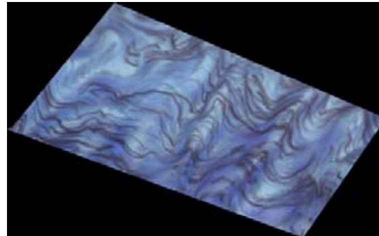
*Completion report on the*

*project*

# "DEVELOPMENT OF DECELLULARISED ANIMAL TISSUE FOR CARDIOVASCULAR APPLICATION"



**FUNDED BY DEPARTMENT OF BIOTECHNOLOGY, NEW DELHI**

**2008-2011**

*Collaborative project between*

**Sree Chitra Tirunal Institute for Medical Sciences and Technology,**

**Thiruvananthapuram**

**and**

**Kerala Livestock Development Board,**

**Government of Kerala**

**December, 2011**

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## I. Project title: "Development of decellularised animal tissue for cardiovascular application"

## II. Investigators


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## III. Introduction

Animal tissue such as bovine pericardium and bovine jugular vein has been used in cardio-vascular surgery after treating them with glutaraldehyde. Problems such as calcification and glutaraldehyde toxicity were observed in such cases. To circumvent this problem, attempts were made to develop techniques which totally avoids glutaraldehyde or which neutralizes the residual glutaraldehyde.

In this project, a technique for de-cellularisation of bovine pericardium and bovine jugular vein followed by cross-linking, neutralization of residual chemicals, anti-mineralization and preservation was developed as a solution to the above problem.

De-cellularised bovine pericardium produced by the technique developed was observed remodelable and less calcifying and it allowed autologous tissue in-growth with ECM laying. Moreover, the graft integrated into the host tissue structurally. This implies that the decellularised bovine pericardium may eventually grow along with the patient following implantation, which will be of utmost importance in pediatric population.

Fabrication of animal tissue based devices requires quality tissue which is to be sourced from a 'low risk herd or 'well monitored herd'.

Through this project, standardization of the processes involved in the production of quality animal tissue sourced from animals meeting the specific criteria and which can be used for biomedical applications was successfully undertaken. The project involved standardization of

1. Procedures for harvest of animal tissue with least microbial load.
2. Procedures for processing the animal tissue such as de-cellularisation, cross-linking, anti-mineralization treatment, sterilization and preservation.
3. Validation of elimination and/or inactivation of virus and/or transmissible agents based on International standard EN 12442-3:2000
4. Procedure for storage of processed animal tissue until further use.
5. Pre-clinical evaluation of the decellularised bovine pericardium qualifying it for human use.

## IV. Objectives:

(1). Standardization and validation of:

- Procedure for harvest of animal tissue conforming to the requirements of EN 12442-2:2000.
- Procedures for processing the animal tissue such as de-cellularisation, cross-linking, anti-mineralization treatment, sterilization and preservation.
- Elimination and/or inactivation of virus and/or transmissible agents based on International standard EN 12442-3:2000.

(2). Preclinical *in-vitro* and animal evaluation of :

- Processed Bovine pericardium for use as a patch material in cardio vascular surgical reconstructions.
- Processed Bovine jugular vein for use as a valve conduit in congenital heart surgery (RV to PA conduit).
- To produce 50 numbers each processed bovine pericardium and jugular vein which will meet the safety requirements needed for its use in the surgical treatment of cardio-vascular diseases in human patients.

## V. Results

### I. Establishment of facility and procedures for collection /handling of animal tissue of biomedical quality based on ISO 22442-part2

Bovine pericardium was sourced from Kerala Livestock Development Board (KLDB), a Government of Kerala Organization which has modern cattle farms with ISO 9001. Infrastructure for collection of bovine pericardium was established at KLDB unit Kulathupuzha, which was 72KM from Trivandrum.

Here bovine pericardium safe for human application was produced by adhering to International standards ISO 22442 (Part 2). The bovine pericardium was sourced from a 'low risk herd' or 'well monitored herd' in which for at least the previous six years

- There was documented veterinary monitoring
- There was no case of Bovine spongiform encephalitis (BSE)
- There was no feeding of mammalian derived protein
- There was a fully documented breeding history
- Each animal was traceable
- Genetic material was introduced only from herds with the same BSE free status and
- No brain penetrating stunning methods are used for slaughter.

KLDB unit, Kulathupuzha

Bovine pericardium was collected with minimum bioburden from only those animals which are healthy and certified 'fit for human consumption' by a veterinarian following antemortem and postmortem inspections. Traceability of each animal, its health status as well as traceability of collected bovine pericardium was thoroughly maintained. Collected tissue was handled in separate area with appropriate level of cleanliness and environmental protection. Care was taken to avoid cross contamination at any stage during collection or transport of tissues. Random samples of serum and brain samples are sent to Southern Regional Disease Diagnostic laboratory, Hebbal Bangalore for screening BSE and other relevant bovine diseases such as Brucellosis, Johnes Disease, Infectious bovine Rhinotrachitis and Peste des petite ruminants (PPR).

Tissue handling at KLDB unit Kulathupuzha

The collected bovine pericardium was transported to Biomedical Technology Wing of SCTIMST, Trivandrum in closed containers to avoid any contamination.

All the above activities are done following approved 'Work procedures' with prompt documentation and periodic auditing.

Any nonconforming tissue was promptly rejected at all stages of processing.

## II. Decellularisation of bovine pericardium (BP) and its further processing for reducing immunogenicity, calcification while improving mechanical strength and constructive remodeling

Glutaraldehyde treated xenografts of porcine and bovine origin has been extensively used as medical implants. By glutaraldehyde treatment stable cross-links in cellular and extra-cellular matrix proteins are formed which reduces graft immunogenicity. However it has been shown that such tissues have altered mechanical property and early mechanical failure because of calcification, cytotoxicity and incomplete suppression of immunological recognition. Glutaraldehyde treated xenografts are not advised for surgical applications in pediatric patients due to increased risk of calcification.

A better alternative to glutaraldehyde treated tissue is de-cellularised tissue. Use of de-cellularised xenografts is an approach where acellular tissue matrices are produced by selective removal of cellular components that are believed to promote calcification and immunogenicity. The different de-cellularisation techniques include chemical, enzymatic and mechanical removal. These acellular matrices promote remodeling of the prosthesis by neo-vascularisation and re-cellularisation by the host. The remodeled de-cellularised xenografts may functionally and structurally integrate into the host tissue.

De-cellularisation is a critical process and inadequate procedure will lead to undesirable results such as calcification or immune reaction. The immune response against incompletely de-cellularised xenografts was seen much more prominent compared to isografts or even allografts.

A process based on non-detergent methods that can be used to de-cellularise allogeneic or xenogenic tissues such as bovine pericardium, porcine pericardium, bovine jugular vein, bovine saphenous vein, bovine aorta and carotid artery was developed and patented. This technique utilized physical methods such as rapid freeze-slow thawing for cell lyses followed by enzymatic treatment for removal of nuclear elements in the tissue. The decellularised tissue was further subjected to removal of soluble proteins by physical methods, followed by stabilization of collagen and fixation of residual protein by short duration low concentration glutaraldehyde

treatment. This was followed by neutralization of residual glutaraldehyde, extraction of lipids, sterilization and preservation. The tissue thus prepared was less immunogenic, less calcifying, have least adjuvant effect, allows cell in-growth and was degradable thus allowing *in-vivo* remodeling as demonstrated in animal implantation studies.

decellularised BP

a. **Characterization of Decellularised Bovine pericardium (Dcl BP)**

i. **Confirmation of decellularisation**

Decellularisation was confirmed by demonstrating absence of nuclear remnants using routine HE staining as well as by use of specific nuclear stain such as Hoechst 33258. Besides this, residual DNA was extracted from 100mg decellularised BP using DNA- XPress Kit (Hi-Media) and concentration was estimated by estimated spectrophotometrically by studying absorption at 260nm/280nm. This was followed by demonstration in Agarose gel electrophoresis. The decellularised BP had a residual DNA content of 7ng/mg tissue which could not be demonstrated in agarose gel electrophoresis probably because of extensive DNA fragmentation.

Minimal criteria of decellularisation are <50ng/mg tissue DNA content, <200bp DNA fragment length and absence of nucleus demonstrated by light microscopy.

The decellularised BP produce by this technique meets this requirement.

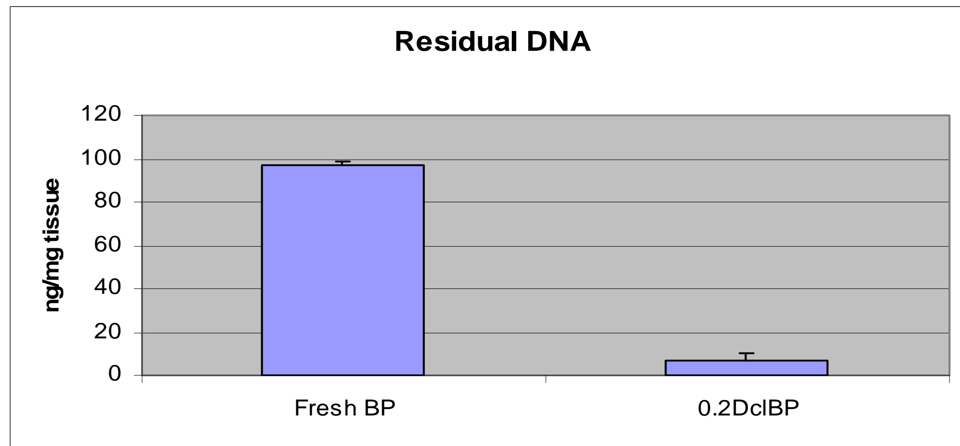
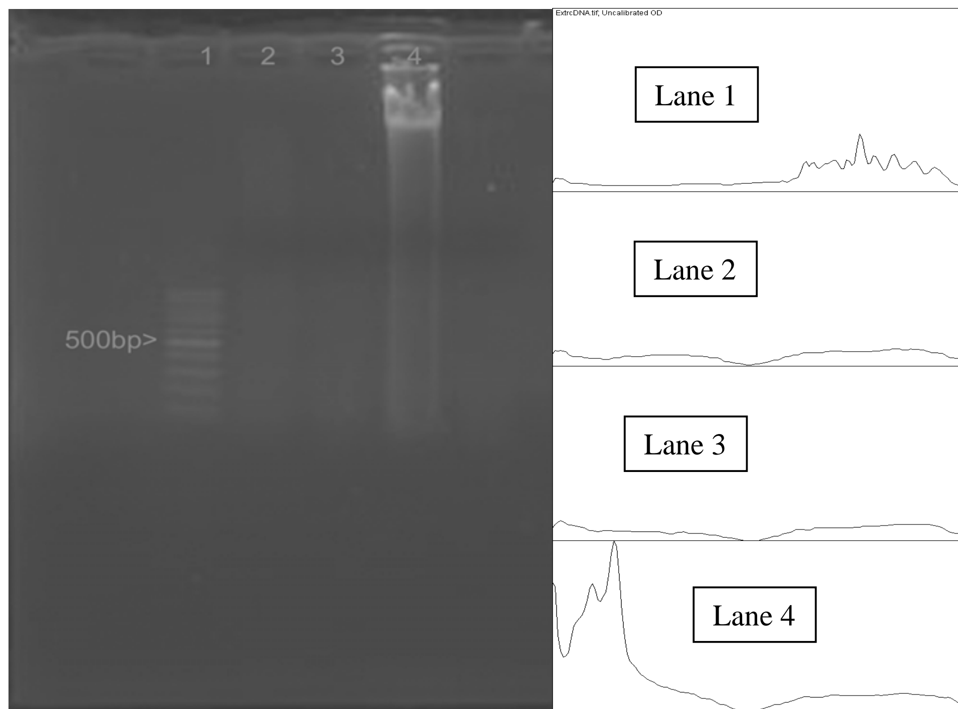
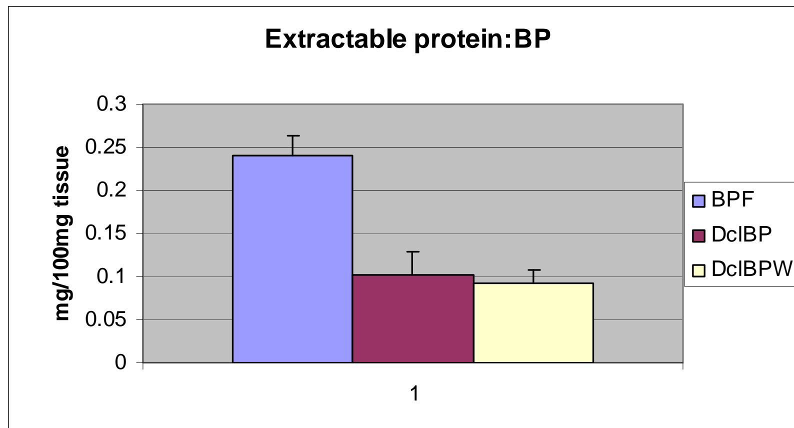


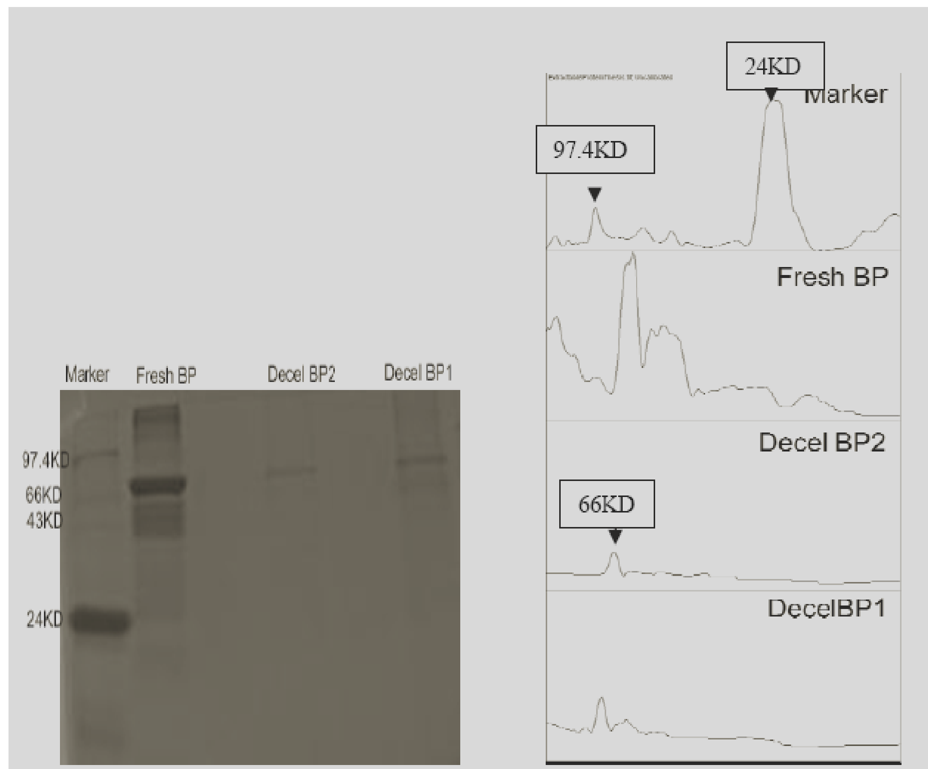

Chart showing substantial reduction in extractable DNA following decellularisation.




Residual DNA in 0.2Dcl BP (lane 2 and 3) were seen fragmented and it may not be immunogenic as un-methylated DNA (CpG sequences) of less than 300 base pair size was reported to be non-immunogenic. Lane 1 is molecular weight marker and lane 4 is DNA extracted from fresh bovine pericardium.

ii. **Estimation of residual non structural protein which can be immunogenic:** For this 100mg sample was homogenized at 700RPM for 20 minutes in ice bath, the homogenate was centrifuged and the protein content of the supernatant was estimated using Lowry's method. The decellularised bovine pericardium has an extractable protein concentration of 0.09± 0.015 mg /100mg wet tissue compared to fresh bovine pericardium which has 0.241±0.02 mg/100mg wet tissue.



The residual protein found in decellularised BP had a molecular weight around 66KD.
Lower extractable protein concentration might induce less immune response. Furthermore this extractable protein was made even less immunogenic by short duration glutaraldehyde cross linking.

### iii. Confirmation of glutaraldehyde cross-linking

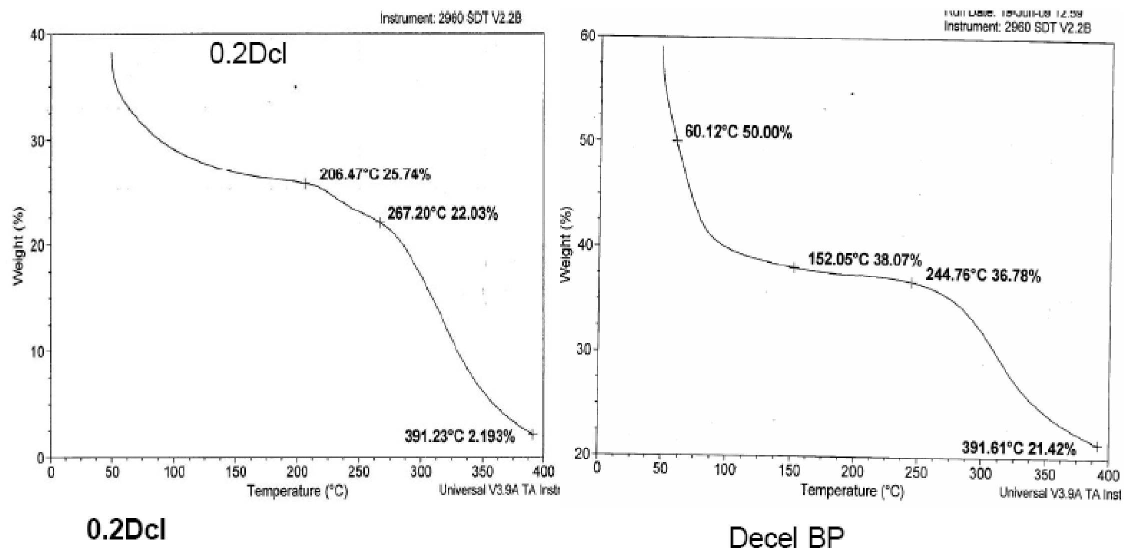
Cross linking was confirmed by estimating shrinkage temperature as well as by determining mass loss of partially cross-linked decellularised BP in comparison to uncross linked decellularised BP by methods of thermogravimetry using Universal V3.9A TA Instruments.

- **Shrinkage temperature**: The temperature at which the denaturation of collagen started was visualized as shrinkage and it was measured in un-crosslinked decellularised bovine pericardium,0.2Dcl (partially cross linked decellularised bovine pericardium) and standard gluteraldehyde treated bovine pericardium. 30x6mm sized samples (n=6) were immersed in water taken in a beaker under constant load by keeping under a glass slide. Water heated up to $90^0$C was pumped into the beaker at 38-48mL/minute using a positive displacement pump (Masterflex, Cole Parmer, model 7518-60, USA). The temperature at which shrinkage was observed was recorded using a digital thermometer. (Accurad 2020, Radix, India).

  Un-cross linked sample showed minimum shrinkage temperature of $61.3 \pm 1.3^o$C. This has significantly increased to $64.6 \pm 0.5^o$C in minimally cross linked decellularised bovine pericardium (0.2Dcl) indicating partial crosslinking. Maximum shrinkage temperature of $77.6 \pm 0.91^o$C was noted in completely glutaraldehyde cross linked bovine pericardium.
- **Thermogravimetry** indicated initiation of structural degradation around $152^0$C in un-cross linked decellularised bovine pericardium (Edcl) compared to $206.4^0$C in minimally glutaraldehyde cross linked sample (0.2Dcl). The initial weight loss noticed in both the samples can be due to water loss. Likewise the temperature of final structural

degradation has increased from 244.76$^{0}$C in un-cross linked sample (Edcl) to 267.2$^{o}$C in 0.2Dcl (minimally cross linked sample, 0.2Dcl). This shift in the degradation temperature observed in minimally cross linked sample can be due to formation of chemical cross links by short duration glutaraldehyde treatment.

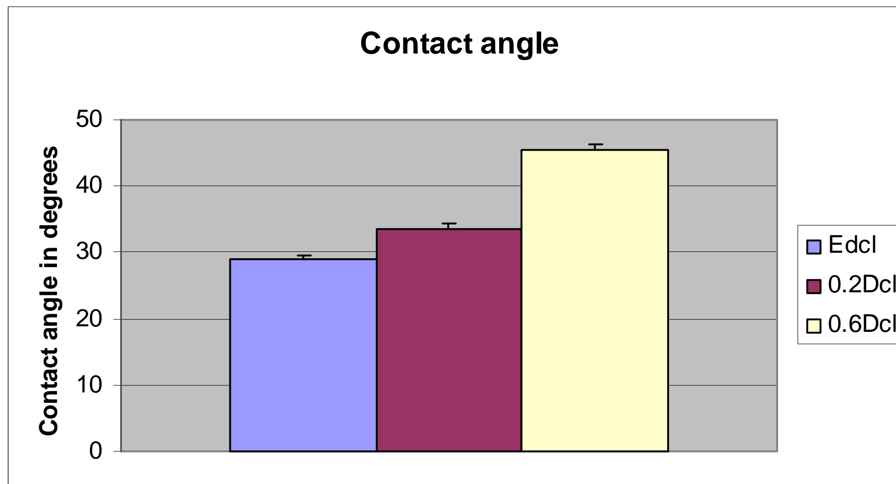
TGA: to show cross-linking




There was significant difference in the loss of sample mass between uncross linked decellularised BP (Decel BP) compared to 0.2Dcl group with the temperature of final structural degradation increasing to the tune of
22.47 $^{0}$C in cross linked sample (0.2Dcl) again indicating partial chemical cross-linking.

#### iv. Contact angle measurement for studying hydrophilic nature of surface

Contact angle was studied using a 'Contact angle Goniometer' by measuring the angle of contact of a small drop of distilled water (sessile drop method) which is placed over the material (decellularised BP). The 0.2Dcl BP has a contact angle of 33.5± 8.8$^{0}$, indicating that it has a hydrophilic surface.




DclBP (0.2Dcl) showing slight increase in contact angle compared to unprocessed decellularised BP (Edcl) on account of partial crosslinking

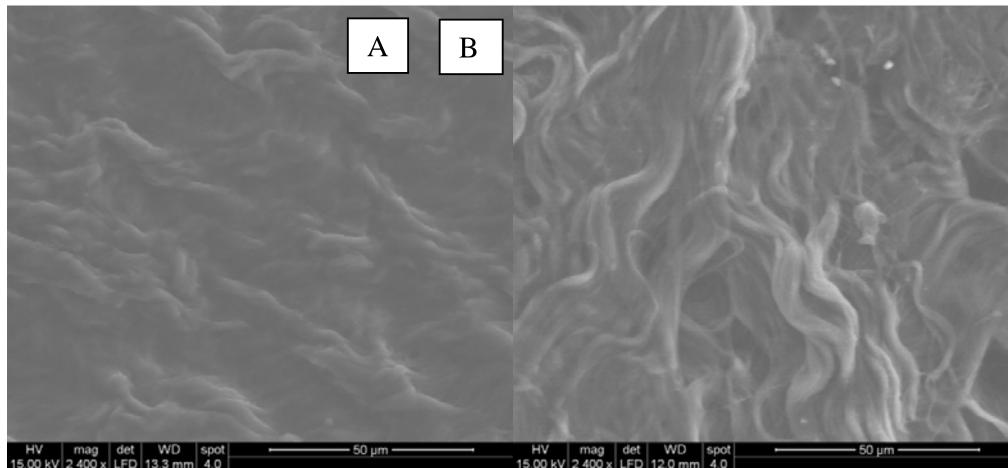
It is reported that cells adhere and proliferate preferentially on hydrophilic surfaces with contact angle below $57^0$.

v. **Structural analysis**:

Decellularised pericardium mainly consisted of well separated collagen bundles with a few elastin fibers as demonstrated by Movats Pentachrome staining (Figure below). Image analysis revealed a collagen content of approximately 92% and elastin content of 8%. Surface study using ESEM revealed morphological differences between the normal bovine pericardium and the decellularized pericardium.

Decellularised BP showing separated collagen bundles (stained yellow)with a few elastin fibers (stained black). Movats Pentachrome200X.

The normal bovine pericardium had a smooth surface which was having corrugations probably on account of glutaraldehyde fixation. The decellularized pericardium showed eroded surface with many crevices and fiber architecture was visible as linear structures more or less arranged in a uniform pattern resembling collagen bundles. Collagen was identified as the most abundant protein within mammalian ECM and was mainly constituted by collagen type I which was also widely used for therapeutic applications (Vanderrest and Garrone 1991). Bovine pericardium was reported to contain 90% collagen and consisting predominantly of type 1 collagen (Schoen *et al* 1986, Naimark *et al* 1992) by hydroxyproline estimation method.

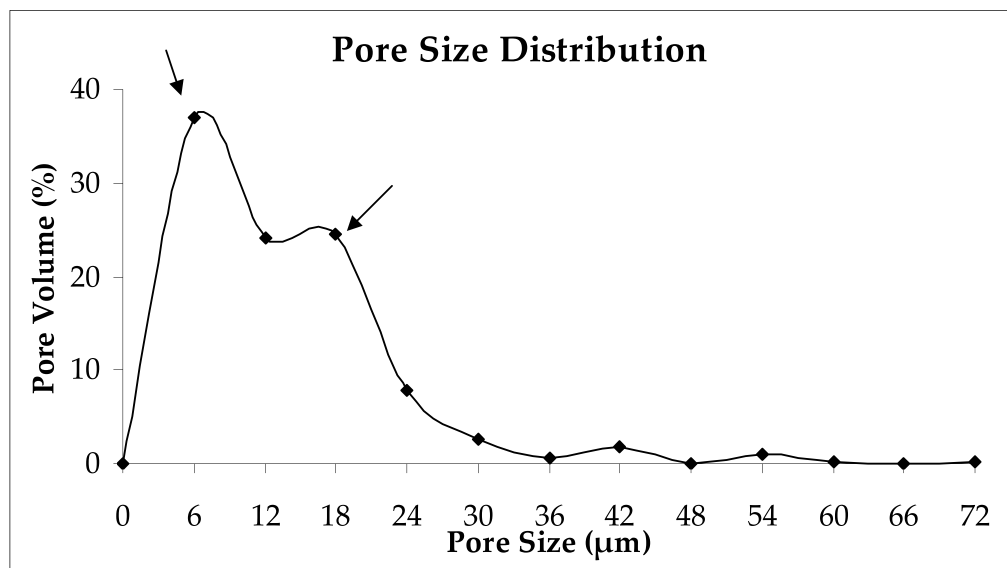



ESEM analysis of pericardial surfaces, A: Normal pericardial surface, B: Surface of decellularised bovine pericardium.

vi. **Porosity evaluation using Micro CT examination.**
(Instrument model = Micro-CT 40 ,Manufacturer = Scanco Medical AG, Switzerland)

Micro-CT imaging of decellularised bovine pericardium tissues were done using x-ray source of 45kVp and 177 µA. Two dimensional cross-sectional images were captured at a resolution of 6µm and reconstructed using cone beam convolution back projection algorithm to generate morphological and structural datasets such as porosity, average pore size distribution, etc. During the reconstruction appropriate gaussian filters were used to suppress

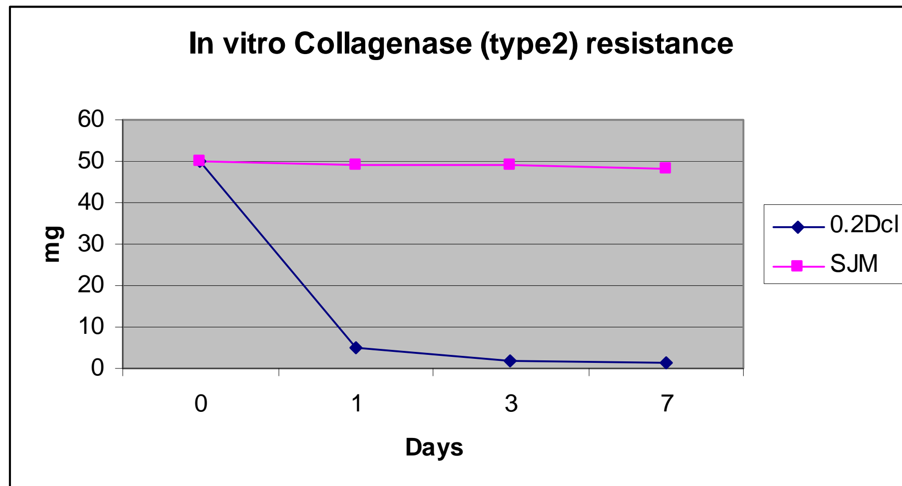
the background noise and around 300 cross-sections were reconstructed for each specimen.




0.2Dcl BP has a pore size ranging from 6 to 30 micron size with nearly 24 to 37% of pore volume constituted by 18 to 6 micron size pores.

#### vii. *In vitro* Collagenase susceptibility

In vitro degradation study was conducted using Collagenase from *Clostridium histolyticum* (125u/mg) (Sigma Aldrich). 50 mg (wet weight in triplicates) of 0.2Dcl, 0.6Dcl and Biocor™ bovine pericardium (St. Jude Medicals) were used for the experiment The samples were incubated at 37 $^0$C for 1 h with constant shaking. Collagenase solution was added in to the vials so as to make the final concentration to 2U/ mg of tissue. The vials were incubated at 37 $^0$C for 24 h, 72h and 7 days. At the end of which, the samples were centrifuged at 12000 rpm for 20 min at 4$^0$C and the remaining tissue samples were weighed after blotting. The weights loss was determined by comparing with the initial weight of the samples.




0.2Dcl BP showed 90.2% weight loss in 24 hours compared to 2% in SJM Biocor™ bovine pericardial patch. At 72 hours 0.2Dcl BP showed 96.5% weight loss, whereas SJM BP showed 2% weight loss. After 72 hours the weight loss rate has slowed down in 0.2Dcl group with only an additional 1% weight loss seen in about 7 days. SJM BP continued to lose weight at very low rate reaching about 4% at 7 days.

The above data clearly indicates that 0.2Dcl BP was fast degrading compared to commercially available product (SJM Biocore™) which was practically resistant to Collagenase degradation. Increased susceptibility to Collagenase degradation may make 0.2Dcl BP more amenable to in-vivo remodeling compared to SJM Biocore™) which is least likely to undergo remodeling in the in-vivo situation.

### viii. Evaluation of *in vitro* response (Cell cytotoxicity and macrophage activation)

*In vitro* toxic responses of glutaraldehyde treated BP and decellularised BP were assessed by cytotoxicity studies using L929 fibroblast cells by direct contact method as well as by studying the effect of the extract of material on L929 fibroblast metabolism by MTT assay. *In vitro* inflammatory response was studied by macrophage activation studies.

- **Cytotoxicity testing: Direct contact method**

Cytotoxic potential of glutaraldehyde treated BP and decellularised BP on account of their direct contact or due to leachables was evaluated by Direct contact method (ISO 10993-5). The test samples were rinsed thrice with normal saline before cytotoxicity evaluation. Test samples, negative control (High density poly ethylene -USP), and positive control (Copper) in triplicate were placed on subconfluent monolayer of L-929 mouse fibroblast cells. After incubation of cells with test samples at $37\pm2^0$C for 24±1 hours, cell culture was examined microscopically for cellular response around the samples. Cellular responses were expressed as non-cytotoxic, mildly cytotoxic, moderately cytotoxic and severely cytotoxic.

Decellularised BP samples were seen non cytotoxic to L929 fibroblasts by direct contact method (Fig. 3). Glut BP sample was seen moderately cytotoxic probably because of residual Glutaraldehyde. Positive control was severely cytotoxic and negative control was non-cytotoxic.

- **Cytotoxicity testing on extract:**

In-vitro MTT assay on extract of Glut BP and Dcl BP in physiological saline was performed to measure the effect of extract on the metabolic activity of L929 fibroblast cells. The ability of the fibroblast cells to metabolize yellow colored tetrazolium salt 3-(4,5-Dimethyl thiazol -2-yl)-2,5-diphenyltetrazolium bromide to purple colored formazan was measured as an indication of its activity. Different dilutions (50% and 25%) of extracts of Glut BP sample, Dcl BP, positive control (dilute phenol), negative control (high density poly ethylene (USP) and reagent control in triplicate were placed on subconfluent monolayer of L929 cells. After incubation of cells with the extract at $37\pm2^0$C for 2h, extract and control medium were replaced with 50μL MTT solution (1mg/mL in medium without supplements) wrapped with Aluminium foils and were incubated at $37\pm2^0$C for 2h. After discarding the MTT solution, 100μof Isopropanol was added to all wells and swayed the plates. The color developed was quantified by measuring absorbance at

540nm using microplate reader. The data obtained was compared with negative control.

The MTT assay of L929 cells after contact with 50% and 25% extract of Decellularised BP showed 96% and 97% metabolic activity respectively compared to Glut BP which had 14% and 15.1% metabolic activity respectively. This clearly showed that the extract of glutaraldehyde treated BP samples has significantly affected the metabolic activity of fibroblast cells and hence it is toxic in nature compared to Dcl BP.

- **Macrophage activation**

Macrophage activation was studied by estimating inflammatory cytokine released by adhered macrophages on contact with materials belonging to different groups. THP-1 (human acute monocytic leukemia cell lines) cells were grown in RPMI 1640 (Sigma) containing 10 % v/v Fetal Bovine Serum (Gibco), 160 U/ml benzylpenicillin and 100 U/ml streptomycin (Sigma). The cells were maintained at 37 °C in an atmosphere of 95% air and 5% $CO_2$ at 90% relative humidity. The differentiation into macrophages was induced by treating THP-1 cells in a 24-well plate for 24 h with RPMI-1640 containing 20 ng/ml Phorbol 12-myristate 13- acetate (PMA). Thereafter fresh medium was added and cells were grown for another 24 h under similar conditions. Release of pro-inflammatory cytokines (IL-1β;, IL-6 and TNF-α;) from THP 1 cells upon contact with triplicate samples of Glut BP and Dcl BP and positive control (bacterial lipopolysaccharide) at 20 pico gram) were carried using ELISA. The concentration of cytokines in culture supernatant was quantified by specific ELISA kit (U-CyTech biosciences, Netherlands) as per manufacturer instructions.

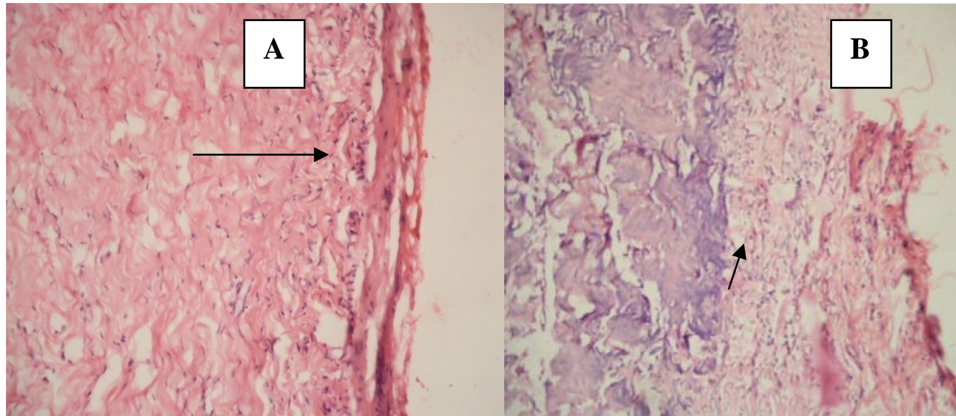
Cytokine release profile of activated macrophage in response to exposure to different groups is given in table 2. Positive control group showed the highest cytokine response. With respect to the TNFα and IL1β, highest macrophage activation was seen in Glut BP group. This was seen statistically significant compared to Dcl BP group. In the case of IL6, Dcl BP group showed a higher response when compared to Glut BP group (p0.004).

### ix. Evaluation of *in vivo* response

The tissue response, calcification response and immune response of decellularised bovine pericardium (0.2Dcl) were studied using juvenile Wistar rat by subcutaneous implantation for 60 days. Glutaraldehyde treated tissue, SJM Biocore™ was used as control. The results of the experiments are presented below. Each group had 5 animals and each animal was implanted with 6 implants of 1X1cm$^2$ size.

- **Tissue response: Rat subcutaneous model (n=15)**.

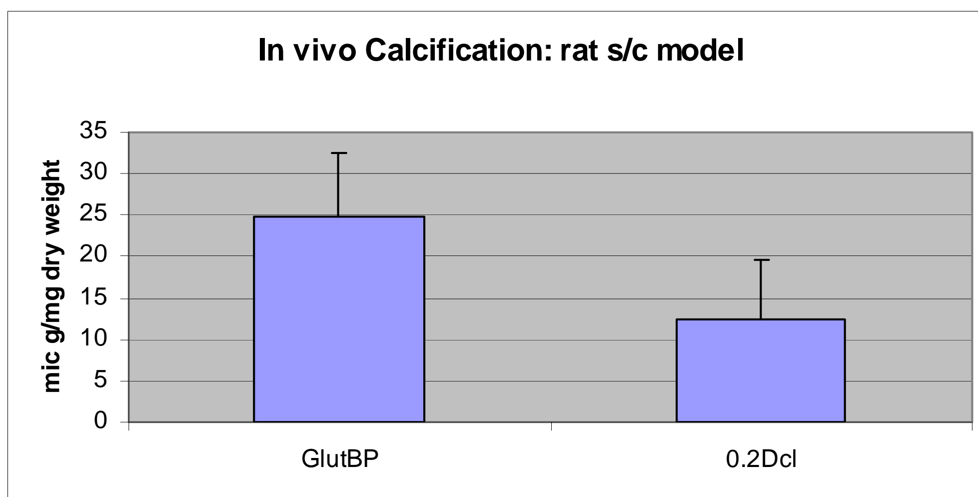
The tissue response studied on haematoxylin and eosin stained paraffin sections is presented below. The sections were qualitatively assessed for peri-implant necrosis, inflammatory response, calcification, peri-implant fibrosis, tissue incorporation and angiogenesis into the implanted material. Dcl BP implant did not show any peri-implant necrosis. A thin fibrous capsule could be appreciated around the implant. Mild to moderate diffuse infiltration of mononuclear cells in to the implant is noticed indicating chronic inflammation. Macrophages could be identified at the site of implant degradation. Implant Calcification could not be observed. Implant showed excellent ingrowth of cells with fibroblast morphology with evidence of newly laid collagen. Angiogenesis is noticed in the implant as well at the periphery.
SJM Biocor™ bovine pericardial patch explants showed thick capsule around the implant. Certain areas showed peri-implant necrosis. Angiogenesis was seen only in the periphery of the implant. Focal mild to moderate chronic inflammation observed as mononuclear cell infiltration could be seen at the periphery of implant. The implant interior was remarkably acellular. Calcium deposit could be observed in many sections.




. Tissue response to different implants at 60 days in rat subcutaneous implantation;

**A**: DCl BP group minimum inflammatory response, uniform host cell incorporation and angiogenesis (arrow) within implant and thin capsule formation HE 400X. **B**: Glut BP group, inflammatory response (arrow) in the interphase and remarkably acellular interior and thicker capsule HE 200X.

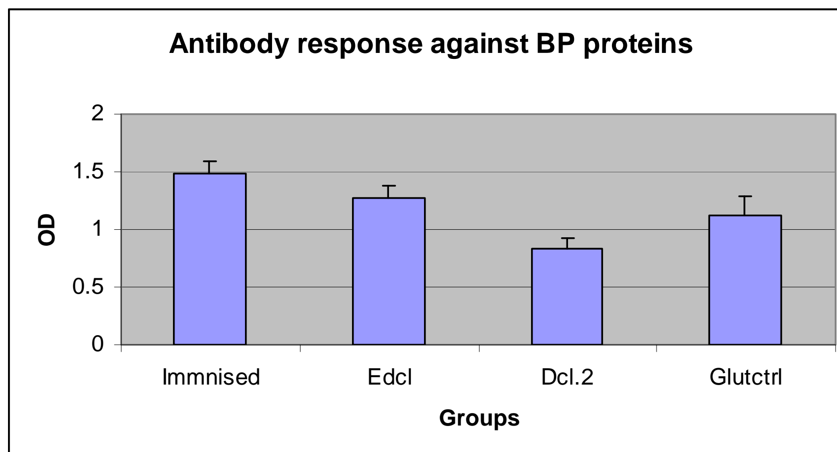
- **Calcification response (rat subcutaneous implantation model, n=6)**: The explanted samples (n= 6 ) were digested in concentrated HCl. The supernatant was neutralized by 0.1N of NaOH followed by estimation of Calcium using the colorimetric method of O-Cresolphthalein complexone obtained as a standard kit. The result of the calcification study is given below.

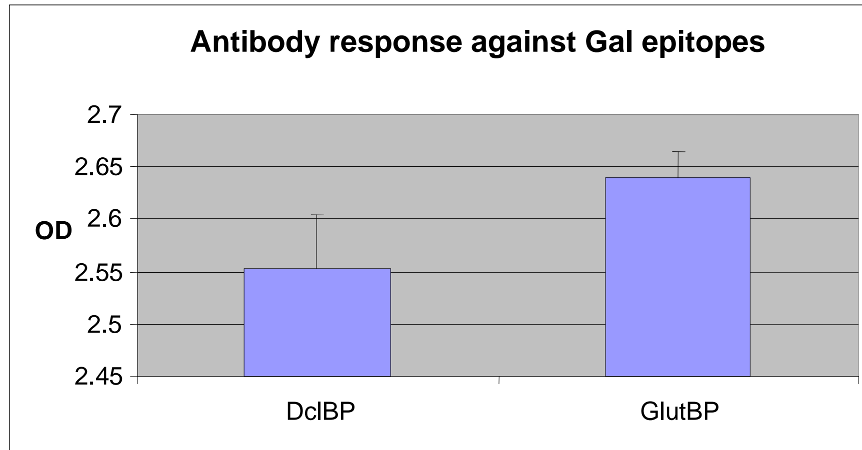


0.2Dcl group had a calcium content of 0.1±0.059mg/100mg wet tissue compared to 1.58±0.73mg/100mg of wet tissue in glutaraldehyde treated tissue.

- **Antibody response** (rat subcutaneous implantation model, n=5)

The objective of this study was to evaluate the antibody response elicited in different groups of animals against the implanted samples (decellularised BP and glutaraldehyde treated BP). For this, antibody response against extractable protein of fresh bovine pericardium in PBS, and GAL α1-3 Galβ1-4 GLC-BSA (Dextra Laboratories Ltd, UK were studied using Indirect ELISA method. Antibody (IgG, IgM and IgA) in the serum samples obtained from decellularised BP and glutaraldehyde BP implanted animals were compared. The ELISA technique was standardized using Rabbit polyclonal to rat IgG+IbM+IgA (HRP) secondary antibody (Abcam ab8521). Serum from animals which is immunized with BP proteins using Freund's adjuvant was used as positive control. Dcl BP group had significantly less antibody response compared to glutaraldehyde treated group (p=0.002). Antibody response in BP protein immunized animal as well as animals implanted with un-processed decellularised BP (Edcl) was higher than glutaraldehyde control group.



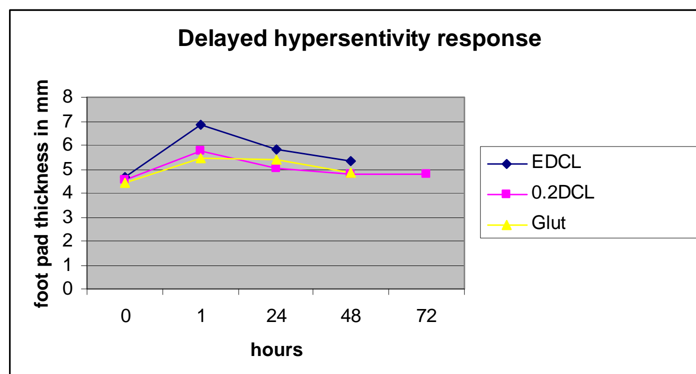



Less antibody response of 0.2Dcl group compared to Edcl ($p < 0.004$) indicate reduction in antibody production in response to partial cross linking. Less antibody response in 0.2Dcl when compared to immunized animals also indicate its minimal adjuvant effect. With respect to antibody response against Gal epitopes, decellularised BP showed significantly lesser response compared to glutaraldehyde treated BP.

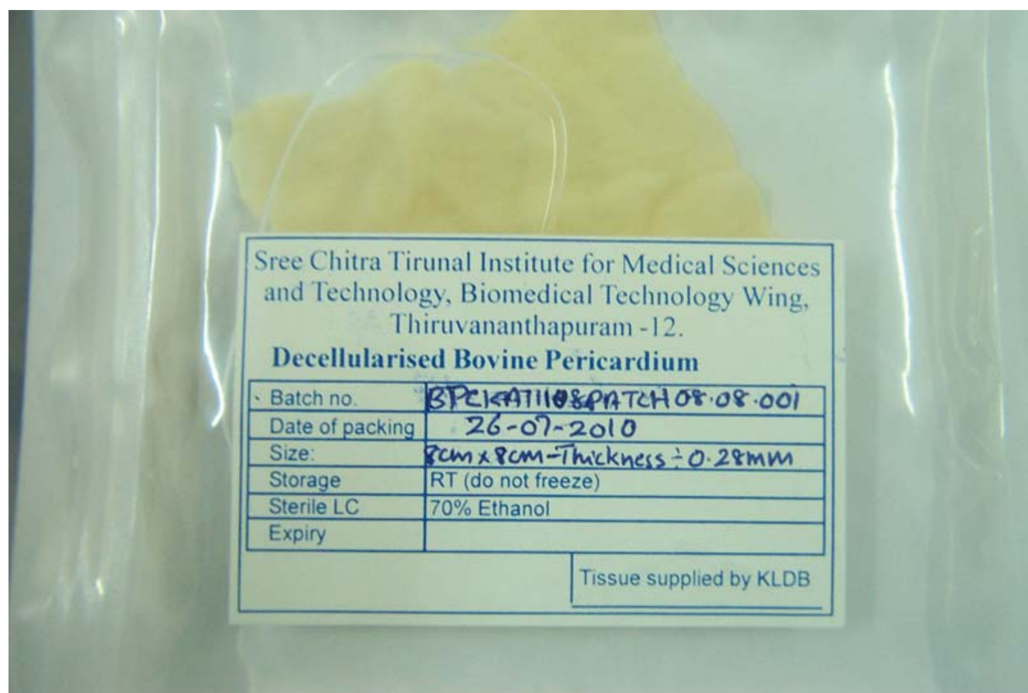
- **Cell mediated immune response: Delayed hypersensitivity**

Delayed type hypersensitivity study:(rat subcutaneous implantation model). Here the implanted animals were challenged with 100μg extractable protein from fresh BP by injecting into foot pad in 20μL PBS. The increase in footpad thickness is measured before injection, one hour, 24 hours, 48 hours and 72 hours after injection. 0.2Dcl BP, (short duration glutaraldehyde crosslinked decellularised BP)showed response comparable to fully crosslinked BP (SJM Biocore™) activation of cell mediated immune response denoted by minimum increase in foot pad thickness is observed.

III.



### III. Preclinical evaluation of Decellularised bovine pericardium




1. **Trace element and heavy metal analysis of 0.2Dcl Bovine pericardium** was done using Optical emission spectroscopy with inductively coupled plasma (Perkin Elmer OES-ICP model 5300DV). The list of elements to be tested was arrived based on the list of chemicals used in the process as well as metals which can cause adverse reactions. The results are presented below which shows that trace elements levels are well within safe range.

| No | Element | Result 0.2Dcl (ppm) | Remarks |
|---|---|---|---|
| 1 | Iron(Fe) | 38.42 | |
| 2 | Lead (Pb) | 0.25 | |
| 3 | Arsenic (As) | BDL | Detection limit is 0.053 |
| 4 | Nickel(Ni) | 0.37 | |
| 5 | Manganese (Mn) | 0.25 | |
| 6 | Cadmium(Cd) | 0.06 | |
| 7 | Mercury(Hg) | BDL | Detection |

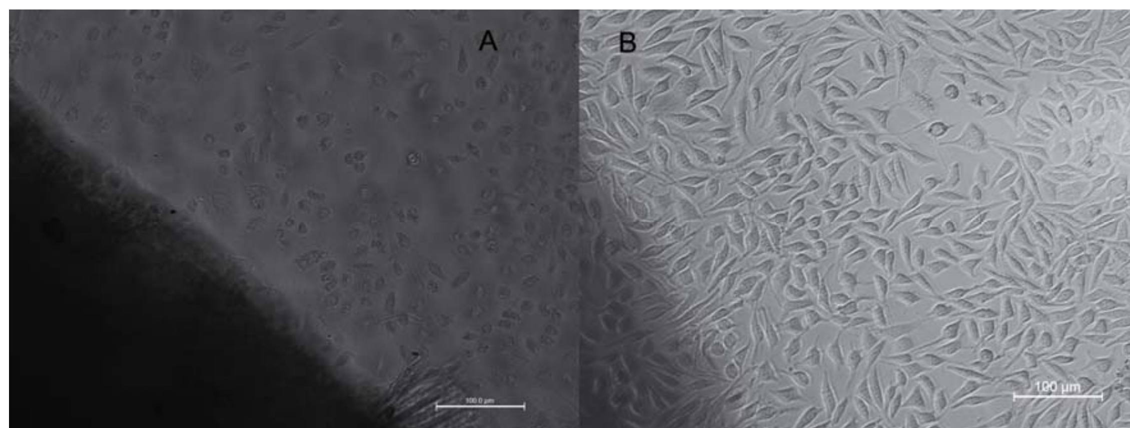
| | | | limit is 0.0610 |
|---|---|---|---|
| 8 | Chromium(Cr) | 2.3 | |
| 9 | Copper(Cu) | 0.425 | |
| 10 | Zinc(Zn) | 1.875 | |
| 11 | Magnesium(Mg) | 84.28 | |
| 12 | Calcium(Ca) | 141.05 | |

2. **Toxicological studies:**

   a. **Cytotoxicity testing: Direct contact method**

Cytotoxic potential of Glut BP and Dcl BP groups on account of their direct contact or due to leachables was evaluated by Direct contact method (ISO 10993-5). The test samples were rinsed thrice with normal saline before cytotoxicity evaluation. Test samples, negative control (High density poly ethylene -USP), and positive control (Copper) in triplicate were placed on subconfluent monolayer of L-929 mouse fibroblast cells. After incubation of cells with test samples at $37\pm2^0$C for 24±1 hours, cell culture was examined microscopically for cellular response around the samples. Cellular responses were expressed as non-cytotoxic, mildly cytotoxic, moderately cytotoxic and severely cytotoxic.

Dcl BP samples (B) were seen non cytotoxic to L929 fibroblasts by direct contact method . SJM Biocor™ sample was seen moderately cytotoxic probably because of residual Gluteraldehyde. Positive control was severely cytotoxic and negative control was non-cytotoxic.




**b. Cytotoxicity testing on extract:**

In-vitro MTT assay on extract of Glut BP and Dcl BP in physiological saline was performed to measure the effect of extract on the metabolic activity of L929 fibroblast cells. The ability of the fibroblast cells to metabolize yellow colored tetrazolium salt 3-(4,5-Dimethyl thiazol -2-yl)-2,5-diphenyltetrazolium bromide to purple colored formazan was measured as an indication of its activity. Different dilutions (50% and 25%) of extracts of Glut BP sample, Dcl BP, positive control (dilute phenol), negative control (high density poly ethylene (USP) and reagent control in triplicate were placed on subconfluent monolayer of L929 cells. After incubation of cells with the extract at 37±2$^0$C for 2h, extract and control medium were replaced with 50μL MTT solution (1mg/mL in medium without supplements) wrapped with Aluminium foils and were incubated at 37±2$^0$C for 2h. After discarding the MTT solution, 100μof Isopropanol was added to all wells and swayed the plates. The color developed was quantified by measuring absorbance at 540nm using microplate reader. The data obtained was compared with negative control. The MTT assay of L929 cells after contact with 50% and 25% extract of Dcl BP showed 96% and 97% metabolic activity respectively compared to Glut BP which had 14% and 15.1% metabolic activity respectively. This clearly showed that the extract of Glut BP samples has significantly affected the metabolic activity of fibroblast cells and hence it was considered toxic compared to Dcl BP.

### c. Acute systemic toxicity testing (ISO 10993-11)

This test was done to evaluate the systemic response of mice following intraperitoneal and intravenous injection of cotton seed oil and physiological saline extract of the test samples respectively. The test samples were prepared by washing the samples in sterile normal saline for 3 wash cycles, with each cycle lasting for 10 minutes. The samples were dried at 37$^{o}$C for 24 hours. 2g each of Glut BP and Dcl BP samples were extracted with 10 mL each of physiological saline and Cotton seed oil for 72 hours at 50$^{o}$C. 50mL/Kg body weight of the above extracts were injected through intravenous (n=5) and intraperitoneal route (n=5) respectively in separate groups of randomly selected health active mice. Injection of plain extraction media in another set of animals served as control. The animals were observed immediately and after 4h, 24h, 48h and 72 h for evidence of abnormalities such as convulsions, prostrations, loss of body weight more than 2g and death.

Administration of cotton seed oil extract of the Glut BP sample through intra peritoneal route did not produce systemic toxic effect evidenced as loss of body weight more than 2g, presence of any abnormalities or death. However intravenous administration of physiological saline extract of the Glut BP sample produced abnormalities and death immediately after injection in three mice, indicating systemic toxicity of this sample. The control animals did not show any signs of systemic toxicity. Cotton seed oil extract and physiological saline extract of Dcl BP sample did not produce any abnormalities, loss of weight or death following intraperitoneal and intravenous administration respectively. This indicated that the DclBP extract do not cause systemic toxicity.

### d. Intracutaneous (Intradermal) reactivity test (ISO 10993-10)

This test was done to assess the potential of the material to produce irritation following intradermal injection of the material extract. The test samples were prepared by washing the samples in sterile normal saline for 3 wash cycles, with each cycle lasting for 10 minutes. The samples were dried at 37$^{o}$C for 24

hours. 2g each of Glut BP and Dcl BP samples were extracted with 10 mL each of physiological saline and Cotton seed oil for 72 hours at 50$^{o}$C. 0.2mL (per each site) of the extract was injected into 5 sites with appropriate controls in randomly selected healthy, active New Zealand white rabbits. The animals were observed for erythema and oedema which was scored as 0 (nil), 1 (very slight), 2 (well defined oedema/erythema), 3 (moderate) and 4 (severe). The requirement of the test is met if the difference between the test sample mean score and control sample mean score is 1.0 or less. All the procedures were done aseptically.

Physiological saline extract of the Glut BP sample produced an average irritation score of 1.18 which is a positive reactivity for this test. At the same time the cotton seed oil extract showed 0 score of irritation indicating no intracutaneous irritation. The physiological saline and cotton seed oil extract of Dcl BP samples produced irritation score of 0.3 and 0.1 respectively which indicated the samples are non-irritating as per the set criteria for this test.

#### e. Sensitization (ISO 10993-10:2002/Amd.1:2006:E)

Cotton seed oil extract of test sample with Freunds' adjuvant was injected intra dermally for induction. Seven days after this, induction was repeated by topical application of test sample extract for 48h. 14 days after this, challenge dose was applied topically for 24h and the animals were observed and scored at 24 h, 48h and 72 h for erythema and oedema. A reaction of grade 1 or above is considered as positive. Cotton seed oil extract of Decellularised BP meets requirement of the test as per ISO 10993-10:2002/Amd.1:2006(E)- Biological evaluation of medical devices: Part 10. Test for irritation and delayed type hypersensitivity: Clause 7.4 Maximization test for delayed hypersensitivity.

#### f. Endotoxin testing

Kinetic Chromogenic method using Endosafe PTS endotoxinwas conducted . Less than 0.5EU is considered non-pyrogenic. Test sample has <0.1EU/mL. Hence meets requirement of USP23NF21<85>, bacterial endotoxin test.

### g. Genotoxicity testing (ISO 10993-3, ISO-12, OECD 471)

In vitro Bacterial Reverse Mutation (Ames) Assay was done. The test material was washed in sterile physiological saline to remove the preservative 70% ethanol. The extraction was done using physiological saline and tested at single concentration both with and without S9 (metabolic) activation against all 5 strains of *S.typhimurium* TA98,100, 1535, 1537 and 1538.

Under condition of test, the polar extractant in physiological saline did not show an mutagenic effect against all the five *S.typhimurium* strains at the concentration tested both with and without metabolic activation. Hence the test material( DclBP) is no-mutagenic by in-vitro *S.typhimurium* reverse mutation assay (Ames test).

### 3. Haemocompatibility evaluation

In-vitro Haemocompatibility (ISO 10993-4:2002 (E): This test consists of exposing the test material to anticoagulated human blood and platelet, RBC, WBC counts are taken. Percentage hemolysis, leukocyte adhesion, activation of coagulation system by studying PTT, fibrinogen assay and platelet activation study are conducted. The observed values in comparison to a clinically approved material (PET) is presented in the table below.

| Parameter | Dcl BP | PET |
|---|---|---|
| Platelet consumption: | 6.31±3.24% | PET has platelet consumption 18.64±2.28%, |
| Leukocyte consumption: | 4.14±1.05%. | Leukocyte consumption 18.36±1.67%, |
| Erythrocyte consumption: | 1.03±0.7% | |
| Increase in clottable fibrinogen: | 4.9%±4% | |
| Decrease in PTT: | 24.8±2.4% | PTT decrease |

| | | 26.15±2.57 |
|---|---|---|
| % hemolysis: | 0.12±0.025% | |
| Platelet activation: | 0.13±0.005% | |

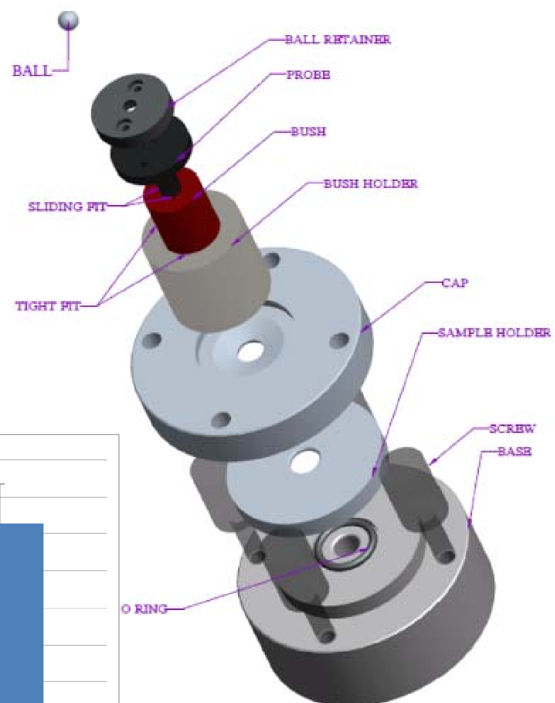
As PET is an approved material for human clinical use, the values reported for decellularised bovine pericardium is assumed safe for human clinical use.

## 4. Mechanical characterization

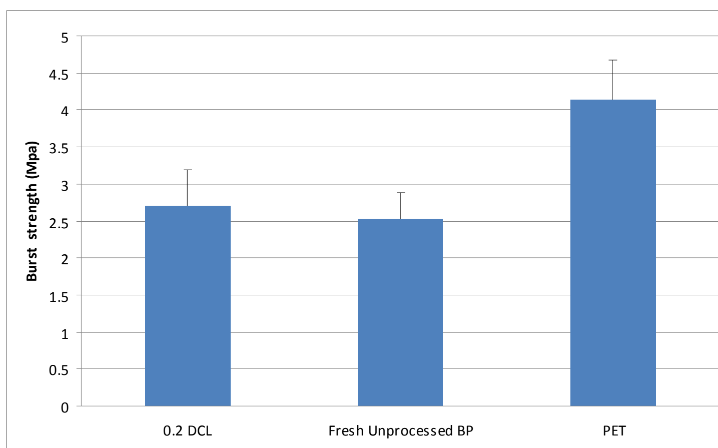
Characterization of the decellularised BP samples were carried out to assess their

i. Burst strength
ii. Tensile properties
iii. Suture retention

i. **Burst strength** : . The test was carried out using Universal Testing Machine (M/s. Instron, UK) employing 500N compressive load cell and spcialised fixture developed for the purpose

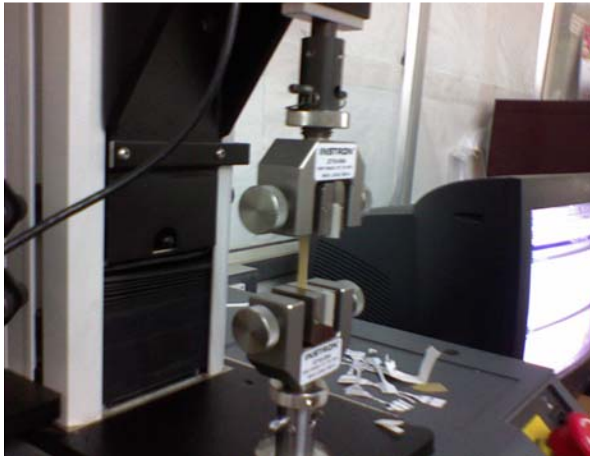



The load at which the central plunger penetrates the specimen is used to assess the burst strength of the material. The results are provided in table below.

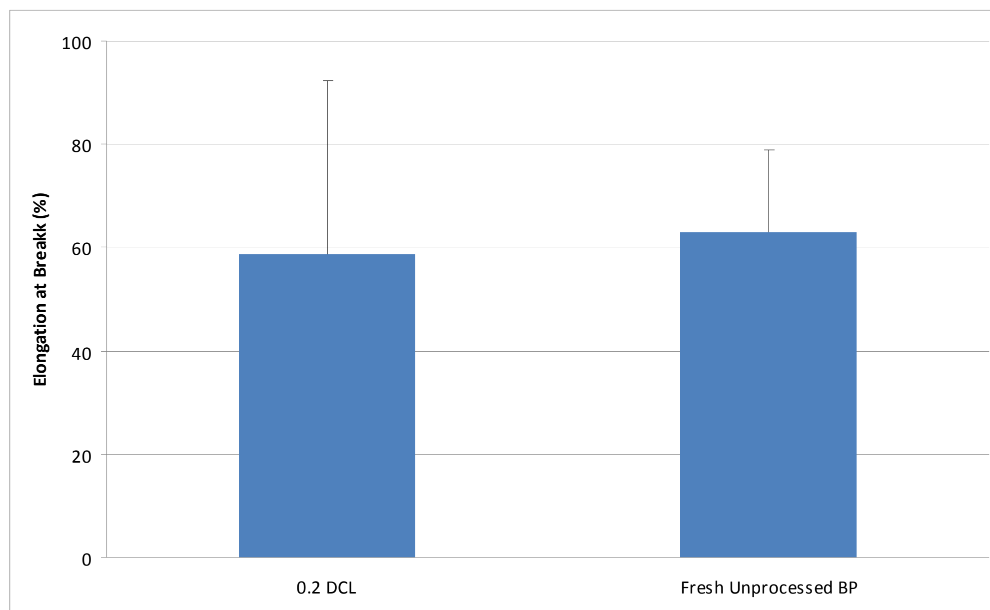


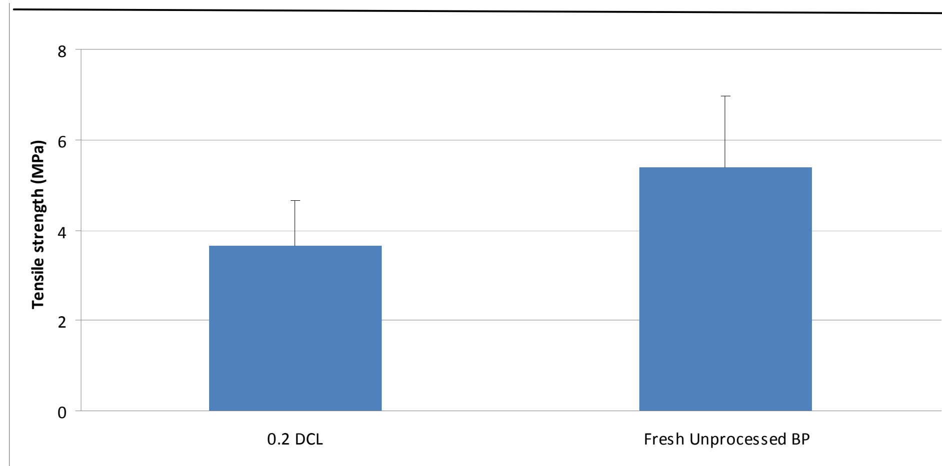
The decellularised bovine pericardium has adequate burst strength for the proposed application.

ii. **Tensile Properties :**



Tensile characterization was carried out using Universal Testing Machine (M/s. Instron,, UK) employing 100N tensile load cell. The sample (25 x 6mm) is clamped in between two jaws and the load is applied at the cross head speed of 10mm/min. The tensile strength and elongation at break are estimated from the load Vs strain characteristics. Tensile properties of the decellularised BP meets the requirement.






### iii. Suture retention:

Suture retention test is done using Instron 3365, Universal testing machine with 100N load cell. Decellularised BP (0.2Dcl) strips of 10x50mm size (n=5) were used. 5/0Prolene suture with taper needle was used to take bite at 2mm distance from the edge. The sample was held in the lower grip and the threaded suture was held in upper grip and pulled at a constant speed of 5mm/minute until failure (material tear or breaking of suture). The suture retention strength was defined as the peak force obtained to during this procedure. The data was compared with a normal pericardium. Decellularised BP has suture retention strength of 5.76±1.73N which was found adequate for the proposed application.

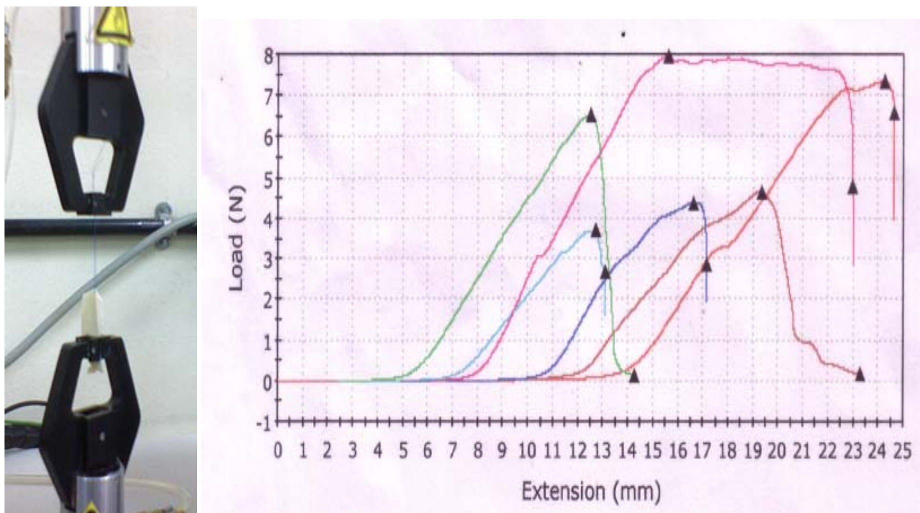



Figure showing experiment set-up and graphical data

## 5. Decellularised tissue: Bioburden evaluation, sterility testing and validation and viral inactivation studies

Objectives

1. Validation of Procedures for sterilization and preservation which includes Bioburden estimation and sterility test.
2. Elimination and/or inactivation of virus and/or transmissible agents based on International standard EN 12442-3:2000.

**Methodology**

**Standardization of sterility testing of the decellularised animal tissue for cardiovascular application.**

For sterilization of medical devices / materials several factors need to be considered. They are the initial bioburden analysis, Analysis of Bioburden levels at various stages of processing and finally sterility check of the finished product.

The sterilization protocol of the finished product also has to be fixed so that product is sterile for the duration of its shelf life.

### i. Bioburden estimation

Summary of the test

Three units of sample are analysed initially and at each stage of the processing for Bioburden estimation.

Methodology: The methodology followed was as per ISO 11737-1: 1995 (E) - Estimation of population of microorganism on products.

The estimation of bioburden was done at each point in triplicate by membrane filtration method and cfu (colony forming units) of colonies formed estimated. The microorganisms isolated were also identified.

114 samples were subjected to Bioburden analysis as listed below.

## REPORT OF BIOBURDEN ESTIMATION

| Stage of processing | No. of units | BC | FC |
|---|---|---|---|
| Immediately after collection | 3 | 178 | 2 |
| | 3 | 20800 | 4466.6 |
| | 3 | 72 | 0 |
| | 3 | 102.6 | 1.33 |
| | 3 | 4 | 5.33 |
| | 3 | 29.33 | 0 |
| | 3 | 34.67 | 0 |
| | 3 | 6.67 | 0 |
| | 3 | 4 | 0 |
| After decellularisation | 3 | Beyond countable limits | 0 |
| | 3 | 0 | 12133.33 |
| | 3 | $4.89\times10^5$ | 2400 |
| | 3 | 93066.67 | 10200 |
| | 3 | Beyond countable limits | 0 |
| | 3 | Beyond countable limits | 0 |
| | 3 | Beyond countable limits | 5.33 |
| | 3 | Beyond countable limits | 0 |
| | 3 | $6.167\times10^4$ | 0 |
| | 3 | $231.33\times10^2$ | 0 |
| After washing | 3 | $199.2\times10^5$ | 0 |
| | 3 | 10.67 | 0 |
| | 3 | 5.33 | 1.33 |
| | 3 | 29.3 | 0 |

| | | | |
|---|---|---|---|
| | 3 | 2200 | 0 |
| Before sterilization | 3 | 2 | 6 |
| | 3 | 0 | 0 |
| | 3 | 42.66 | 86.67 |
| | 3 | 0 | 0 |
| | 3 | 0 | 0 |
| | 3 | 222.67 | 0 |
| | 3 | 80 | 0 |
| | 3 | 13.33 | 0 |
| | 3 | 0 | 0 |
| | 3 | 1.33 | 0 |
| | 3 | 4 | 1.33 |
| | 3 | 2.67 | 1.33 |
| | 3 | 5.33 | 0 |
| | 3 | 0 | 0 |

### ii. Sterility testing

Protocol followed: United States Pharmacopoeia: USP 31 /NF 26,<71>

Summary of the test : At various stages of processing samples were collected and sent for Sterility testing. Also various parameters of the sterilization process were tested for optimization of sterilization process.

The test was conducted by direct transfer method. Sample is directly transferred to Fluid Thioglycollate (FT), and Tryptone Soya broth (TSB) and incubated. FT was incubated at 35.5 $\pm$ 2.5 $^{0}$C and TSB at 22.5 $\pm$ 2.5 $^{0}$C. The period of incubation was 14 days and examined for growth.
From the results the optimal conditions of sterilization was determined.

**REPORT ON STERILITY TEST**

| TEST REQUEST NUMBER | Customer Sample Code and sample details | Report |
|---|---|---|
| p-8016 Sterility test-1 | 1) Valve Decel 121<br>2) Valve Decel 118<br>3) Valve 0.2% Glutaraldehyde control | 1) Test material passed the test of sterility<br>2) ) Test material failed the test of sterility<br>3) Test material passed the test of sterility |
| Request No.007A/22-9-09 | Sample 1,2,3<br>(After sterilization, >30 days at room temperature) | Sample 1-Failed sterility testing<br>Sample 2-Failed sterility testing<br>Sample 3-Failed sterility testing |
| Request No.023/07-1-10 | Sample 1 (After sterilization at room temperature) | Test material passed the test of sterility |
| Request No.035/25-1-10 | Sample 1 (During sterilization,30$^{th}$day at 37$^{0}$C) | Test material passed the test of sterility |
| Request No.037/25-1-10 | Sample 1(During sterilization,30$^{th}$day at 30$^{0}$C) | Test material passed the test of sterility |
| Request No.039/27-1-10 | Sample 1(During sterilization,30$^{th}$day at 30$^{0}$C) | Test material passed the test of sterility |
| Request No.041/27-1-10 | Sample 1(During sterilization,30$^{th}$day at 37$^{0}$C) | Test material passed the test of sterility |
| Request No.059/15-2-10 | Sample 1(During | Test material passed the |

| | | |
|---|---|---|
| | sterilization at $37^0$C) | test of sterility |
| Request No.063/18-2-10 | Sample 1(During sterilization at $30^0$C) | Test material passed the test of sterility |
| Request No.065/18-2-10 | Sample 1(During sterilization at $37^0$C) | Test material passed the test of sterility |
| Request No.077/11-3-10 | Sample 1(During sterilization at $37^0$C) | Test material passed the test of sterility |
| Request No.078/11-3-10 | Sample 1(During sterilization at $30^0$C) | Test material passed the test of sterility |

### iii. Validation of Sterilization Process

The sterilization process for the decellularised materials were validated based on the International Standard ISO 14160: Sterilization of single-use medical devices incorporating materials of animal origin —Validation and routine control of sterilization by liquid sterilants.

The purpose of sterilization processing is to inactivate the microbiological contaminants and thereby transform the non-sterile items into sterile ones. The inactivation of a pure culture of microorganisms by physical and/or chemical agents used to sterilize medical devices often approximates an exponential relationship; inevitably this means that there is always a finite probability that a microorganism can survive regardless of the extent of treatment applied. For a given treatment, the probability of survival is determined by the number and types of microorganisms and by the environment in which the organisms exist during treatment. It follows that the sterility of any one item in a population of items subjected to sterilization processing cannot be guaranteed and the sterility of the processed population of items has to be defined in terms of the probability of there being a viable microorganism present on the device.

**Product description :**

| | |
|---|---|
| Physical description | The product is decellularised bovine pericardium / jugular vein meant for use as implant. |
| Intended use | For replacing diseased / damaged structures in the body like, cardiac atrial / ventricular septum |
| SUD / RUD | Single Use Device |
| Design characteristics and selection of sterilization process | The material is of biological origin and hence conventional sterilization process like steam, ETO, radiation sterilization technique cannot be employed in this case. The chosen sterilant is 70% alcohol in type II reagent water. Historical data exist for the selection of the sterilization process. |
| Raw materials and microbiological quality | The raw materials used in the product as well as the packaging are cleaned and dried to ensure minimal bioburden. None of the materials are known to have complications related to biofilm formation. |
| Manufacturing conditions and microbiological quality | All manufacturing processes are carried out at controlled work area and the final stages of assembly and packaging are carried out at class 10,000 and class 7 clean work spaces. |
| SAL requirements | Being a permanent implant, $10^{-6}$ (MSI=6) is the expected SAL for the product |
| Packaging | The product are packed in polyethylene // Tyvek pouches (conforming to ISO 11607-1 and EN868-5). The product is placed in the container in lay flat form to ensure minimal wrinkles on storage. |
| Loading pattern | The loading of the product is designed to ensure that at least 50% of the chamber volume is free of the |

| | |
|---|---|
| | product load. |
| Compatibility with process | Has been established prior to development of sterilization process. |

**Sterilization Process** :

The sterilant used was 70% ethyl alcohol in type II reagent water. The standardized process was preservation of the material in the sterilant at specific volume to weight ratio (2.5 ml sterilant /g of product) for 30 days, with intermediate sterilant replacement after every 4 days.

**Process Control:**

A sterilization process employing liquid chemical sterilants usually involves a number of phases:

- preparation of the liquid chemical sterilant;
- exposure of the product to the sterilant at a controlled temperature for a specified time.

Since it was not a terminal sterilization process, there are two additional phases which need attention.

- Preparation and sterilization of the primary package and any storage solution in which product is presented
- Aseptic transfer of the product from the sterilant into its primary pack.

Adequate process control measures established ion all these phases to ensure that the sterilization process is always under operational control.

**Validation Process:**

The validation process had three phases including

- Installation Qualification (IQ): To demonstrate that the sterilization equipment meets the specification with the evidence of calibration activities carried out at the time of installation / commissioning of the unit.

- Operational Qualification (OQ): To demonstrate that the equipment will perform consistently, and within pre-defined limits, as specified and installed. Following tests were performed in OQ
  - Temperature distribution measurement at empty and 90% load conditions
  - Humidity distribution measurement at empty and 90% load conditions
  - chamber leak testing
- Performance Qualification (PQ): to demonstrate the efficacy and reproducibility of sterilization process. Most challenging load expected during normal sterilization cycle shall be selected and specified in PQ. Load configuration specification shall include
  - Loading pattern
  - Placement of items

**Validation Protocol Features**:

- The development of a sterilization process was carried out with product compatibility assessment, optimization of the process prior to validation for establishing efficacy. These included
  - determination of the critical process variables and the limits for these variables within the sterilization process
  - estimation of the bioburden levels from which the challenge presented to the sterilization process
  - performance qualification and routine monitoring
- The following points were taken into consideration while the process was established and validated
  - range of conditions which can be achieved with the sterilizing process
  - sterilization processes already in use for other similar products
  - requirements for levels of residuals
- The process validation was segmented into different phases of experiments including ones for
  - determining components which represent the greatest challenge to liquid chemical sterilant – In this case since the product was layered

sheet with minimal crevices and shadow areas the entire surface was chosen as the critical area
  - validating the method of recovery / detection of the test microorganism from the product selected component;
  - determining the inactivation kinetics of the test organism(s) in the presence of product.
- Bioburden estimations were carried out on various processing stages including:
  - Immediately after collection
  - After decellularisation
  - After washing process
  - Immediately prior to sterilization
- The objective of bioburden estimations were:
  - to establish the nature of contaminating microorganisms present on the product
  - to establish the number of microorganisms present on the product
  - to establish the extent of variation and thus the consistency of the contamination by comparing the numbers found on consecutive batches
  - to establish the spread in the bioburden levels during process perturbations..
- During a liquid chemical sterilization process, the material get subjected to environmental stresses and could react with the sterilants used. The validation is addressed to ensure that the functionality and safety are not compromised by exposure to the anticipated changes in sterilization process conditions. The sterilization efficacy studies were carried out for following perturbed process conditions to ensure that the sterilization process is robust and hardened to ensure repeatable sterile assurance levels are achieved.
  - 25, 30 and 35 days of sterilization duration
  - 30°C, 37°C, 40°C temperature conditions

NOTES:

1. Manufacturing processes for medical devices containing animal tissues frequently include exposure to chemical / biological agents which can significantly reduce the bioburden on the medical device. This validation do not take account of the effects of other bioburden reduction steps employed prior to sterilization.
2. Liquid chemical sterilants traditionally employed to sterilize animal tissues in medical devices may not be effective in inactivating the causative agents of transmissable spongiform encephalopathies such as bovine spongiform encephalopathy (BSE). Satisfactory validation in accordance with other relevant international standards has been carried out to ensure that absence of such agents in the final product.

### iv. Virus inactivation assays.

Use of animal tissues or derivatives for medical applications is based on the premise that they would give performance characteristics superior to materials like metal, plastics or textiles. But the use of biological tissues comes with its own control parameters different from non-biological materials. The most important of these is the fact that starting from sourcing of the material each step of processing would contribute to contamination by bacteria, fungi, viruses and transmissible agents. Bacterial and fungal contamination is assessed by Bioburden analysis and ensured by sterility testing. But viral contamination assessment is not so easy as their presence can be assessed only by complicated means provided we know the range of viruses to be assayed for and have permissive cell lines for them. So the method of choice here is Viral inactivation assays.

So for the safety of biological medical devices there are two complimentary approaches (EN12442)

1. selecting the source material for minimal contamination with agents
2. testing the ability of the production processes to remove or inactivate transmissible agents.

Viral inactivation is the process by which the ability of virus to cause infection or pathogenic reaction is reduced and is expressed mathematically in terms of reduction factor. This is done using model viruses (RNA, DNA, enveloped and non-enveloped viruses).

Here two model viruses Reo virus III and Parainfluenza virus were chosen as model viruses. Their permissive cell line is Vero cell line. Elimination or inactivation studies involve deliberate addition or spiking of an agent and after process of sterilization assaying for presence of viable virus by ability to show cytopathic effect on permissive cell line- here vero cell line monolayer. Inactivation was absence of cytopathic effect on permissive cell lines.

***Figure: Stages in viral inactivation assay.***

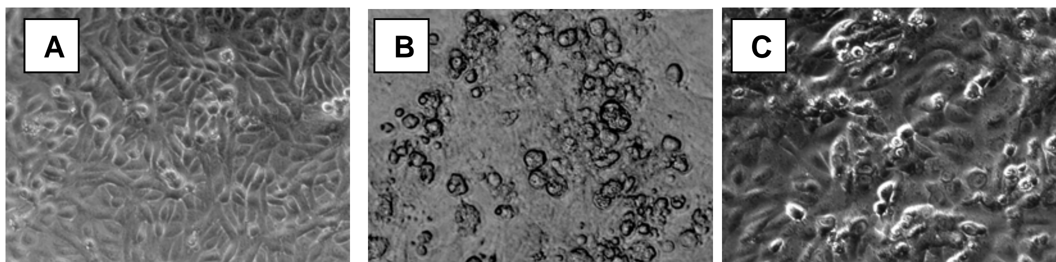



**Figure: A : Monolayer of Vero cell. B- Parainfluenza infected monolayer of Vero cells showing cytopathic effect. C- Reo virus II infected Vero cell monolayer showing cytopathic effect.**

The sterilization method was shown to inactivate both Reo virus III and Parainfluenza virus.

## IV. PRECLINICAL ANIMAL EVALUATION BY PIG AORTIC AND LEFT ATRIAL IMPLANTATION

### Test Device

Sample ID: BPCKA10742PATCH.10.16.001, Mar2010

Sample ID: BPCKA10760PATCH.06.12.001, Mar 2010

Sample ID: BPCKA10661PATCH.10.10.002, Jan 2010

Sample ID: BPCKA10207PATCH.08.12.002, Mar2010

### Control device

SJM Biocor™ Bovine pericardium

### Methodology

The test and control device of approximately 1-2cm X 2-4cm size were implanted in the descending aorta and left atrium as patch for duration of one month, six months and 12 months as per the experimental design given below. Hematology, serum biochemistry, clinical evaluation and gross histopathological evaluation on termination were performed in these animals. Surgeons opinion on the handling qualities of the material was also obtained.

### Experimental design

| Duration | No. of Device implants |
|---|---|
| One month | Test device 6<br>Control device 3 |
| 6 months | Test device 6<br>Control device 3 |
| 12 months | Test device 2<br>Control device 3 |

### Summary of clinical evaluation

- Structural failure of graft was not noticed in both test and control graft at all periods.
- All animals completed the implantation period un-eventfully.
- Blood parameters were within normal range in both test and control animals at all periods

**Pathological evaluation**

**One month**

At one month post implantation, grafts are seen in the aortic arch and left atrial appendage area. Aortic grafts are identified grossly but atrial grafts are identified as fibrosed area.

In the ***aortic patch***, tissue graft is identified as focal granulomatous area of inflammation. Thick neointima formation is noted with smooth muscle cells and collagen. Lumen is lined with endothelial like cells in all cases. Inflammatory cell infiltration in neointma is observed, mild in three case, two tests (TSPAT 001.Y11 and TSPAT 190.Y10) one control (TSPAT 413.Y10C), moderate in three test cases, (TSPAT 191, 199.Y10, TSPAT 002.Y11) and severe in two control cases (TSPAT 200, 202.Y10). Active degeneration of tissue graft is evidenced by infiltration of macrophages, lymphocytes and fibrocytes. Foci of calcification are noted at the anastomoses region in the native vessel in five cases. In two test cases, calcification is noted in the tissue graft region (TSPAT 199.Y10, TSPAT 001.Y11) and in one test case micro spots of calcification are noted in the degrading tissue graft (TSPAT 002.Y11).

In the ***atrial patch*** region, tissue graft is sparsely seen. Active degeneration of tissue graft is seen and replaced by fibrous connective tissue. Focal area of granulomatous inflammation noted in all cases. Inflammatory cell infiltration is observed in the graft region, mild in one test case (TSPAT 190.Y10), moderate in three cases, two test (TSPAT 191, TSPAT 199.Y10) and one control TSPAT413.Y10) and severe in two control cases (TSPAT 200, TSPAT 202.Y10). There is no evidence of calcification in four cases, three test (TSPAT 190.Y10, TSPAT 191, TSPAT 199.Y10) and one control (TSPAT413.Y10). In two test cases (TSPAT 001.Y11,

TSPAT 002.Y11), foci of calcification are noted in the degrading graft region as micro spots and in two test cases in the native atrial wall at anastomoses (TSPAT 001.Y11, TSPAT 002.Y11).

### Six months

At six month post implantation grafts are seen in the aortic arch and left atrial appendage area. Aortic grafts are identified grossly but atrial grafts are identified as fibrosed area.

In the ***aortic patch***, the tissue graft is histologically demarcated from host aorta on either side as a strip of cellular/ acellular/ hypocellular tissue lacking elastic fibers. The luminal side is lined by endothelial like cells in all cases. Thick neointima formation is noted with sub endothelial layer of smooth muscle cells and deep layer of fibrocytes and collagen with neo-vascularisation in all cases. Mild inflammatory cell infiltration is noted in the sub-endothelial region in all cases. At the anastomotic region on sides, degradation and recellularisation of tissue graft is observed with infiltration of macrophages, lymphocytes and fibrocytes in all cases. Medial elastic fiber degeneration and granulomatous inflammation is seen, especially around suture in all cases. Severe calcification of tissue graft is noted in one test case (TSPAT 301.Y10) and one control (TSPAT 320.Y10). Focal calcification of tissue graft is noted in four cases , three test (TSPAT 300.Y10, TSPAT 305.Y10, TSPAT 306.Y10) and one control (TSPAT 339.Y10). Calcification is absent in three cases, two tests (TSPAT 299. Y10 and TSPAT340.Y10) and in one control (TSPAT 321Y10).

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In the ***atrial patch***, the tissue graft is histologically well demarcated from surrounding host atrial tissue as a focal, thick area of cellular/ acellular/ hypocellular fibrous tissue without cardiac muscle fibers in all cases. Degradation and recellularisation of tissue graft is observed. At the graft – host atrial wall anastomoses region, collection of mononuclear cells are seen, especially around sutures in all cases. Foci of mononuclear infiltration surrounding the tissue graft are noted in all cases. Thick neo-endocardium is appreciated with severe fatty infiltration in all cases. Mild inflammatory cell infiltration is noted in the graft region in all cases. Severe calcification of tissue graft is observed in two cases, one test (TSPAT

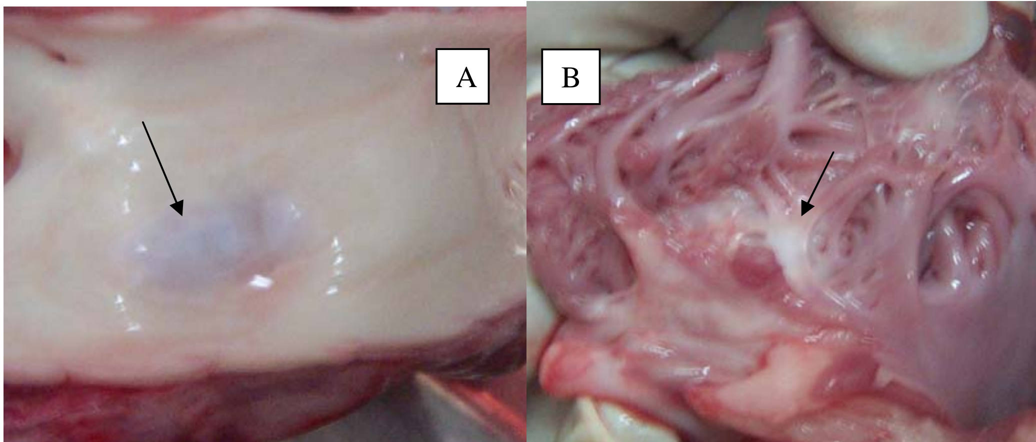
301.Y10) and one control (SPAT 320.Y10). Focal calcification in tissue graft is noted in three cases, two tests (TSPAT 300.Y10 and TSPAT 306.Y10) and one control (TSPAT 339.Y10) Calcification is absent in four cases, three tests (TSPAT299.Y10, TSPAT 305.Y10 and TSPAT 340.Y10) and one control (TSPAT 321.Y10).

**One year**

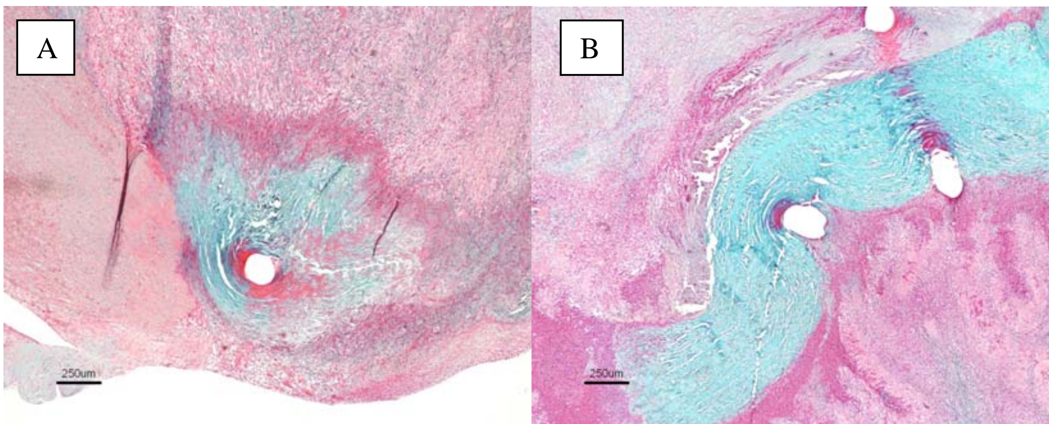
At one year post implantation grafts are seen in the aortic arch and left atrial appendage area. Aortic grafts are identified grossly but atrial grafts are identified as fibrosed area except in one control TSPAT 110.Y11.

In the ***aortic patch***, the tissue graft is histologically demarcated from host aorta on either side as a strip of relatively cellular tissue lacking elastic fibers. The luminal side is lined by endothelial like cells in all cases. Thick neointima formation is noted with sub endothelial layer of smooth muscle cells and deep layer of fibrocytes and collagen with neovascularisation in all cases. Inflammation is absent in the neointima region in all cases. At the anastomotic region on both sides, degradation and recellularisation of tissue graft is observed with infiltration of macrophages, lymphocytes and fibrocytes in all cases. Calcification of tissue graft is seen in three control cases (TSPAT 108.Y11, TSPAT 109.Y11 and TSPAT 110.Y11). Calcification of tissue is observed at anastomoses only and absent in the graft in two test cases (TSPAT 078.Y11, TSPAT 079.Y11). Overall inflammatory response is very less in all cases.

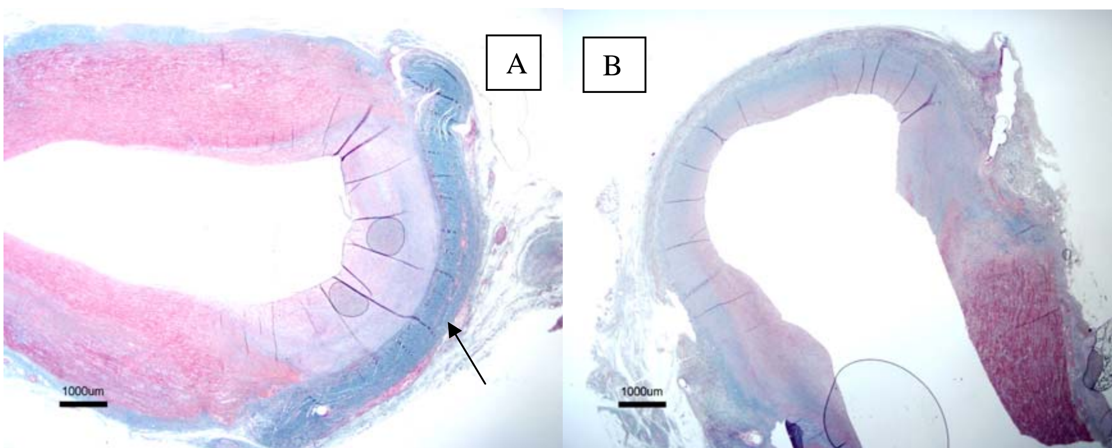
In the ***atrial patch***, the tissue graft is histologically well demarcated from surrounding host atrial tissue as a focal, thick area of cellular/ acellular/ hypocellular fibrous tissue without cardiac muscle fibers in all cases. Degradation and recellularisation of tissue graft is observed. At the graft – host atrial wall anastomoses region, mild collection of mononuclear cells are seen in all cases. Foci of mononuclear infiltration into the tissue graft are noted in all cases. Thick neoendocardium is appreciated with severe fatty infiltration in all cases. Calcification of tissue graft is noticed in four cases, one test (TSPAT 079.Y11) and three controls (TSPAT 108.Y11,TSPAT 109.Y11 and TSPAT 110.Y11). Calcification is absent in one test case (TSPAT078.Y11).



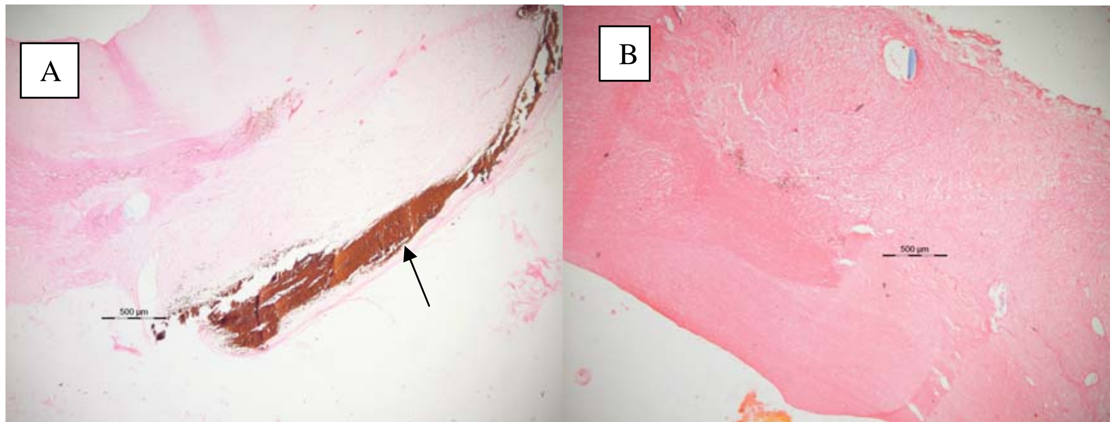

Decellularised bovine pericardium showing structural intactness and excellent healing at aortic position (A) and left atrial position (B).




Decellularised BP (A) showing initiation of graft degradation at one month, whereas SJM Biocor™ (B) showing no graft degradation at all at this period in pig aortic implantation.



Decellularised BP (A) showing thin neointima and integration of graft into native aorta whereas SJM Biocor™ (B) showing thicker neointima, no graft degradation (arrow) and no integration of graft into native vessel at all at 6 months in pig aortic implantation .




A: SJM Biocor™ BP showing graft calcification (arrow) at 12 months

B: Decellularised BP showing no calcification of graft at 12 months.

## V. Summary

Forty five tissue collections were completed from the tissue collection lab established at KLDB farm Kulathupuzha as per ISO 22442 (part 2). Several audits were conducted at KLDB farm and the tissue processing laboratory at SCTIMST to verify the traceability of the animal tissue as well to check compliance to the work procedures and the above standard. The animal tissue was decellularised with the patented technique and further processed using 'Process 0.2Dcl'. Mandatory preclinical testing on decellularised bovine pericardium was completed to qualify the decellularised tissue for human implantation. 'Expression of interest' from the industry for this technology was invited through our website and by personal communication. Interest was shown by TTK Health care Ltd, Trivandrum and Meril Life Sciences Pvt. Ltd.,Vapi, Gujarat. Discussions are initiated with these companies for Technology transfer.

**Any new product/process developed**

- Biomedical quality animal tissue
- Decellularised bovine pericardium as cardiac patch material and
- The process for producing it.

**Any new lead**

- Decellularised bovine pericardium is being evaluated for other applications such as dura substitiute. Animal trial is progressing in this regard.
- Accelerated fatigue testing on decellularised bovine pericardium is ongoing to qualify it as valve leaflet material. Once found suitable, this will be use for the fabrication of Bioprosthetic heart valve. A project proposal is being prepared for funding the proof of concept study.

**Any new technology developed**

- A non-detergent based decellularisation and manufacture of decellularised bovine pericardium suitable for cardiac patch application is developed.

**Any patent taken**

- Complete specification was filed for Indian patent titled 'A process for non-detergent based decellularisation of animal tissue for therapeutic human implantation' Application no. 2113/CHE/2008 dated 29-08-08. Patent application is published and its examination has started this year.

**Future course**

- **MOU with industry for scale-up and production in GMP facility**
- **Complete package validation studies**
- **Ethical clearance for human clinical trial**
- **Explore other applications for decel BP**
- **Develop decel porcine aortic valve using the established process**
- **Explore use of buffalo tissue which is reportedly free of BSE, for biomedical application.**

## Annexure

### Publication from project work


a. Umashankar PR, Arun T , Kumary TV (2011). Short duration glutaraldehyde crosslinking of decellularised bovine pericardium improved biological response. *J Biomed Mater Res*; 97A(3):311-320.

b. Umashankar P.R, Mohanan.P.V and Kumari.T.V. Glutaraldehyde treatment elicits toxic response compared to decellularisation in bovine pericardium. Accepted for publication in Toxicology International.

c. P.R. Umashankar and Suja Leksmi, 'Effect of modification of decellularised bovine pericardium on its in vivo response'. In Stem cells & Regenerative Medicine, International Conference on Biomaterials Implant Devices and Tissue engineering-BIDTE-2012, 6-8 January 2012 at Rejaleksmi Engineering College, Chennai.

# Short duration gluteraldehyde cross linking of decellularized bovine pericardium improves biological response

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**Abstract:** Gluteraldehyde stabilized bovine pericardium is used for clinical application since 1970s because of its desirable features such as less immunogenicity and acceptable durability. However, a propensity for calcification and long term implant failure is reported because of gluteraldehyde treatment. There is also failure of implant to integrate into host tissue because of its resistance to tissue remodeling. Decellularized bovine pericardium, a potential alternative allows tissue remodeling but it has problems such as immunogenicity and chronic inflammatory response. In this study, decellularized bovine pericardium was subjected to short duration, low concentration gluteraldehyde cross-linking at two levels and its biological response (both in vitro and in vivo) was compared with un-crosslinked decellularized bovine pericardium and fully crosslinked normal bovine pericardium. It was observed that both un-crosslinked and partially crosslinked decellularized bovine pericardium to be non-cytotoxic and it caused significantly less inflammatory cytokine release such as TNF alpha and IL1beta from activated macrophages. Among all groups, short duration 0.2% Gluteraldehyde treated decellularized bovine pericardium showed significantly less antibody response and inflammatory response compared to un-crosslinked decellularized pericardium, short duration 0.6% gluteraldehyde treated decellularized bovine pericardium or completely cross linked bovine pericardium in juvenile rat subcutaneous implantation model. Moreover, short duration 0.2% gluteraldehyde crosslinked decellularized bovine pericardium showed minimum calcification, better host fibroblast incorporation, new collagen deposition and angiogenesis within the implant. These attributes may finally lead to better implant remodeling and sustained implant function during clinical use. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 97A: 311–320, 2011.

**Key Words:** decellularized bovine pericardium, short duration gluteraldehyde treatment, immune response, calcification, host tissue incorporation

## INTRODUCTION

Gluteraldehyde stabilized bovine pericardium is in clinical use since 1970s as surgical implants or bioprostheses because of its optimal hydraulic characteristics and durability.[1] However, it was shown that such tissue had altered mechanical property, early mechanical failure, cytotoxicity, incomplete suppression of immunological recognition, and calcification.[2–4] Although gluteraldehyde cross linking renders xenografts immunologically inert this also make it nonresorbable and nonamenable to *in vivo* remodeling because of its resistance to matrix metalloproteinase. Possible sequelae to this failure to remodel *in vivo* can be failure to structurally and physiologically integrate into host tissue. This is of clinical concern since it is reported that optimum scaffold degradation and site appropriate new matrix deposition are crucial for implant function.[5]

An emerging alternative to chemical cross linking for reducing xenograft immunogenicity is decellularization.[6] These acellular matrices promote induced regeneration through remodeling of the prosthesis by neo-vascularization, recellularization and laying of new extracellular matrix by the host. The remodeled xenografts may functionally and structurally integrate into the host tissue.[6] Immunogenicity of decellularized xenografts is still a problem and it is reported that the immune response against in-completely decellularized xenografts was seen much more prominent compared to isografts or even allografts.[7,8] It appears that even decellularization alone is not sufficient since decellularized un-cross linked xenograft is reported to elicit antibody response as demonstrated by immunoblot analysis.[9] The immunogenicity of un-cross linked decellularized xenograft was observed as tissue over growth, inflammatory cell infiltration and incidence of aneurismal dilation by Hilbert et al.[10] through comparing various reported decellularization protocols in long term sheep implantation model. Moreover it was reported that positive results found in short term animal trials of an un-cross linked decellularized xenograft valve had culminated in catastrophic failure during clinical trial.[11] It should be possible to lower the immunogenicity of decellularized tissue by gluteraldehyde crosslinking as it is known to reduce immunogenicity through altering the display of antigenic determinants.[12]

Additional Supporting Information may be found in the online version of this article.
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In this study, it is hypothesized that by suitably cross-linking the decellularized tissue with gluteraldehyde, immuno-suppression, insignificant calcification together with better tissue incorporation and matrix deposition, which are required, for proper implant function can be achieved. In the work presented here, the effect of short duration low concentration gluteraldehyde cross linking of decellularized bovine pericardium on *in vitro* and *in vivo* biological response is reported.

## MATERIALS AND METHODS

### Decellularization

Decellularization of bovine pericardium was achieved using a proprietary process based on a nondetergent method. Decellularization is confirmed by demonstrating absence of nuclear remnants using routine HE staining as well as by use of specific nuclear stain such as Hoechst 33258 on 5 μm paraffin embedded sections ($n = 10$). Besides this, residual DNA is extracted from 100 mg decellularized BP using DNA- XPress Kit (Hi-Media) and concentration is estimated spectrophotometrically ($n = 3$). This is followed by demonstration of extractable DNA by 1% agarose gel electrophoresis. Structural content was evaluated using histochemical staining of 5 μm paraffin embedded sections using Movats Pentachrome staining ($n = 10$).

### Extractable protein

The residual extractable protein after decellularization and three-day wash in sterile normal saline is estimated in triplicate samples. For this ~100 mg (wet weight) of decellularized tissue sample was weighed and washed in PBS for 30 min with constant agitation. Washed tissue is minced into 1 mm pieces and homogenized in 2 mL ice cold sterile PBS at 700 rpm for 20 min in prechilled tubes. The homogenate was aspirated and transferred into fresh prechilled centrifuge tubes and debris was sedimented by centrifugation at 10,000 × g at 4°C for 30 min. The supernatant was collected and aliquoted into sterile tubes and stored at −20°C until estimation. The protein content of the supernatant was estimated using Lowry's method and expressed as mg per 100 mg of wet tissue. This was followed by SDS-PAGE for identifying the molecular weight of the extractable protein. Totally, 10 μg extractable protein from fresh bovine pericardium and decellularized bovine pericardium together with molecular weight marker were subjected to electrophoresis on a 10% (wt/vol) SDS-PAGE gel overlaid with 5% (wt/vol) stacking gel. On completion of the electrophoresis, the gel was removed and the protein bands were demonstrated by routine silver staining.

### Short duration gluteraldehyde cross linking and its confirmation

Decellularized pericardium was washed for three days in normal saline containing antibiotics (Ampicillin and Cloxacillin at 1 mg/mL). This was done under constant shaking in an incubator shaker set at 37°C. The washed tissue was fixed for 10 min in 0.2% (0.2Dcl group) and 0.6% (0.6Dcl group) Gluteraldehyde solution (25%, Sigma Aldrich) in PBS at pH7.4, at 37°C under constant agitation. The Gluteraldehyde fixed tissue was washed thoroughly in several changes of PBS to remove residual Gluteraldehyde. Further inactivation of residual Gluteraldehyde was done by treating with 8% Glutamic acid solution for 24 h under continuous agitation in an incubator shaker. The treated tissue was thoroughly washed in at least three changes of sterile PBS containing antibiotics (Ampicillin-Cloxacillin at 1 mg/mL), with one washing cycle lasting for 10 min. The final tissue was preserved in 70% ethanol (Merck) in a clean sealed container, which also acted as a sterilant. Decellularized bovine pericardium with out any chemical cross-linking (Edcl) preserved in 70% ethanol was used for comparison. Fresh bovine pericardium fixed with 0.6% Gluteraldehyde (25%, Sigma Aldrich) for seven days, prepared in PBS (pH 7.4) followed by storage in 0.2% Gluteraldehyde was used as gluteraldehyde control (Glut control).

Gluteraldehyde cross-linking was assessed by collagenase susceptibility, shrinkage temperature, contact angle measurement, and thermogravimetry.

### Collagenase susceptibility

*In vitro* degradation study was conducted using Collagenase from *Clostridium histolyticum* (125 μm/mg, Sigma Aldrich). Approximately 50 mg (wet weight in triplicates) of 0.2Dcl, 0.6Dcl, Edcl, and Glut control were weighed. To the weighed samples, 0.5 mL of 0.1*M* Tris Chloride (pH 7.4) containing 0.005*M* Calcium Chloride and 0.05 mg/mL Sodium Azide was added. The samples were incubated at 37°C for 1 h with constant shaking. Collagenase enzyme was prepared in 0.1*M* Tris Chloride (pH 7.4) at 37°C and added in to the vials so as to make the final concentration to 2U/mg of tissue. The vials were incubated at 37°C for 24 h, 72 h, and 7 days, at the end of which, the samples were centrifuged at 12,000 rpm for 20 min at 4°C and the remaining tissue samples are blotted in filter paper for 5 min to dry them. They were weighed using Afcoset Electronic Weighing Balance ER120A. The weight loss was determined by paired comparison before and after treatments.

### Shrinkage temperature

The temperature at which the denaturation of collagen started was visualized as shrinkage and it was measured in Edcl, 0.2Dcl, 0.6Dcl and Glut control groups. Totally, 30 × 6 $mm^2$ sized samples ($n = 6$) were immersed in water taken in a beaker under constant load by keeping under a glass slide. Water heated up to 90°C was pumped into the beaker at 38–48 mL/min using a positive displacement pump (Masterflex, Cole Parmer, model 7518-60, USA). The temperature at which shrinkage is observed was recorded using a digital thermometer. (Accurad 2020, Radix, India).

### Contact angle measurement

Contact angle measurement was employed as an indicator of surface chemical modification such as increased hydrophobicity subsequent to cross-linking. For this 1 $cm^2$ triplicate samples of 0.2Dcl, 0.6Dcl, and Edcl groups were dehydrated by keeping in −20°C for overnight followed by

lyophilization. Angle of contact of a small sessile drop of distilled water placed on the material surface was measured on triplicate samples, with three measurements on each sample using Goniometer GII, Kerno Instruments Inc, USA.

### Thermogravimetry

Thermogravimetry was done using the instrument SDT-2960, TA Instruments Inc, Delaware USA. The temperature at which structural degradation of the minimally cross-linked test sample (0.2Dcl group) started was compared with the un-cross linked decellularized bovine pericardium (Edcl group). For this triplicate 1 $cm^2$ samples of the above groups were dehydrated by keeping in $-20°C$ for overnight followed by lyophilization. The samples were studied for weight loss during controlled heating at a range of 50 to 400°C at a heating rate of 10°C/min. Calcined alumina was used as reference material.

### *In vitro* biological response

*In vitro* biological responses of different groups were assessed by cytotoxicity studies using fibroblast cells by direct contact and by macrophage activation studies.

### Cytotoxicity

Cytotoxic potential of materials belonging to different groups on account of leachables or because of their direct contact was evaluated by this study. The test samples (Edcl, 0.2Dcl, 0.6Dcl, and Glut control groups) were rinsed thrice with normal saline before cytotoxicity evaluation. Test samples, negative control (High density poly ethylene -USP), and positive control (Copper) in triplicates were placed on subconfluent monolayer of L-929 mouse fibroblast cells. After incubation of cells with test samples at 37 $\pm$ 2°C for 24 $\pm$ 1 h, cell culture was examined microscopically for cellular response around the samples. Cellular responses were expressed as noncytotoxic, mildly cytotoxic, moderately cytotoxic, and severely cytotoxic.

### Macrophage activation

Macrophage activation was studied by looking for inflammatory cytokine release by adhered macrophages on contact with materials belonging to different groups. THP-1 (human acute monocytic leukemia cell lines) cells were grown in RPMI 1640 (Sigma) containing 10% v/v Fetal Bovine Serum (Gibco), 160 U/mL benzylpenicillin, and 100 U/mL streptomycin (Sigma). The cells were maintained at 37°C in an atmosphere of 95% air and 5% CO2 at 90% relative humidity. The differentiation into macrophages was induced by treating THP-1 cells in a 24-well plate for 24 h with RPMI-1640 containing 20 ng/mL Phorbol 12-myristate 13-acetate (PMA). Thereafter fresh medium was added and cells were grown for another 24 h under similar conditions. Release of proinflammatory cytokines (IL-1β, IL-6, and TNF-α;) from THP 1 cells upon contact with triplicate samples of 0.2Dcl, 0.6Dcl, glut control, and positive control (bacterial lipopolysaccharide at 20 pico gram) were carried using ELISA. The concentration of cytokines in culture supernatant was quantified by specific ELISA kit (U-CyTech biosciences, Netherlands) as per manufacturer's instructions.

### *In vivo* biological response

*In vivo* biological response such as immune response, calcification response and tissue response was studied in juvenile rat sub cutaneous implantation model.

### Animal implantation

Animal protocol was approved by Institutional Animal Ethics Committee. Three week old male Wistar rats were grouped into 0.2Dcl group, 0.6Dcl group, Glut Control group, and Edcl group with each group consisting of five animals. Animals were subcutaneously implanted under the dorsal skin with evenly placed six implants of the same treatment group. The animals were observed daily for 60 days and at the end of which they were euthanized.

### Antibody response

The antibody response (IgG, IgM, and IgA) elicited in different groups of animals implanted with 0.2Dcl, 0.6Dcl, Edcl, and Glut control samples was assessed using Indirect ELISA. For this, extractable protein of fresh bovine pericardium was coated in the 96-well ELISA immuno plate (F96 MAXISORP, NUNC) in PBSN (2 microgram protein per well). Serum samples at 1 in 1000 dilution prepared in blocking buffer from animals belonging to the above groups ($n = 5$) was used as primary antibody. Serum from animals which is immunized with extractable protein from fresh bovine pericardium together with Freund's adjuvant was treated as positive control. Rabbit polyclonal to rat IgG+IgM+IgA conjugated with HRP (Abcam ab8521) was used as secondary antibody. After the reaction, the ELISA plates were read with microplate reader Expert Plus (ASYS Hitech GmbH, Austria). OD readings of micro wells without secondary antibody were taken as negative control. OD values subtracted from the mean negative control values were used for comparison.

### Calcification response

Calcium estimations from explanted samples were done as per the procedure reported earlier.[13] In brief, the explanted 0.2Dcl, 0.6Dcl, Edcl, and Glut control samples ($n = 15$, three samples from each animal) were digested in 2 mL of 2*N* HCl for 48 h at 60°C. The supernatant was neutralized with 2*N* NaOH followed by estimation of Calcium using colorimetric method of O-Cresolphthalein complexone obtained as a standard kit. OD reading were taken at 578 nm using Shimadzu UV-1700 spectrophotometer, Japan.

### Tissue response

The explanted 0.2Dcl, 0.6Dcl, Edcl, and Glut control samples ($n = 15$, three samples from each animal) were fixed in 10% buffered formalin, embedded in paraffin and 5 μm sections were made. The sections were stained by haematoxylin and eosin and a qualitative assessment was made for peri-implant necrosis, inflammatory response, peri-implant fibrosis, tissue incorporation, and angiogenesis in the

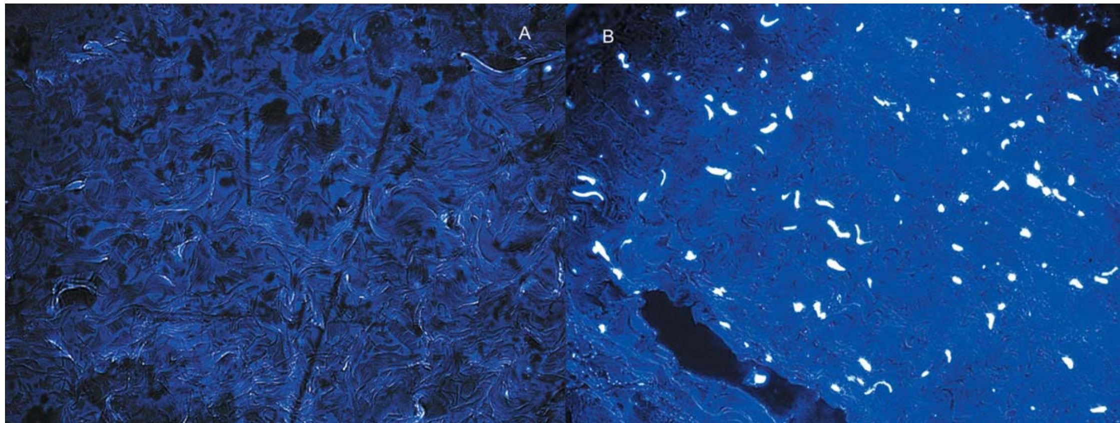


**FIGURE 1.** Confirmation of decellularization with Hoechst 3328 staining. (A) Decellularized pericardium with no nuclear remnants, (B) Normal pericardium showing intact nucleus. Hoechst 33258 400×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

implanted material. Von Kosa stain was used to demonstrate calcium in the paraffin sections.

### Statistics

All quantitative estimations are expressed in mean ± SD. Significance testing for differences of means between groups are done using Single factor ANOVA. This was followed by *F* test for testing homogeneity of variance and Un-paired "*t*" Test for equal/unequal variance as required. Statistically significant difference between means was assumed whenever *p* value was less than 0.05. In the text exact "*p*" value is reported at several instances for the sake of easy comparison.

## RESULTS

### Decellularization

Nuclear remnants could not be demonstrated both by Haematoxylin and Eosin (Supporting Information, Fig. S1) as well as by Hoechst 33258 staining (Fig. 1), indicating satisfactory decellularization. DNA extraction yielded a residual DNA content of 7 ± 3.5 μg per mL in decellularized bovine pericardium compared to 97 ± 2 μg/mL in fresh bovine pericardium (triplicate samples). Extractable DNA from decellularized tissue could not be demonstrated in 1% agarose gel electrophoresis probably because of extensive DNA fragmentation (Fig. 2). Structurally, decellularized pericardium mainly consisted of well separated collagen bundles with a few elastin fibers as demonstrated by Movats Pentachrome staining (Fig. 3).

### Extractable protein

The extractable protein content of decellularized pericardium in comparison to fresh bovine pericardium ($n = 9$) has significantly reduced ($p < 0.001$) from 0.241 ± 0.02 mg/100 mg wet tissue 0.102 ± 0.026 mg/100 mg following decellularization. It was further reduced to 0.09 ± 0.015 mg/100 mg wet tissue following three days normal saline wash; however this reduction in protein content was not statistically significant. SDS-PAGE revealed a faint protein band at around 66 KD region in the case of decellularized bovine pericardium. (Fig. 4). Fresh bovine pericardium showed several protein bands of which the band at 66KD region was the most prominent one.

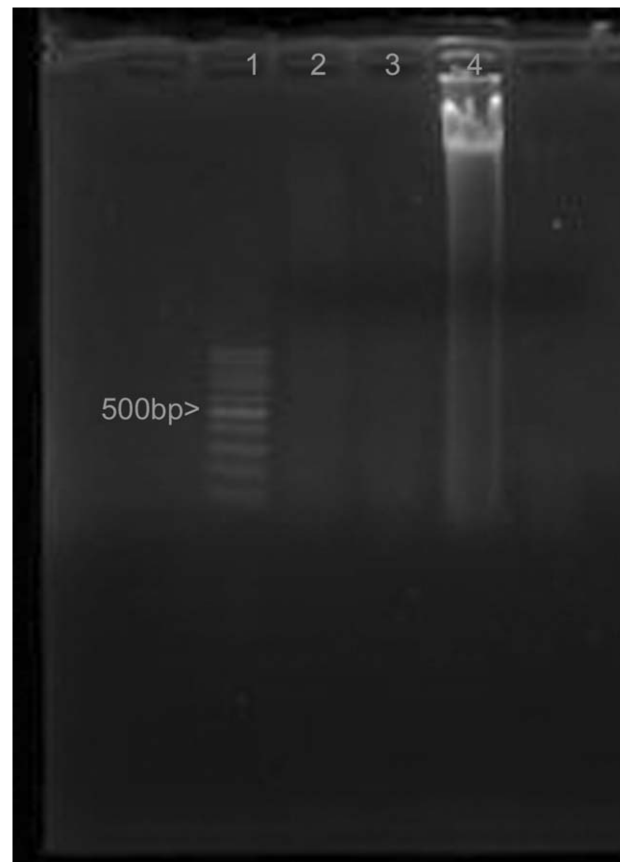


**FIGURE 2.** Extractable DNA from decellularized bovine pericardium; 1st lane: 100bp DNA ladder, 2nd and 3rd lane: DNA from decellularized bovine pericardium, 4th lane: DNA from fresh bovine pericardium.

**FIGURE 3.** Decellularized bovine pericardium showing separated collagen bundles (stained yellow)with a few elastin fibers (stained black). Movats Pentachrome 200×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### Confirmation of gluteraldehyde cross-linking

***Collagenase susceptibility.*** Data on residual weight of different groups following Collagenase II digestion is given in Table I and data on degradation is given in Table II.

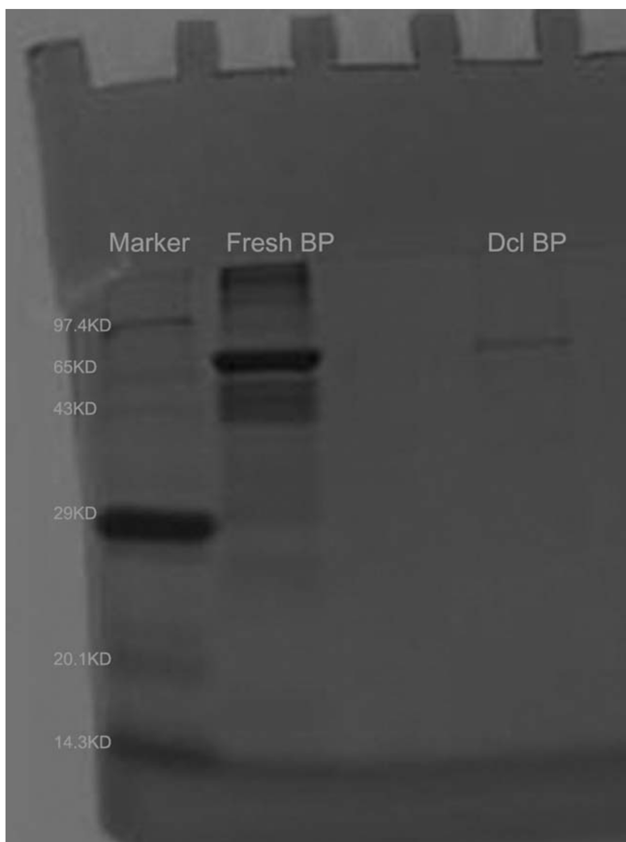


**FIGURE 4.** Extractable protein from decellularized bovine pericardium; 1st lane molecular weight marker; 2nd lane: extractable protein from fresh bovine pericardium; 4th lane: extractable protein from decellularized bovine pericardium.

**TABLE I. Residual Weights in Different Groups with Time Following Collagenase Type II Digestion**

| Residual Weight (mg) | 24 h | 72 h | Seven Days |
|---|---|---|---|
| Edcl | 0.3 ± 0.2 | – | – |
| 0.2Dcl | 4.9 ± 0.4* | 1.8 ± 0.3* | 1.3 ± 0.2 |
| 0.6Dcl | 46.6 ± 1.5* | 35.1 ± 4.3* | 17.9 ± 1.9* |
| Glut control | 49 ± 1* | 49 ± 1 | 48 ± 1 |

* Statistically significant at $p < 0.05$. Comparison was made between groups and between different time periods of the same group.

Following Collagenase type II digestion of 50 mg samples, Edcl group (un-cross linked sample) had maximum degradation (>99% weight loss) in 24 h. In comparison minimally cross-linked group, 0.2Dcl had 90.2% weight loss, indicating chemical cross linking. Moderately cross linked sample, 0.6Dcl group was seen more resistant to degradation with 6.8% weight loss at this period. Glut control group had maximum Collagenase resistance with minimum weight loss (2%). At 72 h residual weight in Edcl group was not measurable. 0.2 Dcl, 0.6Dcl and Glut control groups showed a cumulative weight loss of 96.5%, 29.8%, and 2 indicating progressive degradation in 0.2Dcl and 0.6Dcl groups. At seven days the rate of weight loss has slowed down in 0.2Dcl group with only an additional 1% weight loss was noticed at this time. 0.6Dcl BP showed further weight loss during this period reaching a cumulative weight loss of 64.2%. Glut control continued to loose weight at very low rate reaching about 4% at seven days.

***Shrinkage temperature.*** Data on shrinkage temperature is given in Table II. Un-cross linked sample showed minimum shrinkage temperature of 64.58 ± 0.47°C. This has significantly increased by 1.73°C in minimally cross linked group (0.2Dcl). Moderate cross linking (0.6Dcl group) has further elevated the shrinkage temperature significantly by 3.99°C. Maximum shrinkage temperature of 77.55 ± 0.91°C was noted in completely cross linked sample (Glut control group).

Contact angle formed by the sessile drop of distilled water on chemical cross-linked groups such as 0.2Dcl and 0.6Dcl were 33.5 ± 0.86° and 45.3 ± 0.88° respectively, which were significantly higher ($p < 0.001$) compared with 29 ± 0.57° in Edcl group (noncross linked decellularized BP). This indicates definite structural modification on the measured surface because of short duration gluteraldehyde cross-linking. Furthermore contact angle on 0.6Dcl group was significantly higher ($p < 0.001$) compared with 0.2Dcl group indicating a higher degree of surface change because of treatment. Edcl group with no cross linking was seen as the most hydrophilic one. Contact angle of glut control group could not be measured as sessile drop could not be formed on its surface.

Thermogravimetry indicated initiation of structural degradation around 152°C in uncross linked sample (Edcl)

**TABLE II.** Biological Response (*In Vitro* and *In Vivo*) of Different Treatment Groups

| Treatment groups | Edcl (un-cross linked sample) | 0.2Dcl (minimally cross-linked sample) | 0.6Dcl (moderately cross linked sample) | Gluteraldehyde control (fully cross linked sample) |
|---|---|---|---|---|
| Susceptibility to Collagenase type 2 (*in vitro*)% | >99% degradation in 24 h; no measurable residue left at 72 h | 90.2% degradation in 24 h; 96.5% degradation in 72 h; 97.4% in seven days | 6.8% degradation in 24 h; 29.8% in 72 h; 64.2% in seven days | 2% degradation in 24 h, 2% in 72 h; 4% in seven days |
| Shrinkage temperature,°C | 64.58 ± 0.47* | 66.31 ± 0.48* | 70.3 ± 0.55* | 77.5 ± 0.91* |
| *In-vitro* Cytotoxicity to L929 fibroblast cells | Noncytotoxic | Noncytotoxic | Noncytotoxic | Moderately cytotoxic |
| *In vitro* Macrophage activation**: | | | | |
| IL1 beta (pg) | 85.2 ± 2.9 | 82.4 ± 4.1 | 78.1 ± 7.9 | 144 ± 7.4* |
| IL6 (pg) | 44.4 ± 1.39 | 59.1 ± 3.6* | 46.3 ± 2.9 | 46 ± 0.72 |
| TNF alpha (pg) | 32.1 ± 2.1 | 45.3 ± 6.6 | 37.8 ± 6 | 267 ± 22.9* |
| *In vivo* antibody response (OD) | 1.27 ± 0.1* | 0.83 ± 0.08* | 1.07 ± 0.12 | 1.11 ± 0.16 |
| *In vivo* calcification response (μg/100 mg wet tissue) | 42.7 ± 16.9* | 102 ± 59* | 433 ± 243* | 1579 ± 727 |

* Statistically significant difference at $p < 0.05$.
** LPS activation caused cytokine release at, IL1beta: 255 pg; IL6: 280 pg and TNF alpha: 346pg.

compared with 206.4°C in minimally cross-linked sample (0.2Dcl). The initial weight loss noticed in both the samples can be due to water loss. Likewise the temperature of final structural degradation has increased from 244.76°C in un-cross linked sample (Edcl) to 267.2°C in 0.2Dcl (minimally cross linked sample). This shift in the degradation temperature observed in minimally cross linked sample can be due to formation of chemical cross links by short duration gluteraldehyde treatment (Fig. S2).

### *In vitro* biological response

***Cytotoxicity.*** Test samples 0.2Dcl, 0.6Dcl, and Edcl were seen non cytotoxic to L929 fibroblasts by direct contact method. This indicated that the residual gluteraldehyde following short duration cross linking using both 0.2% and 0.6% Gluteraldehyde could be effectively neutralized by PBS wash and Glutamic acid treatment. Glut control sample was seen moderately cytotoxic probably because of residual Gluteraldehyde. Positive control was severely cytotoxic and negative control was non-cytotoxic. (Fig. S3)

***Macrophage activation.*** Cytokine release profile of activated macrophage in response to exposure to different groups is given in Table II. Positive control group showed the highest cytokine response. With respect to the TNFα and IL1β, highest macrophage activation was seen in gluteraldehyde control group. This was seen statistically significant compared to Edcl, 0.2Dcl, and 0.6Dcl groups. There was no significant difference in response between Edcl, 0.2Dcl, and 0.6Dcl groups with respect to the above cytokines. In the case of IL6, 0.2Dcl group showed a higher response when compared with 0.6Dcl (p0.009), gluteraldehyde control group (p0.004), and Edcl group (p0.005). There was no statistically significant difference between Edcl, 0.6Dcl, and gluteraldehyde control group.

### *In vivo* biological response

***Animal implantation.*** Animal implantation was uneventful and all animals in the groups 0.2Dcl, 0.6Dcl, Edcl, and Glut control survived the duration of the study uneventfully.

***Antibody response.*** Antibody response against extractable protein from fresh bovine pericardium elicited in animals implanted with different treatment groups is given in Table II. Minimally cross linked sample, 0.2Dcl group had significantly lower antibody response against fresh bovine pericardial protein compared with all the other groups ($p < 0.05$). Antibody response in uncross-linked sample, Edcl group was significantly higher than gluteraldehyde control group ($p < 0.05$). 0.6Dcl group with moderate cross-linking also showed significantly lower antibody response compared to Edcl group ($p < 0.05$). Interestingly 0.6Dcl group showed significantly higher antibody response compared to 0.2Dcl group ($p < 0.05$), whereas it was noticed comparable to Glut control group. Reduced antibody response to 0.2Dcl group compared with Edcl indicates reduction in antibody production in response to partial cross linking.

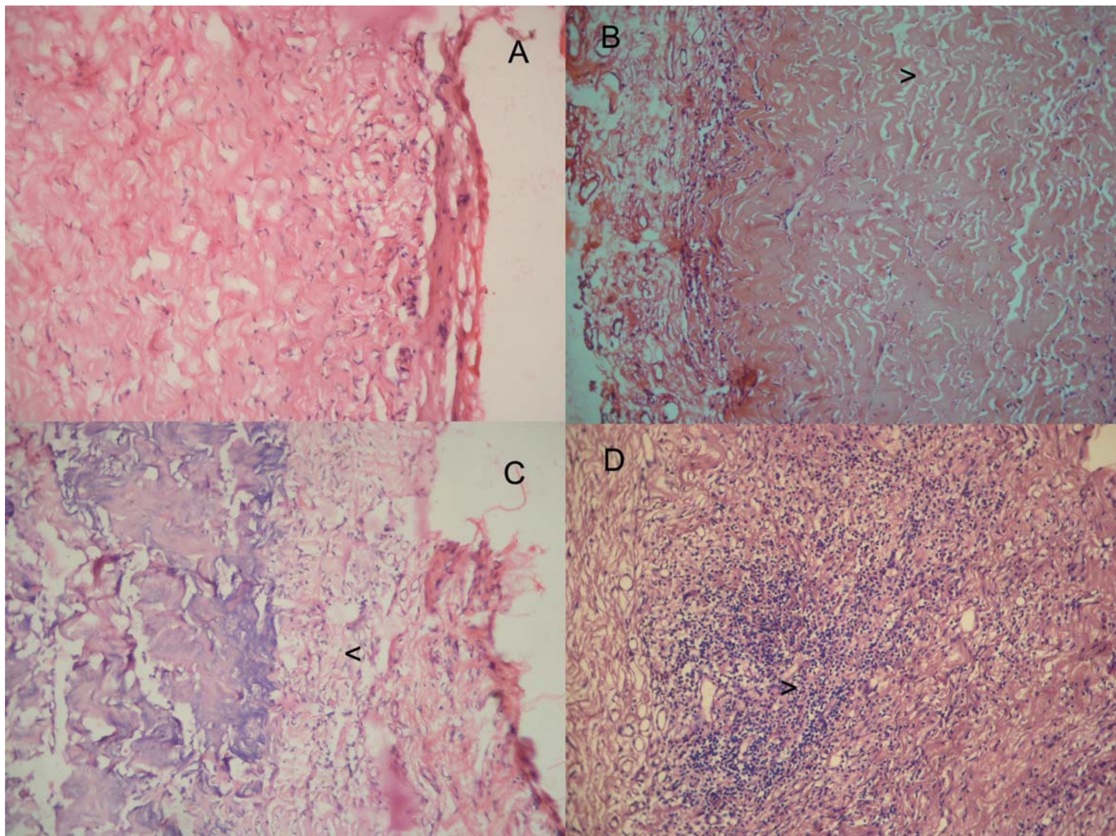


**FIGURE 5.** Tissue response to different implants at 60 days in rat subcutaneous implantation; (A) 0.2DCl group showing thin capsule formation, minimum inflammatory response and uniform host cell incorporation. (B) 0.6DCl group showing thicker capsule formation, focal inflammatory response and acellular interior (arrow). (C) Glut control showing thicker capsule, inflammatory response (arrow) in the interphase an acellular interior. Edcl group: showing moderate to severe inflammatory response in the interior of implant (arrow). HE 200×.

***Calcification response.*** The result of calcification experiment is given in Table II. Edcl group explant had minimum calcium content of 42.7 ± 16.9 μg/100 mg of wet tissue; followed by 0.2Dcl group explant which had a calcium content of 102 ± 59 μg/100 mg wet tissue. 0.6Dcl group showed an intermediate response with a calcium content of 433 ± 243 μg/100 mg of wet tissue. Maximum calcium content of 1579 ± 727 μg/100 mg of wet tissue was noticed in gluteraldehyde treated tissue. All the differences in means noted between the groups were statistically significant at $p < 0.05$.

***Tissue response.*** The tissue response studied on haematoxylin and eosin stained paraffin sections is presented below (Fig. 5). The sections were qualitatively assessed for peri-implant necrosis, inflammatory response, calcification, peri-implant fibrosis, tissue incorporation, and angiogenesis into the implanted material as well as in the periphery.

0.2Dcl implant did not show any peri-implant necrosis. A thin fibrous capsule could be appreciated around the implant. Mild to moderate diffuse infiltration of mononuclear cells in to the implant is noticed. Macrophages could be identified at the site of implant degradation. Implant Calcification could not be observed. Implant showed excellent ingrowth of cells with fibroblast morphology with evidence of newly laid collagen. Angiogenesis is noticed in the implant as well at the periphery.

0.6Dcl explants showed thicker fibrous capsule compared to 0.2Dcl group. No peri-implant necrosis could be observed. Mononuclear cell infiltration of moderate intensity was noticed at several places around the implant. Besides lymphocytes, macrophages and occasional multinucleated giant cells could be identified at these sites. Implant calcification could be observed as small discrete deposits in the collagen fiber. Angiogenesis could be appreciated only in the implant periphery. Some sections showed implant areas devoid of host cell incorporation or angiogenesis. Diffuse mononuclear cell infiltration of mild intensity could be appreciated in the implant.

Edcl implants did not show peri-implant necrosis. A very thin capsule could be appreciated around the implant. There was moderate to severe diffuse infiltration of mononuclear cells into the implant. No focal cell infiltration in the periphery could be appreciated. Implant Calcification could not be observed. Angiogenesis was noticed in the periphery as well as the interior of the implant. Cells with fibroblast morphology could be appreciated in the implant.

*Glut control*. Thick capsule was noticed around the implant. Certain areas showed peri-implant necrosis. Angiogenesis was seen only in the periphery of the implant. Moderate to severe inflammation evidenced as mononuclear cell infiltration could be seen at the periphery of implant. The implant interior was remarkably acellular. Calcium deposit could be observed in may sections.

## DISCUSSION

In this study it is attempted to demonstrate that by decellularization and by optimally cross-linking it is possible to promote positive remodeling of decellularized bovine pericardium. It is reasoned that positive remodeling of the implant can be brought about by reducing immunogenicity, calcification, and by promoting intra implant host tissue incorporation, new matrix deposition and angiogenesis. Decellularization is primarily done to reduce xenograft immunogenicity by selective removal of cellular components where as structural components are preserved. Decellularized tissue made by detergent based methods is reported to cause inflammatory response because of residual detergents.[14,15] Decellularized tissue is also reported to elicit antibody response because of soluble protein antigens retained after decellularization.[9] In this study a nondetergent based decellularization method is employed to avoid inflammatory response due to detergent residues. The decellularization method employed was found adequate as nuclear remnants could not be detected by routine HE staining or by nuclear stain Hoechst 33258. Polyacrylamide gel electrophoresis of residual DNA could not demonstrate DNA bands indicating extensive DNA fragmentation. Extractable protein of around 66 KD molecular weight could be still observed in the decellularized tissue. Partial chemical cross linking through short duration gluteraldehyde treatment was done to avoid immune response against extractable protein antigens remaining in decellularized tissue. Short duration gluteraldehyde fixation is used clinically to augment mechanical strength of autologous pericardium used for aortic valve reconstruction.[16,17] The effect of this treatment on reducing immune response to allograft or xenograft is not reported so far. In this study, different concentrations (0.2% and 0.6%) of gluteraldehyde were used for short duration (10 minutes) fixation of decellularized pericardium and difference in the biological response elicited by them is studied in comparison to un-cross linked decellularized bovine pericardium and totally cross linked normal bovine pericardium. To avoid inflammatory response due to residual gluteraldehyde left after partial cross-lining, it was treated with 8% Glutamic acid as reported earlier.[18] The treated tissue was further incubated in 70% ethanol to extract residual tissue cholesterol and phospholipids which might induce calcification.[19] This step also sterilized the tissue which was confirmed by routine sterility testing. Absence of cytotoxic chemical leachants or endotoxins was confirmed by cytotoxicity study. Partial cross linking of decellularized tissue was assessed by resistance to digestion by Collagenase type II enzyme, measurement of shrinkage temperature, contact angle studies, and thermogravimetric analysis. Increased Collagenase type II resistance, shrinkage temperature, contact angle, and thermal degradation temperature compared to un-cross liked sample indicated that 0.2% gluteraldehyde treatment for 10 min has caused partial crosslinking. An increase in the concentration of gluteraldehyde to 0.6% for the same duration of treatment has significantly increased the above parameters.

*In vitro* data such as cytotoxicity studies and macrophage activation studies clearly suggested a better biological acceptance for partially cross linked samples (both 0.2% and 0.6% short duration gluteraldehyde treated) compared with completely crosslinked sample. *In vitro* cytotoxicity study using L929 fibroblast showed the partially cross linked (0.2Dcl and 0.6Dcl groups) and un-cross linked samples (Edcl) as non-cytotoxic, whereas the fully cross linked sample (gluteraldehyde control group) was moderately cytotoxic. Cytotoxic nature of gluteraldehyde treated tissue was reported earlier.[20] Macrophages as antigen presenting cells have an important role in eliciting specific immune response. Hence macrophage activation study was conducted. This showed a definite increase in the release of inflammatory cytokines such as TNFα and IL1β in completely cross-linked samples (gluteraldehyde control group) compared with both concentrations of partially cross linked samples (0.2% and 0.6% short duration gluteraldehyde treated) and un-crosslinked sample. Partially crosslinked or un-crosslinked did not differ in their macrophage activation potential with respect to the above cytokines. Interestingly, IL6 release was seen highest in the minimally crosslinked sample compared to un-crosslinked, moderately crosslinked and completely crosslinked samples. In contrast to the above observation, a study showed no significant variation between in *vivo* cytokine release of inflammatory and anti-inflammatory cytokines in response to different polymeric materials.[21]

*In vivo* data such as antibody response, calcification response and tissue response demonstrated better biocompatibility for minimally cross linked sample which was treated with 0.2% Gluteraldehyde for 10 min. For studying the immune response, focus was given on antibody response elicited against extractable protein from fresh pericardium as it was assumed that in decellularized tissue the residual protein is the major immunogen. Since there was complete fragmentation of DNA following decellularization, immunogenicity on this account was not anticipated. DNA fragments less than 300bp is reported to be nonimmunogenic.[22] Un-cross linked decellularized tissue elicited the maximum antibody response. This might be due to the presence of residual proteins present in the decellularized pericardium. Short duration chemical crosslinking with 0.2% gluteraldehyde had significantly reduced the antibody response, even compared with fully cross linked pericardium. However use of higher concentration (0.6%) of gluteraldehyde for the same duration had resulted in significantly higher antibody response compared to low concentration (0.2%) gluteraldehyde treatment. Interestingly the antibody response in short duration 0.6% gluteraldehyde treated group was seen similar to the fully crosslinked

gluteraldehyde control group. This indicated that higher concentration of gluteraldehyde is not necessarily immunosuppressive. A study on antigenicity of gluteraldehyde treated bovine serum albumin (BSA) reported that gluteraldehyde caused intermolecular cross-linkages forming soluble aggregates; such modified proteins were seen antigenic and produced antibodies against antigenic determinant of BSA as well as against newly acquired groups arising from this modification.[23] In this study a protein of around 66 KD molecular weight was identified as a prominent constituent of extractable protein from decellularized pericardium. Literature indicates BSA and Allergen bos d6 as two antigenic proteins having molecular weights around this region.[24] The increased antibody response seen in short duration 0.6% gluteraldehyde treated samples might be due to formation of more soluble aggregates of BSA compared to short duration 0.2% gluteraldehyde treated samples. *In vivo* calcification was seen directly linked to the extent of gluteraldehyde treatment. Gluteraldehyde control group was seen most calcifying as previously reported,[15] un-cross linked decellularized pericardium had minimum calcification and the partially cross linked samples (short duration 0.2% and 0.6 % gluteraldehyde treated) had intermediate calcification. Among the partially cross linked samples, short duration 0.2% gluteraldehyde treated sample had minimum calcification compared with short duration 0.6% gluteraldehyde-treated sample. Short duration 0.6% gluteraldehyde treated sample had four times calcification, even though both the groups had similar anticalcifying treatments. A previous study has reported direct relation between calcification and antibody response.[25] This relationship was noticed only in gluteraldehyde crosslinked samples immaterial of whether partially or completely cross linked. This relation was not observed in un-cross linked sample which showed a significant antibody response with minimum calcification.

Un-cross linked decellularized pericardium (Edcl) and completely crosslinked gluteraldehyde control group presented two extreme tissue responses; where as partially crosslinked groups (short duration 0.2% and 0.6% gluteraldehyde-treated groups) showed variations of an intermediate response. Un-cross linked group (Edcl) showed a picture indicating chronic inflammation of moderate to severe intensity with mononuclear cell infiltration within the implant. Presence of tissue remodeling was visibly evident by the presence of phagocytic macrophages and foreign body giant cells together with cells with fibroblast morphology with newly laid collagen. Significantly high antibody response in this group matches with the above observation. Implication of the above observations in a functional situation is not known. On the contrary gluteraldehyde control group showed no host cell incorporation into implant and a thicker fibrous tissue capsule separated the implant from the host tissue. Presence of mild to moderate chronic inflammation is noticed in the implant periphery. There is calcification as well as significant antibody response in this group. These conditions may not favor a positive tissue remodeling.[22] Similar observation was previously reported for gluteraldehyde-treated tissue.[20] Partially cross linked groups (0.2Dcl and 0.6Dcl) showed contrasting tissue responses, with minimally cross linked group (0.2Dcl; short duration 0.2% gluteraldehyde treated) showing desirable tissue response such as thin fibrous capsule, undetectable calcification, implant degradation with laying of new collagen and mild chronic inflammation. All the above observation points to better implant remodeling. It is assumed that the presence of mononuclear cells in the minimally cross linked implant should not be of concern since the antibody response elicited in this group was significantly lower compared with all the other groups. In contrast the moderately cross linked group (0.6 Dcl) showed thicker fibrous capsule, more implant calcification, moderately intense mononuclear cell infiltration in the periphery and angiogenesis restricted to implant periphery. Besides this some sections even showed implant regions devoid of host cell incorporation. These observations are not congenial for an acceptable implant remodeling. Extensive crosslinking with higher concentrations of Genipin has shown similar observations.[26]

## CONCLUSION

Thus it is concluded that decellularized bovine pericardium with short duration 0.2% gluteraldehyde treatment has all the biological attributes needed for positive tissue remodeling which is essential for long term successful outcome as an implant. Nevertheless further studies by orthotopic implantation in larger animal models might give more information on its clinical applicability.

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# Glutaraldehyde treatment elicits toxic response compared to decellularisation in bovine pericardium

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**Running title: Toxic effect of Glutaraldehyde treatment**

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# Glutaraldehyde treatment elicits toxic response compared to decellularisation in bovine pericardium


## Abstract

Glutaraldehyde stabilized bovine pericardium is used for clinical application since 1970s because of its desirable features such as less immunogenicity and acceptable durability. However, a propensity for calcification is reported on account of Glutaraldehyde treatment. In this study, commercially available Glutaraldehyde cross linked bovine pericardium was evaluated for its *in vitro* cytotoxic effect, macrophage activation and *in vivo* toxic response in comparison to decellularised bovine pericardium. Glutaraldehyde treated bovine pericardium and its extract was observed to be cytotoxic and it also caused significant inflammatory cytokine release from activated macrophages. Significant antibody response, calcification response, necrotic and inflammatory response was noticed in Glutaraldehyde treated bovine pericardium in comparison to decellularised bovine pericardium in rat subcutaneous implantation model. Glutaraldehyde treated bovine pericardium also failed in Acute systemic toxicity testing and Intracutaneous irritation testing as per ISO 10993. With respect to healing and implant remodeling, total lack of host tissue incorporation and angiogenesis was noticed in Glutaraldehyde treated bovine pericardium compared to excellent host fibroblast incorporation and angiogenesis within the implant in decellularised bovine pericardium.

In conclusion, using *in vitro* and *in vivo* techniques, this study has demonstrated that Glutaraldehyde treated bovine pericardium elicits toxic response compared to decellularised bovine pericardium which is not congenial for long term implant performance.


**Key words:** Glutaraldehyde treated bovine pericardium, decellularised bovine pericardium, toxic response, immunogenicity, calcification, tissue incorporation.

# Glutaraldehyde treatment elicits toxic response compared to decellularisation in bovine pericardium

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## 1.Introduction

Glutaraldehyde stabilized bovine pericardium is commercially available and used for medical application in the form of patches and bioprosthetic valves on account of its low immunogenicity and excellent durability [1]. Glutaraldehyde treatment produces stable cross-links in cellular and extra-cellular matrix proteins which substantially reduced graft immunogenicity [2]. However it was also shown that such tissue had altered mechanical property, early mechanical failure, cytotoxicity, and incomplete suppression of immunological recognition. [2,3,4]. Besides this severe calcification was noticed in Glutaraldehyde treated bovine pericardium in pediatric patients.[4]. An emerging alternative to Glutaraldehyde treatment for reducing xenograft immunogenicity is decellularisation [5,6]. Use of de-cellularised xenograft is an approach where acellular tissue matrices are produced by selective removal of cellular components that are believed to promote immunogenicity and calcification. These acellular matrices promote induced regeneration through remodeling of the prosthesis by neo-vascularisation, re-cellularisation and laying of new extracellular matrix by the host. The remodeled decellularised xenografts may functionally and structurally integrate into the host tissue [6].

In this study the *in vitro* and *in vivo* toxic effects of Glutaraldehyde treated bovine pericardium in comparison to decellularised bovine pericardium is reported. The *in vivo* effect of this toxic response such as inflammatory potential, immunogenicity, calcification potential and lack of tissue incorporation is discussed as this may have effect on the long term implant performance in clinical situation.

## Materials and methods

**Glutaraldehyde treated bovine pericardium (Glut BP)**

Commercially available Glutaraldehyde treated bovine pericardium approved for clinical use in India was used in this study. This treatment group was named as Glut BP.

**Decellularised bovine pericardium (Dcl BP)**

Decellularisation of bovine pericardium was achieved using a proprietary process based on a non-detergent method. Decellularisation is confirmed by demonstrating absence of nuclear remnants using routine HE staining as well as by use of specific nuclear stain such as Hoechst 33258 on 5 micron paraffin embedded sections (n=10). Besides this, residual DNA was extracted from 100mg decellularised BP using DNA- XPress Kit (Hi-Media) and concentration was estimated spectrophotometrically (n=3). This was followed by demonstration of extractable DNA by 1% agarose gel electrophoresis. Structural content was evaluated using histochemical staining of 5 μ paraffin embedded sections using Movats Pentachrome staining (n=10). Decellularised bovine pericardium was sterilized by a proprietary process and sterility was confirmed by routine sterility testing.

**Collagenase susceptibility**

This study was conducted to assess the ability of the graft to undergo *in vivo* degradation which will pave the way for laying host extracellular matrix. In this test the susceptibility of the test material to Collagenase II activity ( from *Clostridium histolyticum* ,125u/mg, Sigma Aldrich) was studied. Approximately 50 mg (wet weight in triplicates) of Glutaraldehyde treated bovine pericardium (Glut BP) and decellularised bovine pericardium (Dcl BP) were weighed. To the weighed samples, 0.5 ml of 0.1M Tris Chloride (pH 7.4) containing 0.005M Calcium Chloride and 0.05mg/ml Sodium Azide was added. The samples were incubated at 37 $^0$C for 1 hr with constant shaking. Collagenase enzyme was prepared in 0.1M Tris Chloride (pH 7.4) at 37 $^0$C and added in to the vials so as to make the final concentration to 2U/ mg of tissue. The vials were incubated at 37 $^0$C for 24 hrs, 72 hours and 7 days, at the end

of which, the samples were centrifuged at 12000rpm for 20 min at $4^0$C and the remaining tissue samples are blotted in filter paper for 5 min to dry them. They were weighed using Afcoset Electronic Weighing Balance ER120A. The weight loss was determined by paired comparison before and after treatments.

***In vitro* response**

*In vitro* toxic responses of Glut BP and Dcl BP was assessed by cytotoxicity studies using L929 fibroblast cells by direct contact method as well as by studying the effect of the extract on L929 fibroblast metabolism by MTT assay. *In vitro* inflammatory response was studied by macrophage activation studies.

**Cytotoxicity testing: Direct contact method**

Cytotoxic potential of Glut BP and Dcl BP groups on account of their direct contact or due to leachables was evaluated by Direct contact method (ISO 10993-5). The test samples were rinsed thrice with normal saline before cytotoxicity evaluation. Test samples, negative control (High density poly ethylene -USP), and positive control (Copper) in triplicate were placed on subconfluent monolayer of L-929 mouse fibroblast cells. After incubation of cells with test samples at $37\pm2^0$C for 24±1 hours, cell culture was examined microscopically for cellular response around the samples. Cellular responses were expressed as non-cytotoxic, mildly cytotoxic, moderately cytotoxic and severely cytotoxic.

**Cytotoxicity testing on extract:**

In-vitro MTT assay on extract of Glut BP and Dcl BP in physiological saline was performed to measure the effect of extract on the metabolic activity of L929 fibroblast cells. The ability of the fibroblast cells to metabolize yellow colored tetrazolium salt 3-(4,5-Dimethyl thiazol -2-yl)-2,5-diphenyltetrazolium bromide to purple colored formazan was measured as an indication of its activity. Different dilutions (50% and 25%) of extracts of Glut BP sample, Dcl BP, positive control (dilute phenol), negative control (high density poly ethylene (USP) and reagent control in triplicate were placed on subconfluent monolayer of L929 cells. After incubation of cells with

the extract at 37±2$^0$C for 2h, extract and control medium were replaced with 50µL MTT solution (1mg/mL in medium without supplements) wrapped with Aluminium foils and were incubated at 37±2$^0$C for 2h. After discarding the MTT solution, 100µof Isopropanol was added to all wells and swayed the plates. The color developed was quantified by measuring absorbance at 540nm using microplate reader. The data obtained was compared with negative control.

**Macrophage activation**

Macrophage activation was studied by estimating inflammatory cytokine released by adhered macrophages on contact with materials belonging to different groups. THP-1 (human acute monocytic leukemia cell lines) cells were grown in RPMI 1640 (Sigma) containing 10 % v/v Fetal Bovine Serum (Gibco), 160 U/ml benzylpenicillin and 100 U/ml streptomycin (Sigma). The cells were maintained at 37 °C in an atmosphere of 95% air and 5% $CO_2$ at 90% relative humidity. The differentiation into macrophages was induced by treating THP-1 cells in a 24-well plate for 24 h with RPMI-1640 containing 20 ng/ml Phorbol 12-myristate 13- acetate (PMA). Thereafter fresh medium was added and cells were grown for another 24 h under similar conditions. Release of pro-inflammatory cytokines (IL-1β;, IL-6 and TNF-α;) from THP 1 cells upon contact with triplicate samples of Glut BP and Dcl BP and positive control (bacterial lipopolysaccharide) at 20 pico gram) were carried using ELISA. The concentration of cytokines in culture supernatant was quantified by specific ELISA kit (U-CyTech biosciences, Netherlands) as per manufacturer instructions.

***In vivo* response**

*In vivo* toxic response was studied by conducting Acute systemic toxicity testing (ISO 10993-11) and Intracutaneous (Intradermal) reactivity test (ISO 10993-10). *In vivo* response such as immune response, calcification response and tissue response was studied by juvenile rat sub cutaneous implantation for 60 days. The animal test protocols were approved by Institutional Animal Ethics Committee and animal care and maintenance were done as per CPCSEA guidelines.

**Acute systemic toxicity testing (ISO 10993-11)**

This test was done to evaluate the systemic response of mice following intraperitoneal and intravenous injection of cotton seed oil and physiological saline extract of the test samples respectively. The test samples were prepared by washing the samples in sterile normal saline for 3 wash cycles, with each cycle lasting for 10 minutes. The samples were dried at $37^oC$ for 24 hours. 2g each of Glut BP and Dcl BP samples were extracted with 10 mL each of physiological saline and Cotton seed oil for 72 hours at $50^oC$. 50mL/Kg body weight of the above extracts were injected through intravenous (n=5) and intraperitoneal route (n=5) respectively in separate groups of randomly selected health active mice. Injection of plain extraction media in another set of animals served as control. The animals were observed immediately and after 4h, 24h, 48h and 72 h for evidence of abnormalities such as convulsions, prostrations, loss of body weight more than 2g and death.

**Intracutaneous (Intradermal) reactivity test (ISO 10993-10)**

This test was done to assess the potential of the material to produce irritation following intradermal injection of the material extract. The test samples were prepared by washing the samples in sterile normal saline for 3 wash cycles, with each cycle lasting for 10 minutes. The samples were dried at $37^oC$ for 24 hours. 2g each of Glut BP and Dcl BP samples were extracted with 10 mL each of physiological saline and Cotton seed oil for 72 hours at $50^oC$. 0.2mL (per each site) of the extract was injected into 5 sites with appropriate controls in randomly selected healthy, active New Zealand white rabbits. The animals were observed for erythema and oedema which was scored as 0 (nil), 1 (very slight), 2 (well defined oedema/erythema), 3 (moderate) and 4 (severe). The requirement of the test is met if the difference between the test sample mean score and control sample mean score is 1.0 or less. All the procedures were done aseptically.

**Implantation study**

This study was done to evaluate calcification potential, immunogenic potential and tissue response by subcutaneous implantation for 60 days in three week old male Wistar rats. Healthy animals were randomly assigned into Glut BP and Dcl BP groups with each group consisting of 5 animals. The animals were subcutaneously implanted under the dorsal skin with evenly placed six implants of the same treatment group. The animals were observed daily for 60 days and at the end of which they were euthanized. The serum was collected for estimating antibody response against bovine pericardial proteins. One set of explants (n=15) were used for calcium estimation and another set (n=15) was processed for histopathological evaluation.

**Antibody response**

The antibody response (IgG, IgM and IgA) elicited in different groups of animals implanted with Glut BP and Dcl BP samples was assessed using Indirect ELISA. For this, extractable protein of fresh bovine pericardium was coated in the 96 well ELISA immuno plate ( F96 MAXISORP, NUNC) in PBSN (2 microgram protein per well). Serum samples at 1in1000 dilution prepared in blocking buffer from animals belonging to the above groups (n=5) was used as primary antibody. Serum from animals which is immunized with extractable protein from fresh bovine pericardium was treated as positive control. Rabbit polyclonal to rat IgG+IgM+IgA conjugated with HRP (Abcam ab8521) was used as secondary antibody. After the reaction, the ELISA plates were read with microplate reader Expert Plus (ASYS Hitech GmbH, Austria). OD readings of micro wells without secondary antibody were taken as negative control. OD values subtracted from the mean negative control values were used for comparison.

**Calcification response**

Calcium estimations from explanted samples were done as per the procedure reported earlier. [7] . In brief, the explanted Glut BP and Dcl BP samples (n=15, three samples from each animal) were digested in 2mL of 2N HCl for 48 hrs at 60$^{o}$C. The supernatant was neutralized with 2N NaOH followed by estimation of Calcium using

colorimetric method of O-Cresolphthalein complexone obtained as a standard kit. OD readings were taken at 578 nm using Shimadzu UV-1700 spectrophotometer, Japan.

**Tissue response**

The explanted Glut BP and Dcl BP samples (n=15, three samples from each animal) were fixed in 10% buffered formalin, embedded in paraffin and 5 micron sections were made. The sections were stained by haematoxylin and eosin and Von Kosa stain. A qualitative assessment was made for peri-implant necrosis, inflammatory response, peri-implant fibrosis, tissue incorporation, angiogenesis in the implanted material and calcification.

**Statistics**

All quantitative estimations are expressed in mean ± SD. Significance testing for differences of means between groups is done using Unpaired Student t Test for equal or unequal variance depending on the homogeneity of variance tested using F test.. Statistically significant difference between means was assumed whenever p value was less than 0.05.

**Results**

**Confirmation of decellularisation**

Nuclear remnants could not be demonstrated both by Haematoxylin and Eosin as well as by Hoechst 33258 staining (Fig 1), indicating satisfactory decellularisation. DNA extraction yielded a residual DNA content of 7±3.5 µg per mL in decellularised bovine pericardium compared to 97±2 µg /mL in fresh bovine pericardium (triplicate samples). Extractable DNA from decellularised tissue could not be demonstrated in 1% agarose gel electrophoresis probably because of extensive DNA fragmentation (Fig. 2). Structurally, decellularised pericardium mainly consisted of well separated collagen bundles with a few elastin fibers as demonstrated by Movats Pentachrome staining.

**Collagenase susceptibility**

Data on residual weight of different groups following Collagenase II digestion is given in table1 and data on degradation is given in table 2.

Following Collagenase type II digestion for 24 hours, Dcl BP samples had 90.2% degradation with residual weight of 4.9±0.4mg. Glut BP group had maximum Collagenase resistance with minimum weight loss of around 1 mg (2%). At 72 hours residual weight Dcl BP and Glut BP groups showed a residual weight of 1.8±0.3mg (96.5% degradation) and 49±1mg (2% degradation) respectively. At 7 days the residual weights were 1.3±0.25mg and 48±1mg for Dcl BP and Glut BP groups respectively. At this period the rate of weight loss has slowed down in Dcl BP group with only an additional 1% weight loss was noticed in about 7 days. Glut BP group continued to loose weight at very low rate reaching about 4% at 7 days.

***In vitro* response**

**Cytotoxicity: Direct contact**

Dcl BP samples were seen non cytotoxic to L929 fibroblasts by direct contact method (Fig. 3). Glut BP sample was seen moderately cytotoxic probably because of residual Glutaraldehyde. Positive control was severely cytotoxic and negative control was non-cytotoxic.

**Cytotoxicity testing on extract**

The MTT assay of L929 cells after contact with 50% and 25% extract of Dcl BP showed 96% and 97% metabolic activity respectively compared to Glut BP which had 14% and 15.1% metabolic activity respectively. This clearly showed that the extract of Glut BP samples has significantly affected the metabolic activity of fibroblast cells and hence it is toxic in nature compared to Dcl BP.

**Macrophage activation**

Cytokine release profile of activated macrophage in response to exposure to different groups is given in table 2. Positive control group showed the highest cytokine response. With respect to the TNF$\alpha$ and IL1$\beta$, highest macrophage activation was seen in Glut BP group. This was seen statistically significant compared to Dcl BP

group. In the case of IL6, Dcl BP group showed a higher response when compared to Glut BP group (p0.004).

***In vivo* response**

**Acute systemic toxicity testing (ISO 10993-11)**

Administration of cotton seed oil extract of the Glut BP sample through intra peritoneal route did not produce systemic toxic effect evidenced as loss of body weight more than 2g, presence of any abnormalities or death. However intravenous administration of physiological saline extract of the Glut BP sample produced abnormalities and death immediately after injection in three mice, indicating systemic toxicity of this sample. The control animals did not show any signs of systemic toxicity. Cotton seed oil extract and physiological saline extract of Dcl BP sample did not produce any abnormalities, loss of weight or death following intraperitoneal and intravenous administration respectively. This indicated that the DclBP extract do not cause systemic toxicity.

**Intracutaneous (Intradermal) reactivity test (ISO 10993-10)**

Physiological saline extract of the Glut BP sample produced an average irritation score of 1.18 which is a positive reactivity for this test. At the same time the cotton seed oil extract showed 0 score of irritation indicating no intracutaneous irritation. The physiological saline and cotton seed oil extract of Dcl BP samples produced irritation score of 0.3 and 0.1 respectively which indicated the samples are non-irritating as per the set criteria for this test.

**Implantation study**

Animal implantation was uneventful and all animals in the groups Glut BP and DclBP survived the duration of the study uneventfully.

**Antibody response**

Antibody response against extractable protein from fresh bovine pericardium elicited in animals implanted with different treatment groups is given in table 2. Dcl BP group had significantly lower antibody response against fresh bovine pericardial

protein compared to Glut BP group ($p<0.05$). This indicated that Glutaraldehyde treatment which is done to reduce immune response of xenografts has failed to reduce it in comparison to decellularisation.

**Calcification response**

The result of calcification experiment is given in Table 2. Dcl BP group explant had significantly lower Calcium content of 157±31μg/100mg dry weight of tissue in comparison to 434±84μg/100mg of dry weight of Glut BP explant.

**Tissue response**

The tissue response studied on haematoxylin and eosin stained paraffin sections is presented below (Fig. 4). The sections were qualitatively assessed for peri-implant necrosis, inflammatory response, calcification, peri-implant fibrosis, tissue incorporation and angiogenesis into the implanted material. Dcl BP implant did not show any peri-implant necrosis. A thin fibrous capsule could be appreciated around the implant. Mild to moderate diffuse infiltration of mononuclear cells in to the implant is noticed indicating chronic inflammation. Macrophages could be identified at the site of implant degradation. Implant Calcification could not be observed. Implant showed excellent ingrowth of cells with fibroblast morphology with evidence of newly laid collagen. Angiogenesis is noticed in the implant as well at the periphery.

Glut BP explants showed thick capsule around the implant. Certain areas showed peri-implant necrosis. Angiogenesis was seen only in the periphery of the implant. Focal mild to moderate chronic inflammation observed as mononuclear cell infiltration could be seen at the periphery of implant. The implant interior was remarkably acellular. Calcium deposit could be observed in many sections.

**Discussion**

In the present study it is attempted to demonstrate that Glutaraldehyde treatment of bovine pericardium induces toxic response which will ultimately affect its' long term performance in clinical situation. This is because of its cytotoxicity, persistent

inflammation, immunogenicity, calcification and lack of remodeling. It is reasoned that decellularisation of bovine pericardium will bring about positive remodeling of the implant which is brought about by its susceptibility to matrix metalloproteinase which promotes intra implant host tissue incorporation, new matrix deposition and angiogenesis besides it low immunogenicity, inflammatory potential and calcification potential. In this study a commercially available clinically used Glutaraldehyde treated bovine pericardium was studied and hence it was not further characterized. Decellularised tissue produced by a non-detergent based decellularisation method was used for comparison. The decellularisation method employed was found adequate as nuclear remnants could not be detected by routine HE staining or by nuclear stain Hoechst 33258. Polyacrylamide gel electrophoresis of residual DNA could not demonstrate DNA bands indicating extensive DNA fragmentation.

The data clearly indicated that the Glutaraldehyde treated bovine pericardium is resistant to Collagenase which is a major enzyme in the body responsible for extracellular matrix remodeling. In the rat subcutaneous implantation model this was seen as acellular areas in the implants indicating absence to tissue penetration into the implant. In comparison decellularised bovine pericardium was seen susceptible to Collagenase enzyme and correspondingly the *in vivo* data showed excellent host tissue incorporation into the implant.

*In vitro* data such as direct cytotoxicity as well as toxicity of extract clearly suggested cytotoxic nature of the Glutaraldehyde treated bovine pericardium. This is clearly correlated in rat implantation studies where it was observed as areas of peri-implant necrosis around Glut BP samples. Whereas Dcl BP samples or its extracts were seen non cytotoxic and subsequently there was absence of peri-implant necrosis in rat study. Intracutaneous irritation study and systemic toxicity also showed that the physiological saline extract of the Glutaraldehyde treated bovine pericardium to be of toxic nature. Interestingly only the water soluble extract was observed as toxic whereas as cotton seed oil extract was seen non toxic. Earlier studies have reported on

the cytotoxic nature of Glutaraldehyde treated tissue without indicating which fraction is cytotoxic [8] . Macrophages as inflammatory cells as well as antigen presenting cells have an important role in *in vivo* response against biomaterials. Hence macrophage activation study was conducted. This study showed a definite increase in the release of inflammatory cytokines such as TNFα and IL1β in Glut BP samples compared to Dcl BP samples. This clearly indicated that the Glutaraldehyde treated bovine pericardium activates macrophages significantly in comparison to decellularised bovine pericardium. Increased inflammatory response noticed in Glut BP samples of rat implantation study might be a reflection what is observed in *in vitro*. Interestingly, IL6 release was seen higher in the Dcl BP sample compared to Glut BP samples. In contrast to the above observations, a study showed no significant variation between in *vitro* cytokine release of inflammatory and anti-inflammatory cytokines in response to different polymeric materials [9] .

*In vivo* data such as antibody response, calcification response and tissue response demonstrated better biocompatibility for decellularised bovine pericardium compared to Glutaraldehyde treated one. For studying the immune response, focus was given on antibody response elicited against extractable protein from fresh pericardium as it was assumed that in processed bovine pericardium the residual protein is the major immunogen. Since there was complete fragmentation of DNA following decellularisation, immunogenicity on this account was not anticipated. DNA fragments less than 300bp is reported to be non-immunogenic [10] . Decellularised bovine pericardium had significantly reduced antibody response compared to Glutaraldehyde treated one. This indicated that Glutaraldehyde treatment is not necessarily immunosuppressive. A study on antigenicity of Glutaraldehyde treated bovine serum albumin (BSA) reported that Glutaraldehyde caused intermolecular cross-linkages forming soluble aggregates; such modified proteins were seen antigenic and produced antibodies against antigenic determinant of BSA as well as against newly acquired groups arising from this modification [11] . The increased

antibody response seen in the Glutaraldehyde treated samples might be due to formation of soluble aggregates of BSA. *In vivo* calcification was seen directly linked to the extent of Glutaraldehyde treatment. Glutaraldehyde treated samples were seen most calcifying as previously reported [12] . Decellularised pericardium had minimum calcification. A previous study has reported direct relation between calcification and antibody response [13] . This relationship was noticed here also. Decellularised pericardium and Glutaraldehyde treated pericardium presented two extreme tissue responses. Glutaraldehyde treated pericardium showed no host cell incorporation into implant and a thicker fibrous tissue capsule separated the implant from the host tissue. Presence of mild to moderate chronic inflammation is noticed in the implant periphery. There is calcification as well as significant antibody response in this group. These conditions may not favor a positive tissue remodeling [10] . Similar observation was previously reported for Glutaraldehyde treated tissue [8] . Decellularised bovine pericardium showed contrasting and desirable tissue response such as thin fibrous capsule, un-detectable calcification, implant degradation with laying of new collagen and mild chronic inflammation. All the above observations points to better implant remodeling [14] . It is assumed that the presence of mononuclear cells in this group should not be of concern since the antibody response elicited in this group was significantly lower compared to Glut BP group. In contrast the observations noted in Glutaraldehyde treated group such as thicker fibrous capsule, implant calcification, moderately intense mononuclear cell infiltration in the periphery and angiogenesis restricted to implant periphery and visible lack of host cell incorporation into implant are not conducive for sustained implant function. Hence the observed made in Glutaraldehyde treated bovine pericardium are not congenial for an acceptable tissue compatibility as well as implant remodeling which is essential for successful long term clinical performance of a surgical implant.

**Conclusion**

Thus it is concluded that Glutaraldehyde treated bovine pericardium incites significant toxic, inflammatory, immune and calcification responses which is not desirable for long term function as an implant. Furthermore Glutaraldehyde also prevents tissue remodeling on account of its resistance to matrix metalloproteinase resulting in the implant remaining as a separate entity in the body with out physiologically integrating into the host.

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Table 1: Residual weights in different groups with time following Collagenase type II digestion.

| Residual weight (mg) | 24 hours | 72 hours | 7Days |
| --- | --- | --- | --- |
| Dcl BP | 4.9±0.4* | 1.8±0.3* | 1.3±0.2 |
| Glut BP | 49±1* | 49±1 | 48±1 |

* Statistically significant at $p<0.05$. Comparison was made between groups and between different time periods of the same group.

**Table 2: Toxic response (*in vitro* and *in vivo*) of Glut BP compared to Dcl BP.**

| **Treatment groups** | **Dcl BP** | **Glut BP** |
|---|---|---|
| **Susceptibility to Collagenase type 2** (*in vitro*) % | 90.2% degradation in 24 hours; 96.5% degradation in 72 hours; 97.4% in 7 days. | 2% degradation in 24 hours, 2% in 72 hours; 4% in 7 days. |
| ***In-vitro* Cytotoxicity** to L929 fibroblast cells | Non- cytotoxic | Moderately cytotoxic |
| ***In-vitro cytotoxicity of extract*** | Non-cytotoxic | Cytotoxic |
| ***In vitro* Macrophage activation**:** | | |
| IL1 beta (pg) | 82.4±4.1* | 144±7.4* |
| IL6 (pg) | 59.1±3.6* | 46±0.72* |
| TNF alpha (pg) | 45.3±6.6* | 267±22.9* |
| **Systemic toxicity** | | |
| Intravenous route | Non toxic | Toxic |
| Intraperitoneal route | Non toxic | Non toxic |
| **Intracutaneous irritation** | | |
| Cotton seed oil extract | Non toxic | Non toxic |
| Physiological saline extract | Non toxic | Toxic |
| ***In vivo* antibody response** to BP proteins (OD) | 0.83±0.08* | 1.11±0.16* |
| ***In vivo* calcification response** (µg/100mg dry weight) | 157±31* | 434±84* |
| **Tissue response** | | |
| Necrosis | Absent | Present |
| Inflammation | Mild to moderate, diffuse | Focal, mild to moderate |
| Fibrosis (peri implant capsule) | Thin | Thick |
| Tissue incorporation | Present | Absent |
| Angiogenesis within implant | Present | Absent |
| Calcification | Not detectable | Focal deposits seen |

*Statistically significant difference at $p<0.05$ **LPS activation caused cytokine release at, IL1beta: 255pg; IL6: 280pg and TNF alpha: 346pg.

Fig: 1: Confirmation of decellularisation with Hoechst 3328 staining. A: Decellularised pericardium with no nuclear remnants, B: Normal pericardium showing intact nucleus. Hoechst 33258, 400X.

Fig.2: Extractable DNA form decellularised bovine pericardium; 1st lane: 100bp DNA ladder, 2nd and 3rd lane: DNA from decellularised bovine pericardium, 4th lane: DNA from fresh bovine pericardium.

Fig.3: A. Glut BP sample showing moderate cytotoxicity. B: Dcl BP sample showing non-cytotoxic nature.

Fig 4: Tissue response to different implants at 60 days in rat subcutaneous implantation; **A**: DCl BP group minimum inflammatory response, uniform host cell incorporation and angiogenesis (arrow) within implant HE 400X. **B:** Glut BP group, inflammatory response (arrow) in the interphase and remarkably acellular interior. HE 200X.

# The Effect Of Modification Of Decellularised Bovine Pericardium On It's *IN VIVO* Response

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## ABSTRACT

### BACKGROUND

Decellularised tissue is considered as an excellent scaffold for induced regeneration but it still remains a relatively new biomaterial whose biological performance is uncertain and not well understood. A host of healing response ranging from scarring to more desirable regenerative response is observed in the decellularised tissue produced from different sources and by different methods.

### OBJECTIVE

In the present study the effect of modification of decellularised bovine pericardium on its *in vivo* response is evaluated with respect to its regenerative potential.

### METHODS

Decellularised bovine pericardium was modified with known biocompatibility enhancers such as amino PEG-PPG (Molecular weight 1900) and oxidized Dextran 40 through Schiff reaction. The chemical modification was confirmed by measuring contact angle and FTIR spectroscopy. The *in-vivo* response of these material such as antibody response, calcification response and tissue response were studied in juvenile rat subcutaneous model for 60 days in comparison to un-modified decellularised bovine pericardium. The *in vivo* study was further done in rat abdominal regeneration model to compare the effect on regenerative potential between the above groups on account of its modification.

### RESULTS & CONCLUSION

The chemical modifications produced distinct *in vivo* response between groups when compared to un-modified decellularised bovine pericardium. PEG-PPG modification had minimum calcification, minimum antibody response and comparable chronic inflammatory response when compared to Dextran-40 modified and un-modified decellularised pericardium in subcutaneous implantation study. With respect to regeneration study in rat abdominal implantation model, only un-modified decellularised bovine pericardium produced induced regeneration evidenced as skeletal muscle formation within the scaffold. Dextran40 modified group showed negative remodeling evidenced by structural weakening of implant and herniation and PEG-PPG modification showed only fibrotic thickening of implant without any new muscle formation. This indicated that although the above modifications of decellularised bovine pericardium improved biocompatibility in conventional sense, this has also resulted in the loss of induced regeneration which is the ultimate goal for such modifications. Moreover these modifications also produced undesirable *in-vivo* effects such as structural failure or fibrotic thickening.

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