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Technique to process xenogenic tissues for cardiovascular implantation – A preliminary report

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Processing of xenograft, a biomaterial in clinical cardiovascular surgery can influence implantation life and efficiency in desired function. Existing xenografts have a limited service time as they undergo degenerative changes. Degradation of xenografts occurs due to several deficiencies in processing and also due to *in vivo* enzymatic digestion and immune response. The objective of this study is to decellularize xenograft tissues and preserve their architecture using an efficient method. This method can produce decellularized tissues that are durable and sturdy to withstand physiological stresses due to circulation during cardiovascular surgeries. Porcine valves (40 pulmonary and eight aortic), bovine jugular veins (57 with and 41 without valves) and 29 bovine pericardia were treated chemically with different methods of decellularization. Anti-calcium treatment was performed to reduce mineralization and further processing of tissues was performed to prevent blood-protein seepage in the biological tissues during blood circulation. The most effective decellularization process was determined by a microscopic examination of the decellularized tissues. One per cent DCA and enzymatic treatment for 50 and 15 h respectively, decellularized the xenografts in an efficient manner. This decellularization along with enzymatic treatment was found to produce good results in comparison with other detergents. This can make them a suitable xenograft tissue for clinical use. Animal experiments can provide evidence of autologous cell seeding in the same processed tissues after implantation has taken place. These experiments can prove the ability of the tissue to grow cells in individuals inside whom the grafts are to be implanted.

Keywords: Anti-calcium treatment, calcific degeneration, decellularization, xenograft.

ALLOGRAFTS and xenografts have clinical advantages over non-biological synthetic biomaterials. Since allografts are rarely available, dependence on xenografts as implants during surgeries has grown exponentially over the years. Heart valves made of xenografts like porcine valve tissue

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or bovine pericardium, have the advantage of low incidence of thrombogenicity without anti-coagulation[1]. These valves have near normal functional accuracy like the native valves. However, valve failure with structural dysfunction due to progressive tissue deterioration, including calcific degeneration and noncalcific damage is a serious disadvantage, which undermines the advantages of tissue valve substitutes. It has been reported that though xenografts are considered to have more physiological functions like native tissue, its early degeneration and short life span discourage their use[1–3]. None of the commercially available bioprosthetic heart valves mimics the native valve and has a long service life withstanding stresses like the native valve[1,4]. Surgical replacement of the malfunctioning aortic valve with an allograft or another mechanical prosthetic valve is an alternative to the bioprosthetic valve[2]. Therefore, it is important to process biological tissues, in such a manner that the above-mentioned problems do not hinder their clinical use.

Xenograft tissue processing for clinical use has to cover many requirements, which would make it suitable for implantation in humans. It has been reported that calcific degeneration of bioprosthetic materials plays a major role in the failure of bioprosthetic and other biological substitutes[1,2]. Calcium contained in extracellular fluid reacts with the membrane-associated phosphorus to yield calcium phosphate, which mineralizes on the cell membrane[1,5,6]. Cellular residues found in tissues treated with glutaraldehyde (GA) primarily initiate tissue calcification. It has been reported that acellular tissue matrices can be produced by removing cellular components completely, which can prevent calcification in biological tissues through chemical, enzymatic and mechanical means[7].

Antigenicity due to the presence of native cells can be alleviated by decellularization, which is supposed to be a major factor for calcific degeneration and at the same time anti-calcium treatment would reduce the degenerative changes as a whole[2,8]. Prevention of blood protein seepage in the collagen matrix also plays a major role in preserving the life of biological tissue[9]. The objective of this study was not only to make xenograft tissues acellular, but also to keep the tissue architecture intact so as to increase durability and withstand stresses during blood circulation.

Porcine valves (40 pulmonary and eight aortic), bovine jugular veins (57 with and 41 without valves) and twenty-nine bovine pericardia were treated chemically with different detergents of decellularization. Tissue processing procedures were performed with various detergents and enzymes (in combination or sequential manner or in isolation) like formalin, ethyl alcohol, heparin, sodium do-decyl sulphate (SDS), DCA, tert-octylphenyl-polyoxyethylene (TritonX 100) and aluminium chloride. Preliminary experiments were conducted on few of the above tissues to standardize a set of procedures which can make these tissues substantially acellular. After determining a sound method of decellularization, further tissue processing was performed to maintain the sterility and prevent further calcification.

Tissue processing in our study was performed in a sequential manner. Several methods of decellularization as reported by various groups[10–15] were used in our study. Decellularization of allografts and subsequent cryopreservation were reported[16]. Cell-extraction techniques are done sequentially using detergents[10,11] and multistep-detergent-enzymatic procedures to create acellular grafts have been reported[8,12,13,17]. Multistep processing using detergents can ensure that tissues are not only acellular, but can improve their life and performance when used as implants.

Detergents like 1% SDS, 1% DCA and 1% TritonX 100 were used separately (with or without enzymatic digestion) for decellularization. The SDS and TritonX 100 combination[14,15] was not tried, but TritonX 100 alone and also with enzymatic digestion was used in the decellularization technique. Distilled water and Denacol (poly epoxy compound) treatment[18] was also used as a method of decellularization of tissues. In order to standardize the procedure, the detergents used as well as the enzymatic digestions were made to vary in the concentration along with variation in the time period of emersion of the concerned tissues in these chemicals. This procedure was executed under continuous shaking in a shaker. As a baseline before any treatment, basic tissue histology was done to compare with the histological examination findings after processing. Histology using haematoxylene and eosin, and by Van Gieson stains was performed under light microscope with 10 and 40X magnification.

Decellularization was followed by collagen cross-linking and sterilization using 10% formalin in stages. This was subsequently followed by anti-calcium treatment and preservation using 70% ethyl alcohol. Heparin treatment can prevent blood protein seepage in the decellularized matrix, as it conjugates with the collagen through various receptors[9]. Decellularized tissues were split into two groups and another step of collagen stability by treatment with heparin was performed on both tissue groups. Group 1 consisted of processed tissues which were preserved in 70% alcohol, while group 2 consisted of fresh decellularized tissues. Group 1 products were taken out of ethanol preservation and immersed in 100 units/ml of heparin in balanced salt solution to prevent blood protein seepage in xenograft collagen matrix when in contact with the circulating blood and were later placed in a preservation jar. Group 2 of fresh decellularized tissues was subjected to heparin treatment, followed by collagen cross-linking with 10% formalin in stages for 15 h.

This process was followed by an anti-calcium treatment and final preservation in 70% ethyl alcohol (Figure 1 *a* and *b*). Initially, various detergents at different concentrations with or without enzymatic digestion were used in the decellularization procedure.

GA treatment and preservation has been reported to cause collagen cross-linking that is responsible for calcific degeneration and tissue fatigue[19,20]. Calcification can be accelerated by age, GA fixation, and also mechanical stress

on the implanted tissue[1]. Recently, it has been reported that pathological calcification, like bone mineralization is controlled by inductive and inhibitory factors[2]. The most promising preventive strategy for calcification is performed using tissue cross-linking agents other than GA and removal or modification of components responsible for calcification[2,21]. On the contrary, few reports suggest that higher concentration of GA may help in reduction of calcification[19]. Replacing GA with 1-ethyl-3-(3-dimethylaminopropyl; EDC) as a storage solution after aldehyde-capping with alpha amino oleic acid, considerably mitigated the chances of calcification in freestyle Medtronic valves[21]. It has been reported that calcification can be prevented by performing an additional Jeffamine (polypropyleneglyco-bis-aminopropyl ether)/EDC cross-linking step[21].

Tissue becomes toxic to endothelial cells due to GA treatment[19], and there is a possibility that autologous cell seeding may not happen in the implanted xenograft. Considering the above arguments, GA treatment was avoided in our study and 10% formaldehyde was used in staged concentration for 15 h to make the tissues devoid of all infections. Retro viruses in porcine tissues and bovine spongiform encephalopathy (BSE) producing viruses in bovine tissues were the major concerns and formaldehyde treatment removes the chance of any microbial infection. Before standardizing the 10% formalin strength, experiments were performed with 4% formalin, which resulted in faster tissue denaturation. As the tissues are acellular, presence of live viruses is unlikely.

Immune response could result if any cells are leftover after the decellularization process. It has been reported that using cryo-preservation technique leftover cells would be preserved, which might cause immune response[22]. It has been also reported that cryo-preserved homografts are more immunogenic than stented GA-treated xenografts[23], but they survive longer, which indicates that the immunologic process is not the pivotal factor leading to failure of implant while comparing these two valves. However, while examining the cause of homograft failure, there is substantial evidence that cryo-preservation preserves endothelial and smooth muscle cells and enhances viability and antigenicity[16,24]. Short time period between homograft retrieval and cryo-preservation enhances viability, antigenicity and failure rate[25]. It seems that the immunologic reaction rather than cryo-preservation process is responsible for the degenerative process occurring in cryo-preserved homografts[16].

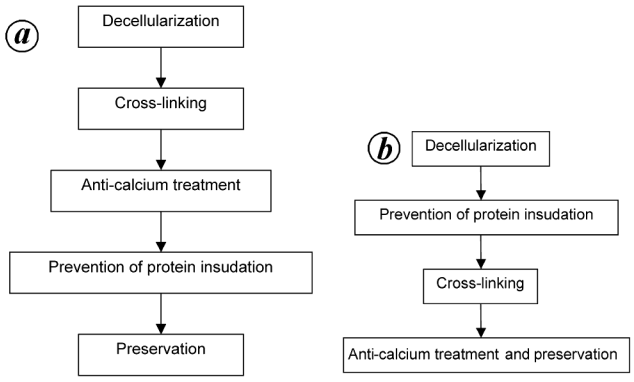

Figure 1. Sequence of processing of group 1 (*a*) and group 2 (*b*) xenogenic tissues.

Seventy per cent ethyl alcohol[26,27], an anti-calcium agent, has been chosen as a preservative for these processed tissues in our study. This had been decided and implemented after using different procedures for preservation. Tissues were treated with aluminum chloride and ethyl alcohol simultaneously by hydraulic separation as one of the anti-calcium measures of arteries, such that aluminium chloride remains only in contact with the outer wall of the blood vessel. Peracetic acid treatment along with alcohol is reported to remove any viral infection in the xenograft, but the toxicity effect of peracetic acid has not been established[28]. As this technique is in the experimental phase, our study did not use this chemical for treatment. Finally, the products were sterilized using gamma rays, according to the International Atomic Energy Commission stipulation.

SDS did not remove any cell nuclei but it denatured the tissue, and the treatment was not repeated. Enzymatic digestion was conducted along with detergents and also sequentially. Effectiveness of decellularization was satisfactory when they were used sequentially. Tert-octylphenyl-polyoxythylen for more than 24 h followed by the same enzymatic digestion separately denatured the tissues with partial decellularization. DCA treatment for 50 h gave adequate decellularization keeping cellular architecture intact, along with separate enzymatic digestion as shown in Figures 2–4.

The above result was more evident in the thick-walled vessels. Bovine pericardium decellularization even with lesser time of immersion in DCA (24 h) was satisfactory. TritonX 100, individually and also with enzymatic digestion, resulted in partial decellularization with less preservation of tissue architecture. Distilled water and Denacol procedures were not as effective as DCA. Thus DCA was identified as the preferred detergent and subsequently other steps were followed. Tissue texture of group 2 was soft and elastic in comparison with group 1, even after completion of the whole procedure up to preservation.

Preservation of tissues by immersing in 4% formalin did not provide clinically satisfactory results. Similarly, anti-calcium treatment of tissues using a combination of ethyl alcohol and aluminium chloride resulted in tissues having a hard texture, and hence the treatment was not repeated. Seventy per cent ethyl alcohol as a preservative kept the tissues sterile, pliable and easy-to-handle. Each step of processing in both the groups had inherent sterilization property which took care of the infectivity of the materials. These materials have been checked for post-processing sterility and kept on culture for 6 weeks with negative culture growth.

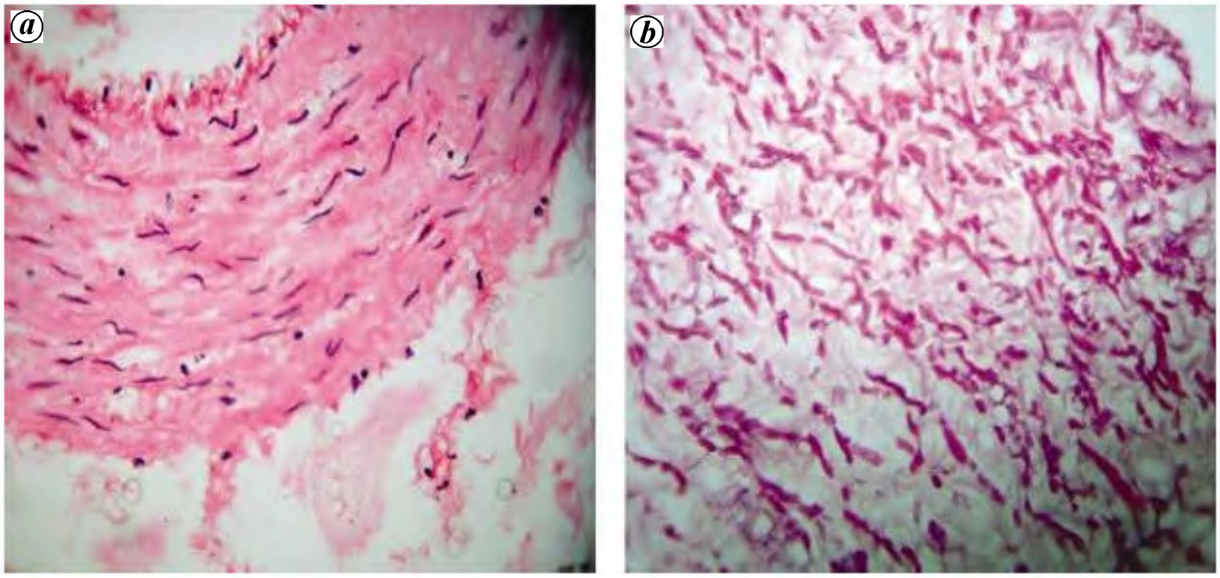

Figure 2. H&E staining of a cut section of BJV with 40X magnification. ***a***, BJV before processing showing nucleated cells; ***b***, BJV after processing showing acellular matrix.

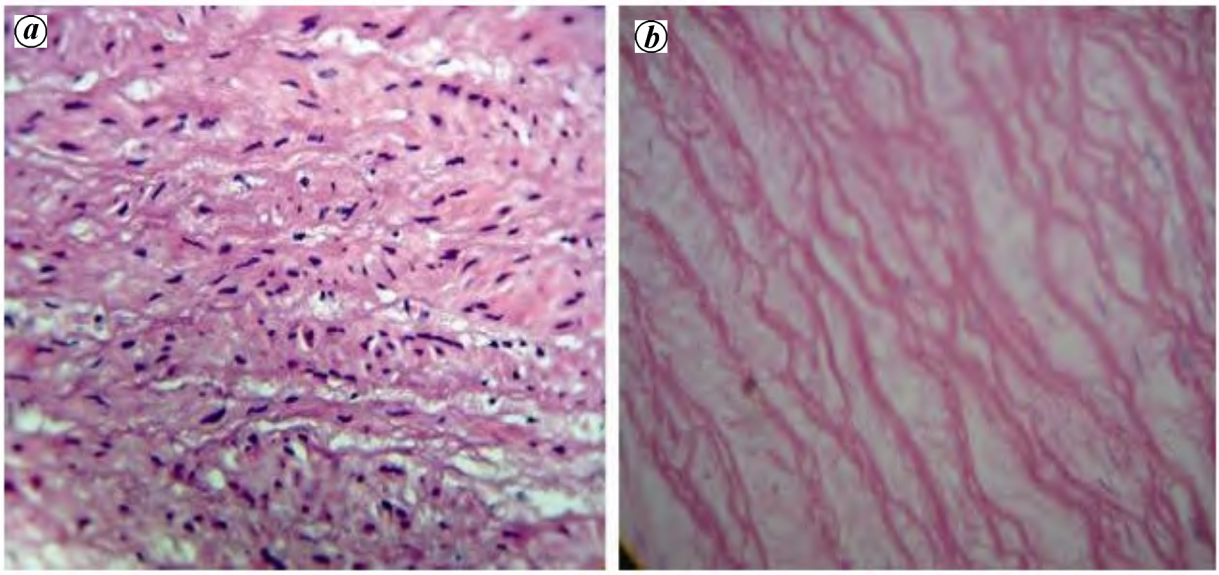

Figure 3. H&E staining of a cut section of porcine pulmonary artery with 40X magnification. ***a***, Porcine tissue before processing showing nucleated cells; ***b***, Porcine tissue after processing showing acellular matrix.

The objective of this study was to standardize a treatment method for decellularization of xenografts and to keep the architecture intact. This can ensure durable and sturdy tissues, which will be able to bear the stress of blood circulation when used as implants in cardiovascular surgeries. Several methods for treating xenografts have been reported in the literature. However, a standard treatment for xenografts to give the graft near-normal life and physiological functioning ability has never been achieved[1,2]. This study used different methods of decellularization that have been reported in the literature, to determine a standard treatment for different xenografts. Keeping in mind the probable complications of commercially processed xenograft materials for clinical use, this study has tried to protect the tissue regarding all aspects.

Efficacy of treatment and enzymatic digestion were used to judge the effective procedure of decellularization. It is evident from the results of our study that 1% DCA was the standardized decellularization procedure. Indwelling time period of tissues in these chemicals was also identified after several trials. After decellularization, group 2 tissues had undergone heparin treatment in balanced salt solution not only to make them antithrombogenic, but also to prevent blood-protein seepage through the interfibrillar space. Heparin treatment just after decellularization gave a better texture to these tissues than the group 1 tissues, which

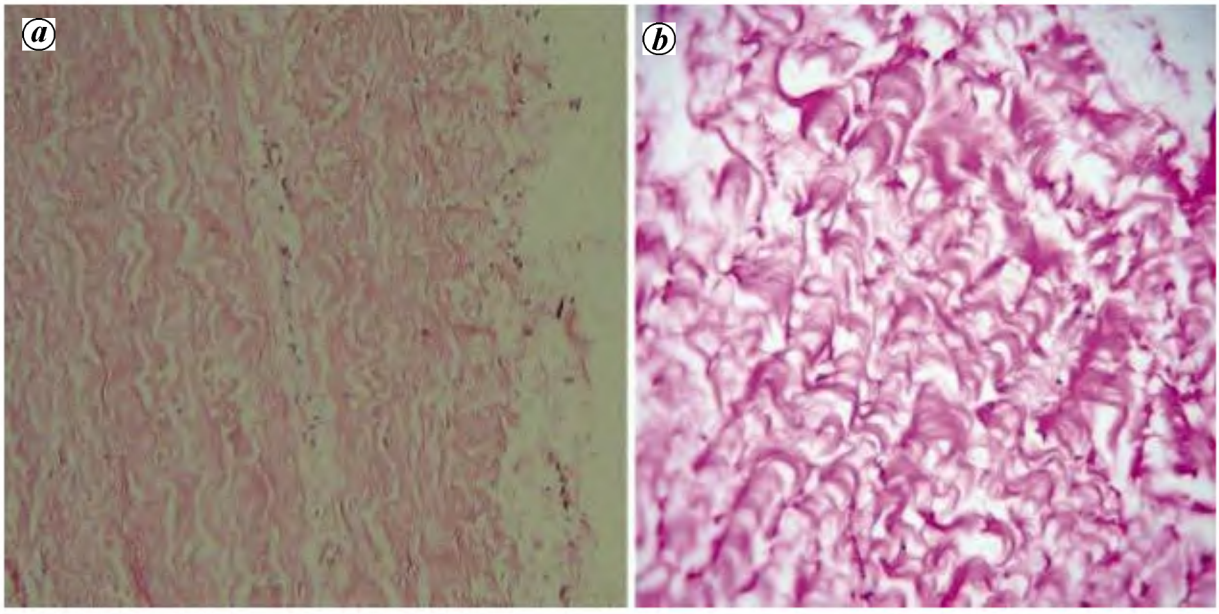

Figure 4. H&E staining of a cut section of BP with 40X magnification. ***a***, BP tissue before processing showing nucleated cells; ***b***, BP tissue after processing showing acellular matrix.

had heparin treatment after preservation in alcohol. However, this tactile observation of tissues, which can be subjective, was later confirmed by results of high-resolution optical microscopy.

Heparin treatment prevents blood-protein seepage, as heparin conjugates with the collagen through various receptors[9]. The lack of epithelial or endothelial barrier allows circulating blood protein to enter through the interstitial spaces of the collagen network. Protein seepage and accumulation causes solidification and thus becomes a hindrance to the free intra and interfibrillary movements. This is a major contributory factor for collagen breakdown with fibrillary ruptures. Moreover, protein impregnation is regarded as an important source of lipid formation, mineralization and late calcification of materials.

Heparin has a high affinity for collagen and forms a strong ionic bond with tissue collagen. This is a well-documented reaction of heparin, thus forming an artificial matrix of heparin–protein complex[9]. Heparin-treatment was followed by formalin cross-linking to prevent viral contamination of tissues and to make it nonantigenic. Formalin concentration was varied with time in order to achieve the desired results. Formaldehyde as the cross-linking agent might have endothelial cell toxicity. Subsequently, these acellular tissues were preserved in 70% ethyl alcohol and hypothetically, it could be expected that such toxic effect was counteracted.

Animal experimentations with these processed tissues were performed by following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India, and obtaining due approval from the Animal Ethical Committee of the institution. Madras red breed sheep were used for experiments. Apparently no local inflammatory response was noticed at the site of implantation in the immediate post-operative period. No evidence of pyrexial reaction and no drop in haemoglobin level were observed. Clinically, animals had the same vigour as they were in during the pre-operative period, i.e. with good appetite and urine output. Only two bovine jugular veins (BJV) which had been implanted as interposition grafts at the left internal jugular vein of the sheep were explanted and partial endothelialization had been noticed in the lumen of the explanted BJV. However, these two belonged to group 1 and the sheep were not on anti-platelet therapy. So far 13 experiments have been performed with groups 1 and 2 tissues and detailed long-term results of the comparative study are awaited. Accountability of mineralization by von Kossa stain and calcium estimation can provide a comparative study between the two groups of tissues.

Fluoroscopy reports confirm that these two explanted specimens did not show calcified opacification, which is a qualitative result of detection of calcification. No inflammation, aneurysm or dystrophic calcification has been noted for the acelluar matrix vascular prosthesis[12] and complete reendothelialization of grafts in canine has been reported[11]. In a marked difference, this study found partial endothelialization of the acellular jugular vein lumen, which is suggestive of autologous cell seeding. Autologous cell seeding is the eventual goal of our study. It is expected that post decellularization, heparin interaction along with anti-calcium measures in 70% alcohol would prevent tissue degeneration. *In vitro* cytotoxicity test of the processed tissues was performed and it was found that they were nontoxic.

Finally the products are preserved in 70% ethyl alcohol, which is considered to be an anti-calcium agent apart from its anti-infective and preservative properties[26].

SEM and TEM of these tissues can provide details about the surface physical properties and collagen conformation. In future, Fourier transform infrared spectroscopy and differential scanning calorimetry to assess collagen property and stability in both the tissue groups can be performed.

Decellularization of tissues using 1% DCA and separate enzymatic digestions has produced clinically satisfactory results than using detergents like SDS, TritonX 100 or polyepoxy compound (with or without enzymatic digestion). Microscopically, these tissues showed satisfactory decellularization with intact architecture. This structural integrity was not observed using other procedures. It was also observed that decellularization was partial in the case of other detergents. Tissues processed using this procedure have been implanted in animals and results on *in vivo* performance are awaited.

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