MATRACELL™ Decellularized Allograft Bio-implants

MATRACELL™ Decellularized Allograft Bio-implants – Critical Applications for Cardiovascular Surgery

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Abstract

Matracell™ decellularized CardioGraft® is human cardiovascular tissue that has gone through a patented and validated process to render the tissue acellular, by removing over 99% of the donor genetic material, while retaining the biomechanical strength of the tissue. It is well documented in the surgical literature that the donor cell component present in cryopreserved cardiovascular allograft tissue is the source of its three primary failure modes: calcification, stenosis and immunogenicity.[1,2,3] The donor cells serve as the nucleation point for calcification, which can lead to stenosis and replacement of the allograft tissue. Another failure mode, immunogenicity, is also well documented in the surgical literature and can be attributed to the donor cells.[3,4,5] LifeNet Health's proprietary and patented decellularization process is a methodology that removes donor cells from allograft tissues while retaining the native biomechanical strength of the tissue as determined by extensive bench and large animal testing. This processing is coupled with LifeNet Health's long history of providing safe allografts for clinicians and their patients.[a] Matracell decellularized cardiovascular tissues are currently indicated for use in the repair of the right ventricular outflow tract.

Introduction

Approximately every 9 in 1,000 live births are diagnosed with a congenital heart defect yearly.[6] Of those, 26% require surgical or catheter procedures in the first year of life.[3] These infants often require the use of donated human cardiovascular tissue (allograft/homograft) to repair and/or reconstruct their heart valves and associated outflow tracts.

These young patients are challenging surgical cases due to their small size. As such, cryopreserved cardiovascular allografts are the primary material used to treat these children due to its compliance and malleability.[7] The biomechanical compliance of allograft tissue is similar to the compliance of the recipient tissue into which it is being sewn, in contrast to that afforded by synthetic materials.[8] With all of the positive reasons associated with the use of allograft cardiovascular tissues, there are also drawbacks.

The cardiovascular surgical literature is replete with references to the "ideal" cardiovascular repair product and it is often described as possessing the following characteristics[9]:

- Long-term patency
- Availability in a range of sizes
- Excellent handling characteristics
- Compliant and flexible
- Long-term valve function (if valved)
- Low-to-moderate costs
- Low infectious potential
- Non-thrombogenic
- Potential for growth
  (in particular for pediatric patients)

It has been hypothesized that some of these advantages could be better realized through further processing of cryopreserved cardiovascular allografts to render them acellular; however, the processing must be well conceived.

Cardiac tissues are comprised of three distinct tissues: 1) myocardium, 2) conduit and 3) leaflet. These tissue types vary in their thicknesses, cell types, matrix proteins, biomechanical characteristics and function. Therefore, the decellularization procedure should be designed to be stringent enough to completely decellularize the thick

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myocardium yet gentle enough to not compromise the biomechanical strength of the conduit and delicate leaflets. The end result of the decellularization procedure is an extracellular matrix comprised of collagen, elastin and proteoglycans. The scientific rationale utilized in developing an effective decellularization process should involve the following:

1. Use of reagents that will leave the remaining extracellular matrix biocompatible and biomechanically sound;

2. Characterization of reagents residuals;

3. Characterization of biocompatibility;

4. Characterization of the biomechanical strength of the resultant extracellular matrix;

5. Assessment of the function and durability in a large animal model (ISO 5840); and

6. Validation of manufacturing procedures to reproducibly decellularize the tissue.

Multiple strategies have been employed by researchers and three will be further discussed.

ENZYME BASED DECELLULARIZATION STRATEGIES

Enzyme mediated decellularization strategies are based upon controlling the enzymatic degradation of the constituent cells so as not to negatively affect the extracellular matrix that provides cardiovascular allografts with their biomechanical strength.[10] The most often employed combination is trypsin and EDTA. Trypsin is utilized during routine cell culture to release adherent cells from the flask in which they are being grown and thus adapted to decellularization for the same general purpose – cell removal from the extracellular matrix. Trypsin is a serine protease that cleaves proteins at the arginine or the lysine amino acid residue on the carboxyl side except when followed by proline. This is advantageous as proline is one of the main constituent amino acids in collagen and hence the collagen extracellular matrix is generally not as susceptible to trypsin degradation. EDTA is employed to inactivate the intracellular proteases that may be released as the cells are being

trypsinized as these proteases can degrade the extracellular matrix.[11] However, there are multiple disadvantages to this approach:

1. Trypsin is still capable of degrading the extracellular matrix;

2. EDTA may not be able to inhibit all the proteolytic activity of the intracellular proteases released from the cells;

3. The decellularization process is performed at 37°C which is the optimal temperature for proteolytic enzyme activity; and

4. The decellularization process does not employ the use of endonucleases to degrade DNA and facilitate its removal from the extracellular matrix.

Thus, due to all four of these factors, the biomechanical integrity of the extracellular matrix could be adversely affected.

HYPOTONIC LYSIS

Decellularization accomplished by hypotonic lysis is currently being utilized to produce decellularized pulmonary allograft heart valves by CryoLife (Kennesaw, GA) that are commercially available in the United States. The PowerPoint available on the CryoLife website states that the genetic (DNA) content of tissue is reduced by >99% as assessed spectrophometrically (slide 9).[12]

The decellularization procedure follows routine dissection and disinfection according to the PowerPoint (slide 7) available on the company's website.[12] According to US Patent 7,318,998 the decellularization might be accomplished by:

1. Multiple incubations of the tissue in hypotonic solution over the course of two weeks;

2. DNAse (derived from bovine pancreas) and RNase incubation of the tissue to degrade the donor DNA and RNA;

3. Sequential washing of the tissue; and

4. Routine Cryopreservation of the tissue.

This decellularization process has several disadvantages:

- The decellularization process requires the tissue to be in solution for an extended period of time that could result in hydrolysis of the collagen fibers, which may negatively affect the biomechanical integrity of the tissue;
- Lack of a detergent to facilitate cell remnant removal could result in cellular remnants being left in the tissue, potentially causing an inflammatory response; and
- The endonuclease is derived from a bovine source and thus there exists the risk of prion disease transmission.

DETERGENT MEDIATED DECELLULARIZATION

Detergent mediated decellularization has been attempted by many groups. There are two main classes of detergents that have been utilized: non-ionic and anionic. Detergents have a hydrophilic head and hydrophobic tail, and thus, can penetrate the extracellular matrix and cell membranes as a result of their ability to reduce the surface tension of the local environment.[13] However, non-ionic detergents like Triton X 100 have been demonstrated to lack sufficient strength to fully decellularize cardiovascular tissues.[14]

Anionic detergents have also been utilized to decellularize cardiovascular tissues.[15] Anionic detergents are stronger solubilizing agents than non-ionic detergents due to the charged moiety on their hydrophilic head. However, with this added strength can come some detrimental effects. One detergent, sodium dodecyl sulphate (SDS), has been widely employed; however, due to its known ability to denature proteins, may not be a good candidate detergent.[16] As such, SDS has the potential to result in reduced biomechanical strength, and thus, predispose the allograft to aneurysm formation once *in vivo.* Additionally, denaturation of the extracellular matrix proteins such as collagen could increase the immunogenic potential of the allograft by exposing amino acid residues that are commonly found in the interior of the molecule such as glycine. Another

anionic detergent, N-Lauroyl sarconsinate (NLS), has been successfully utilized to decellularize pulmonary artery patch grafts in conjunction with a recombinant endonuclease to degrade the DNA/RNA[17, 18] and is described in U.S. Patent 6,743,574. NLS is different from SDS in that it is not denaturing; however, it is an efficient solubilizer, thus, effecting a rather complete decellularization. Additionally, NLS possesses bactericidal properties which have led to its use in toothpaste, cosmetics and shampoo and can be thought of as another means by which the tissue can be further disinfected.[19, 20]

Detergent mediated decellularization has potential disadvantages if the correct detergents are not selected for the decellularization process, thus, resulting in an incompletely decellularized extracellular matrix (non-ionic detergents), use of overly aggressive detergents (SDS) that may negatively affect the biomechanical integrity of the tissue and not optimizing the detergent concentration/contact time and quantification of the detergent residuals.

NUCLEASES

Several, but not all of the decellularization methodologies outlined above utilize endonucleases to degrade the constituent DNA/RNA because detergents alone are insufficient to degrade and remove nucleic acids. Some of these enzymes are extracted from bovine tissues, and thus, there is the potential for prion disease transmission. Other options to degrade DNA are recombinant endonucleases. Two commercially available recombinant endonucleases are Benzonase® by Merck and Pulmozyme® by Genetech.

LIFENET HEALTH'S MATRACELL™ PROCESS

LifeNet Health's Matracell process was developed to minimize the amount of reagents and reagent contact time required to render the cardiovascular tissue acellular. Additionally, a thorough characterization of the processing reagents that could possibly remain

associated with the tissue was performed. Taking advantage of knowledge gained from the decellularization technologies described above, Matracell processed tissue is rendered acellular in a solution of non-denaturing anionic detergent (N-Lauroyl sarcosinate, NLS), recombinant endonuclease (Benzonase®) and antibiotics (Polymixin B, Vancomycin and Lincomycin). Furthermore, following decellularization, the tissue is rinsed of the decellularization reagents by circulating the rinse fluid through a bed of anion exchange resin and hydrophobic adsorbent resin to continually regenerate the rinse fluid, thus, allowing maximal reagent removal. The last step entails treating the decellularized tissue with glycerol to remove and replace the water volume with glycerol prior to freezing the tissue. The Matracell process is carried out in a closed system inside a class 100 cleanroom. The Matracell process has been fully validated to reproducibly render cardiovascular tissue acellular as assessed by the DNA content being reduced by >99%. Figure 1 below illustrates traditionally cryopreserved tissues and Matracell processed tissue.

MATRACELL™ PRE-CLINICAL TESTING

The Matracell process has been rigorously characterized and investigated to understand the optimal conditions for decellularization. The resultant Matracell decellularized tissue has been thoroughly assessed via analytical methods, biomechanical analyses, *in vivo* analysis in a large animal model and lot release testing of every lot of donor tissue processed.

Characterization of Processing Reagents

The concentration and contact time for the NLS exposure were experimentally determined by conducting binding and release experiments with radiolabeled NLS. This same approach was utilized to determine how extensively to rinse the tissue to reduce the day one reagent residuals. The final amount of NLS and Benzonase associated with the tissue were quantitatively determined. The amount of NLS was below the cytotoxic threshold and the amount of Benzonase was below the detection limit of Merck's Benzonase® ELISA assay, and thus, below the cytotoxic threshold.

DNA Content

The DNA content of tissue decellularized using the MATRACELL™ process is reduced by >99% and is assessed for every lot (one donor = one lot of tissue) of tissues using a validated DNA assay in LifeNet Health's CLIA certified laboratory. The DNA assay was validated as described in the International Committee on Harmonization document Q2, "Validation of Analytical Procedures: Text and Methodology." The assay utilizes a fluorometric dye, PicoGreen (Invitrogene) that has a lower limit of detection of 0.7 ng DNA/ml and lower limit of quantitation of 2.7 ng DNA/ml. This fluorometric assay is minimally 100 times more sensitive than standard spectrophotometric methods used by manufacturers of other decellularized cardiovascular products.
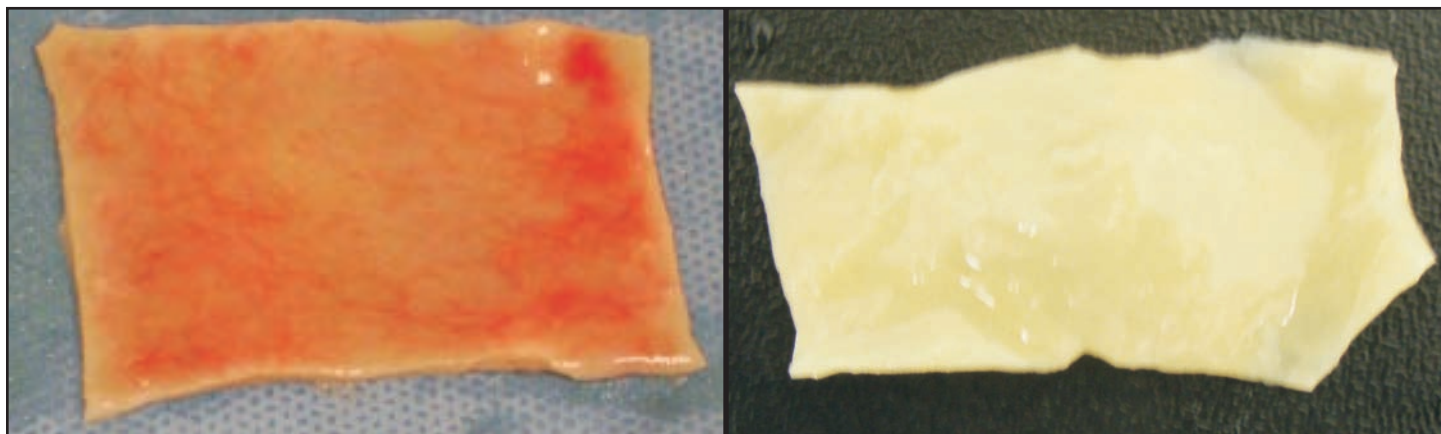

Figure 1. The tissue on the left is representative of tissue prior to decellularization with MATRACELL™ Process and the tissue on the right is representative of tissue after decellularization with MATRACELL™ Process.

Microbiological Assessment

Every lot (one donor = one lot of tissue) of Matracell processed allograft tissue is co-processed with a representative piece of conduit tissue, of the same approximate size and from the same donor. This representative piece of tissue is microbiologically assessed for its microbial culture status according to USP<71> test methodologies. Also, the last solution in contact with the tissue is microbiologically assessed according to USP<71>. The end-point testing was fully validated using the organisms required by USP<71>, and additional organisms known to frequently occur on cardiovascular tissue were utilized during the test method validation to ensure the methodology was sufficiently robust to detect any organisms, which may have been present on the tissue. Thus, every lot of Matracell™ processed tissue must be culture negative per USP<71> standards to be released for implantation.

Histological Assessment

As shown is Figure 2, representative histological analysis of cardiovascular tissue rendered acellular by the Matracell process corroborates the DNA assay results, in that there are no visible cells or genetic material, Figure 2.
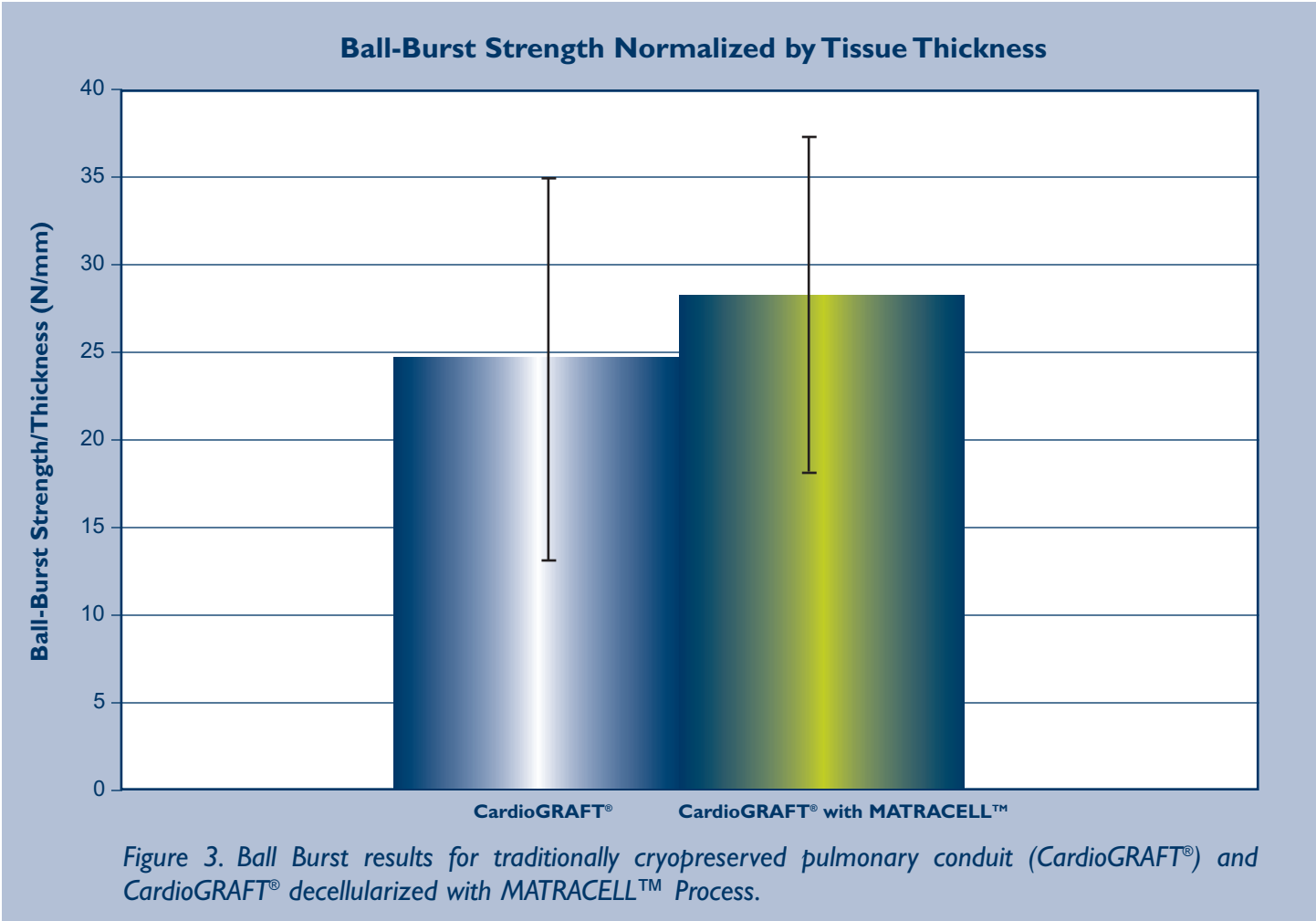
Biomechanical Assessments

Two different biomechanical assessments were performed, ball burst testing and suture pull-out testing, to confirm the Matracell processing did not adversely affect the biomechanical strength of the tissue. Biomechanical testing according to ASTM D 3797-89, "Standard Test Method for Bursting Strength of Knitted Goods, Constant-Rate-of-Transverse (CRT) Ball-Burst Test," was utilized to assess the biaxial strength of the conduit tissue post-decellularization.[21] Testing the biaxial strength of the tissue, allows susceptibility to aneurysmal formation to be assessed by stressing the collagen fibers in a manner similar to that of high pressure blood flow. Data replicating the aneurysmal failure mode are not readily attained by standard uniaxial tensile testing due to the unidirectional force that is applied to the tissue, parallel to the collagen fibers, versus biaxial testing where the pressure is applied perpendicular and parallel to the collagen fibers. The ball burst strength of traditionally cryopreserved cardiovascular conduit tissue was compared to conduit tissue rendered acellular using Matracell Process and found not to be statistically significantly different at the 95% confidence interval, $p>0.05$ (Figure 3).
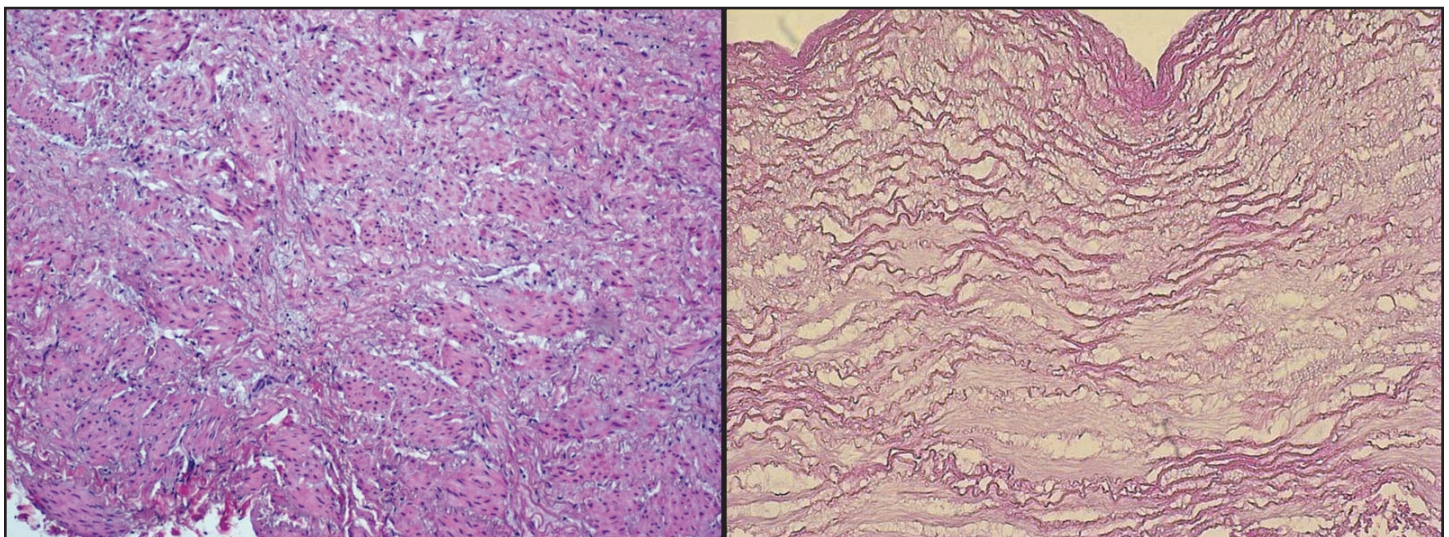
Fresh Conduit Tissue **MATRACELL™ Processed Conduit Tissue**

Figure 2. Hematoxylin and Eosin (H&E) staining of pulmonary conduit tissue. On the left is fresh, cellular conduit tissue as evidenced by the blue staining DNA inside the cells and on the right is tissue decellularized using MATRACELL™ Process demonstrating removal of cells and cellular remnants as evidenced by a lack of blue staining elements.

Ball-Burst Strength Normalized by Tissue Thickness

Figure 3. Ball Burst results for traditionally cryopreserved pulmonary conduit (CardioGRAFT®) and CardioGRAFT® decellularized with MATRACELL™ Process.

These data were transformed to evaluate the burst strength of traditionally cryopreserved pulmonary conduit tissue and pulmonary conduit tissue decellularized with Matracell Process relative to the pressure exerted on a vessel under stage 1 hypertension at 140 mmHg. The data presented in Table 1 demonstrate that traditionally cryopreserved tissue and tissue decellularized with Matracell. These data were transformed to evaluate the burst strength of traditionally cryopreserved pulmonary conduit tissue and pulmonary conduit tissue decellularized with Matracell Process relative to the pressure exerted on a vessel under stage 1 hypertension at 140 mmHg. The data presented in Table 1 demonstrate that traditionally cryopreserved tissue and tissue decellularized with Matracell Process are thirty times stronger than required to withstand 140 mmHg.

	MATRACELL™ Pulmonary Tissue	Cryopreserved Pulmonary Tissue
Ball Burst Pressure	0.58 +/- 0.14MPa	0.54+/-0.24 MPa
Type I Hypertension	0.0187 MPa (140 mmHg)	
Ball Burst Strength	25.20 ± 9.20 N/mm	21.77 ± 8.22 N/mm
Ball Burst Strength p-value	P=0.37	

Table 1. Ball Burst Pressure Transformed into Burst Pressure.

To further characterize pulmonary conduit tissue decellularized by Matracell process, suture retention testing was conducted. The results demonstrated no difference in suture retention strength between cryopreserved pulmonary tissue and Matracell decellularized pulmonary tissue, p>0.05, Figure 4. The sutures were applied in the three directions: parallel, perpendicular and at a 45° angle to the collagen fibers.

In Vivo Durability and Functional Assessments

An *in vivo* pre-clinical evaluation was performed in the female juvenile sheep model according to ANSI/ISO/AAMI 5840, "Cardiovascular Valve Prostheses." This model was used to replicate the human cardiovascular system, as sheep provide a similar hemodynamic and load environment to test human cardiac devices. The Matracell processed test device was allograft (sheep) pulmonary conduit tissue. The in-life duration was 20-weeks. During the in-life portion of the study, the animals' blood was assessed regularly to determine any local organ toxicity and inflammatory response to the device. The functionality of the device was assessed by echocardiography post-implant, mid-study and prior to sacrifice. At sacrifice, the animals were given a full necropsy to assess the health of the animal and potential adverse effects of the device on the internal organs. The explanted devices (two per animal) were assessed. The first explant was assessed for total calcium content by inductively coupled optical emission spectroscopy (ICP-OES), Table 2. The second explant was assessed histologically for inflammation, recellularization, calcification, presence of phenotypically correct cells and apoptotic cells. The Matracell process test articles resulted in in-life and explant data that raised no new concerns of safety relative to the predicate device and traditionally cryopreserved materials.

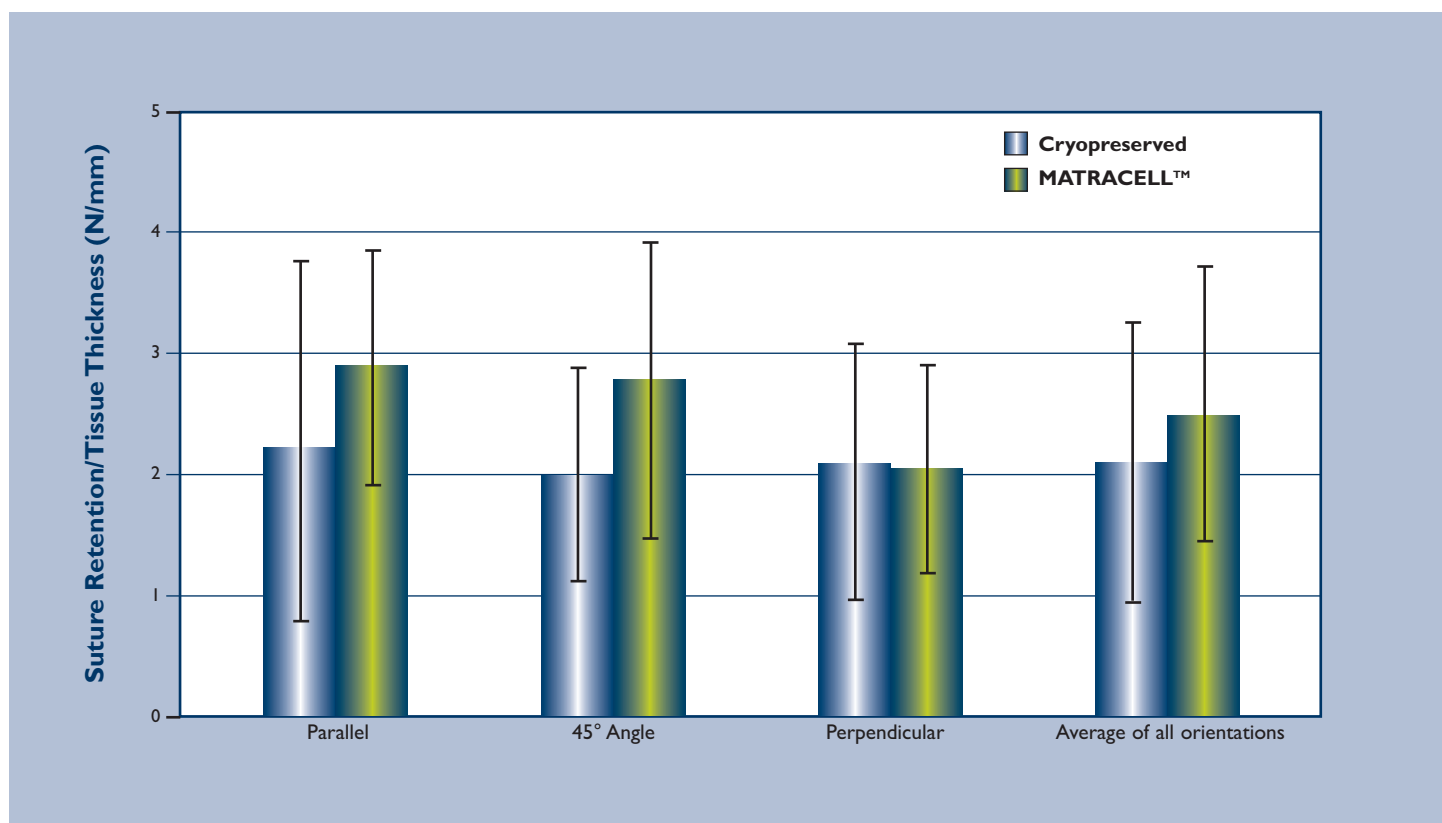**Suture Retention Strength Normalized by Tissue Thickness**

Figure 4. Suture retention strength results for traditionally cryopreserved conduit (CardioGRAFT®) and CardioGRAFT® decellularized with MATRACELL™ Process.

Sample	Calcium (µg/mg tissue)	Standard Deviation
Traditionally cryopreserved (whole explant)	65,855	44,240
Traditionally cryopreserved (center of explant)	32,719	n=2
MATRACELL™ (whole explant)	4,665	5254
MATRACELL™ (center of explant)	140	74
Fresh cellular tissue (non-implanted)	196	72
MATRACELL™ processed (non-implanted)	111	6

Table 2. Total Calcium Content by Inductively Coupled Optical Emission Spectroscopy (ICP-OES). The tissues were explanted, divided into nine sections (1-4 and 6-9 contained sutures and section 5 from the center did not), freeze dried, individually digested in neat nitric acid, assessed for calcium content and the data normalized by dividing the total calcium content by the dry weight of the sample. The fresh cellular tissue and MATRACELL™ tissue controls represent the baseline calcium value expected in the tissue.

WHY CHOOSE A MATRACELL™ CARDIOGRAFT® BIOIMPLANT?

LifeNet Health's Matracell Decellularization Process was scientifically developed by understanding how and why the decellularization process works and then fully validating the process along with end-point testing for every donor lot of tissue to ensure the highest possible quality allograft tissues. Table 3 is a comparison between Matracell processed allografts and other commercially available decellularized allografts for right ventricular outflow tract reconstructions.

Table 3. Key Comparator Table

Key Comparators	MATRACELL™ Process	Other Commercially Available Decellularized Tissue
Decellularization Requires Only Two Days (reduced opportunity for water mediated lysis of the tissue's collagen and elastin scaffold)	✔	Requires one - two weeks in solution
Decellularization in a Closed System within a Class 100 Cleanroom (significant reduction in opportunity for contamination of the tissue)	✔	Unknown
Decellularization employs multiple disinfecting agents targeting hexokinase, cell wall synthesis, protein translation and degradation of genetic material (multifaceted approach to tissue disinfection)	✔	Only one
Decellularization Employs Recombinant Endonuclease (elimination of prion disease transmission risk from endonuclease)	✔	Uses bovine derived endonucleases
Quantitative Analysis of Processing Reagent Residuals (scientifically sound process development)	✔	No known data
Validated Decellularization Process (reproducible process)	✔	✔
Assess DNA Content Post-Decellularization of Every Donor Lot of Tissue (100% verification of the decellularization process for all grafts in addition to the process being validated)	✔	No DNA Assay Validated
DNA Assay Validated (ensures validity, accuracy and precision of resultant data)	✔	Unknown
Sensitivity of DNA Assay <1ng DNA/mL (ensures highest level of detection for potential remaining DNA)	✔	No – spectrophotometry limit of detection >50ng DNA/mL
Validated endpoint microbial testing (ensure ability to detect microorganisms if present)	✔	✔
Endpoint microbial validation included common cardiovascular microorganisms not required by USP<71> (ensure ability to detect microorganisms if present)	✔	Unknown
Microbiological assessment of tissue representative sample and last solution in contact with the tissue (ensure ability to detect microorganisms if present)	✔	Unknown

Conclusions

Matracell decellularization is a validated process that was developed to minimize contact time with the decellularization agents by fully characterizing and optimizing the process. The decellularization reagents were chosen with consideration for patient safety with respect to maintenance of graft structural integrity, ease of removal and eliminating the risk of prion disease transmission. The results are Matracell processed allografts, a validated decellularization process that reproducibly results in allograft tissue in which the DNA content has been reduced by greater than 99%. As an added measure of safety, tissue from every donor lot is assessed for DNA content in LifeNet Health's CLIA certified laboratory, using a validated DNA assay as part of the release criteria. All of the research efforts employed to develop the Matracell process are predicated on years of LifeNet Health's commitment to developing allografts that will further extend the donated gift while providing surgeons access to allografts that will meet their expectations and those of the patient.

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68-20-009 REV 000