Modulus and Strength: The response to forces applied to stretch the membrane, dry and wet, under physiologically relevant conditions was quantified. Starting tensile forces ranged from 0.1 N (100 mmHg), 0.225 N (225 mmHg) and 0.35 N (335 mmHg) and oscillation rates of 1 to 20 rad/s (10 – 192 BPM) were applied, Figure 5.

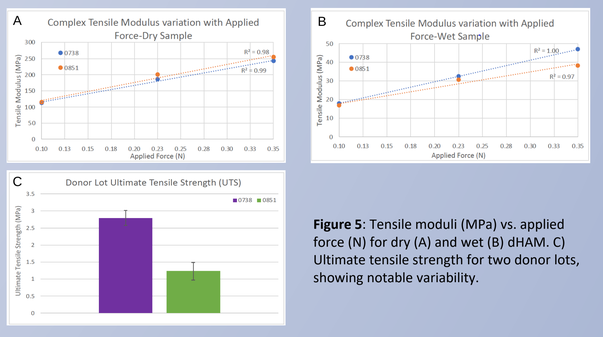


Figure 5: Tensile moduli (MPa) vs. applied force (N) for dry (A) and wet (B) dHAM. C) Ultimate tensile strength for two donor lots, showing notable variability.

Compressive and Shear Modulus: To quantify the resistance to compressive forces, e.g., compression of a wound covering on a foot ulcer during walking, or the resistance to the layers delaminating (shearing apart), a stack of six 20 mm circles (to increase sample height) of hydrated membrane was compressed to a steady state of compression (0.2 – 0.35 N) at 27.5% – 30% compression. A dynamic shearing rate of 1 to 20 rad/s was applied to quantify shear modulus, Figure 6.

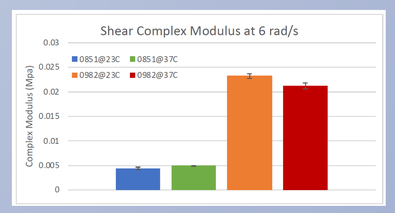


Figure 6: A) Comparison of shear modulus (G*) for two sample lots at a physiologically relevant shear rate (6 rad/s, 1 Hz, 60 BPM).

Ultimate Suture Strength (USS): This tensile force-related measure quantifies the strength required to tear a suture from a hydrated graft during tension pulling, using an interrupted 2-0 prolene suture with a pull rate of 50 µm/s until the suture completely tore from the membrane, Figure 7. Pull forces were extrapolated for various suture sizes, Table 2.

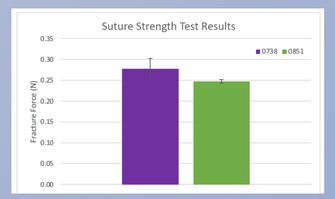


Figure 7: Suture strength, quantified by the force required to completely tear the suture from the membrane. There is no significance between the two lots.

Ultimate Suture Strength (USS) table

suture size (USP)	suture diam. (mm)	suture area (mm2)	USS (MPa)	**suture force (N)**
6-0	0.07	0.005	5.6	**0.03**
5-0	0.10	0.008	5.6	**0.04**
4-0	0.15	0.012	5.6	**0.07**
3-0	0.20	0.016	5.6	**0.09**
2-0	0.30	0.024	5.6	**0.13**
0	0.35	0.027	5.6	**0.15**

Table 2: Ultimate Suture Strength (USS) determination and suture force based on suture size.

Discussion

We performed a broad characterization of the biocompatibility, porosity and mechanical properties to scientifically understand how dHAM functions as a wound covering and how it improves healing outcomes compared to Standards of Care.

Biocompatibility was confirmed by comparable ATP production for fibroblasts cultured with or without dHAM. Fluorescent viable cells were visualized under confocal microscopy, readily distinguished from the dehydrated epithelial cells resident in the membrane.

Antimicrobial activity was not detected by the simple disk diffusion test. An initial review of a deep proteomics analysis of the dHAM demonstrate the presence of several AMPs (data in review), suggesting that these peptides either do not diffuse out of the membrane or are biologically inactive. Research by Ramuta et al., demonstrated that these AMPs are present and bioactive when solubilized from the membrane by homogenization.

As a wound covering and structural barrier, dHAM must have structural strength. The absence of through pores and presence of nanometer sized blind and open pores confirm that the epithelial cells and basement membrane suggest preservation of the tight junction and barrier functions. Variability in the volume or true density is expected with donor variability, as do several mechanical characterization results.

Exploring the fundamental biocompatibility and mechanical properties of dHAM, will increase understanding of how best to use these products with Standard of Care to improve clinical or surgical outcomes. Dehydrated amniotic membrane is under increasing scrutiny by regulatory bodies, to ensure inaccurate or inappropriate claims are not made that may put a patient at risk.

Next Steps

An in-depth analysis of the 7,678 proteins identified from dHAM proteomics is underway, identifying key AMPs, growth factors, cytokines and other proteins to increase understanding of its biological functions when applied as a wound covering and tissue wrap, including further characterization of AMP activity. A comparison characterization study will be performed with dHAM from different sources e.g., vaginal sources and other comparator membranes.

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Disclosure Statement

All information presented herein is represented as pre-clinical, research use only data and is not meant to alter any claim or intended use of the Axolotl Graft™ or DualGraft™ product. Conflict of interest statement: All authors are paid employees of Axolotl Biologix.

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