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360° in Making Acellular and Biocompatible Xenografts for Surgical Applications

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Abstract

There goes a famous saying - "We can judge the heart of a man by his treatment of animals". Now these animals help a man save his heart and vital organs through xenografts. The concept for xenograft is an explosive phenomenon in regenerative and tissue engineering. It has a wide application in many fields of medicine-Cardiology, Orthopedics, Dentistry, Gastrointestinology, Opthalmology and Dermatology. This comprehensive review presents in detail the current methods and procedures followed in the preparation of a xenograft. It helps us in the optimal selection of a suitable method the potential barriers and challenges faced during processing and clinical application with a view towards the future direction.

Keywords: Xenograft; Decellularization; Cross-linking; Detoxification

Introduction

Grafting is the fundamental element on which the magnanimous fields of regenerative and transplantation medicines have been laid. Grafts differ from flaps on the absence of their own blood supply. The basis of grafting is directed differentiation. The four main categories of grafts based on their origin can be–Autograft (from same person), Isograft (between genetically identical twins), Allograft (between same species), Xenograft (between two different species).

Xenotransplantation refers to any procedure that involves the transplantation implantation, or infusion in to a human recipient of either 1) live cells, tissues, or organs from a nonhuman animal source or 2) human body fluids, cells, tissues, or organs that have had *ex-vivo* contact with live nonhuman animal cells tissues or organs The potential targets for xenograft harvesting are–Chimpanzee, baboon, pig and cattle. Xenografts are widely used for tissue engineering applications. Of these, porcine and bovine sources have vast applications (Figure 1). They are less expensive and readily available than homografts and allografts. The porcine dermis, pulmonary artery, aorta, submucosal membrane and bovine bone, pericardium, jugular vein are popular sources of tissue harvesting. Acellular xenografts have wide application in several fields like;

- **Cardiology:** Bioprosthetic heart valve constructed from bovine pericardial leaflets or porcine pulmonary valve are widely used in cardiac surgeries [1,2] .The valvular defects, aneurysm repair and the septal defects can be repaired with xenografts valves and pericardial patches.
- **Orthopedics:** Demineralized and deantigenized xenograft bone matrix can be used for orthopedic applications [3]. Likewise Krackow Achilles tendon repair can be augmented with use of xenograft like porcine dermal matrix [4].
- **Dentistry:** Xenograft is also used in dentistry for reconstruction of defects or replacement of missing teeth and can be used in combination with autogenous grafts [5].
- **Gastrointestinology:** Acellular xenograft has been used for hernia repair. The occurrence of infection, wound complication, gastrointestinal complication and recurrence of hernia is much lesser with xenograft mesh implants in comparison to synthetic mesh [6].
- **Ophthalmology**: It can also be used for complex scalp defects and eyelid defects [7]. The possibility of implanting decellularized porcine cornea as a xenograft in place of a defective cornea is being explored. Bovine pericardium has fewer complications when used as a wrap for hydroxyapatite implants to enable attachment of extraocular muscles in the artificial eye [8].
- **Dermatology:** Porcine skin has similar composition, pain reducing traits, hemostasis function and collagen content as the human skin [9]. It is used in cosmetology and burn wounds to ameliorate scar formation and infection. It can also be used to patch up an area after tumor removal, reducing chance of infection and fluid loss. In deep burns and scars, application of meshed acellular xenograft and the split-thickness skin autograft can induce tissue regeneration [9,10]. The application of xenograft provides a micro-environment that initiates migration and proliferation of epithelial cells. There are many commercially available xenografts in ready-to-use form like Apligraf® (skin construct comprised of neonatal foreskin keratinocytes and a matrix made from bovine collagen and fibroblasts), Integra® (bilaminate dermal substitute composed of an outer silicone layer and an inner layer made of cross-linked bovine collagen-glycosaminoglycan matrix) [11,12].
- Furthermore, use of Fetal Bovine Acellular Dermal Xenograft with tissue expansion has been currently implicated for Staged Breast Reconstruction (Pectoral extender), oncological hypopharyngeal defects reconstruction and interposition grafting in urological studies [13,14].

However pretreatment and intensive processing is necessary to remove antigenic factors, to reduce the rate of tissue rejection and inflammatory response triggered by the heterograft. The review deals with the processing of the xenograft starting from the collection of sample, decellularization, fixation and detoxification making it fit for clinical application.

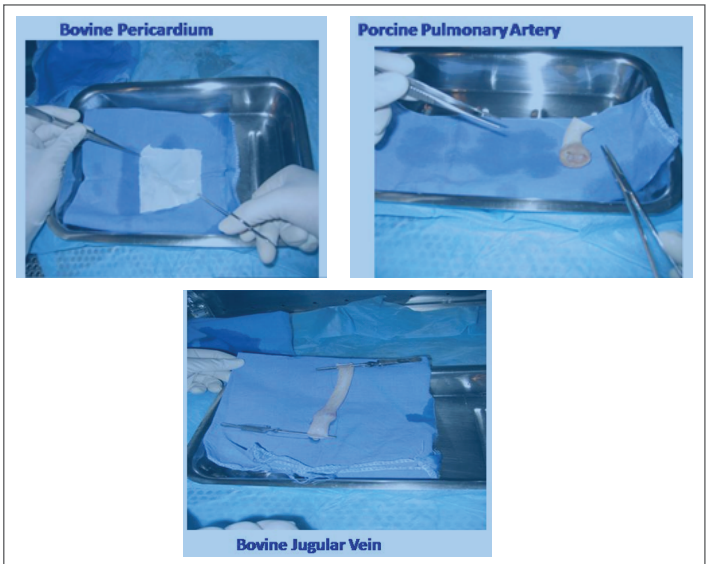

Figure 1: Xenograft products

Tissue harvesting and transportation

The animals selected for tissue harvesting are pre screened for zoonotic diseases and other risk factors. Desired tissue is collected under strict asepsis. The primary factor determining the success of this harvesting depends on the transport media. An ideal transport media should fulfill the following needs-maintain the viability and pH of cells, nourishment, inhibit microbial growth. However the choice of media mainly depends on the nature of tissue and the time of transportation. Some of the widely used media in various time-line are listed below for optimal selection.

Jee et al. [15] used Hanks' Balanced Salt Solution (HBSS) to store bovine pericardium at 4°C along with antibiotic cocktail of Cifran, Gentamycin, Streptomycin, Cephalosporin, Amphotericin B.

Balasundari et al. [16] used HBSS with cocktail of antibiotics like Cefuroxime, Gentamycin, Ciprofloxacin, Vancomycin, Amphotericin to transport the tissues at 4°C.

Phosphate buffered saline (PBS) with antibiotics like penicillin and streptomycin has been used to transport porcine aortic valve conduits [17].

Lee et al. [18] used 0.1M PBS pH 7.4 for the transportation of bovine pericardium.

0.9% Cold saline can be used to transport tissues from the slaughter house to the destination [19-22].

Roswell Park Memorial Institute medium [RPMI] can also be used as a transport media for the graft [23].

Golan et al. [24] used ice-cold RPMI to transport bovine fetal and human intestinal tissue.

Histocon is used as preservative-transport medium at 0-4°C. It is a cryoprotective and membrane-stabilizing agent [25].

Thomas et al. [26] had studied the efficacy of filtered coconut water as a transport medium, for avulsed tooth. It is sterile, containing a varied cocktail of sugars, amino acids, minerals, electrolytes. The preserving efficacy was compared with HBSS and milk. Results denote that coconut water is a better transport media compared to HBSS when storage is for more than 15 min and milk was inferior to HBSS [27].

The aim should be to deliver the tissue at the lab within 48 hours. The choice of media and additives added can be modified based on individual needs. However normal saline with antibiotics can be used if the transport time is less than two hours.

Decellularization

Decellularization is the initial step in preparing a xenograft. It involves removal of cells and debris present in the native tissue that might create an imbalance in calcium transport, increasing the intracellular calcium concentration. Which triggers Ca_2^+ ions binding to cell membrane phospholipids and form calcium phosphate crystals. Thus tissue decellularization reduces *in-vivo* calcification a critical phenomenon in surgical application [28]. Furthermore, it also removes the cellular antigenic components responsible for immunorejection specifically α-Gal and non-α-Gal T antigens [29].

Decellularization of graft tissues are generally performed using Sodium Dodecyl Sulphate, Triton X 100, Sodium Deoxycholate, Nuclease and Trypsin. However they are harsh to the tissues and even trace quantity remains in the tissue even after repeated wash. It has been reported that, these the trace quantity of detergens potentially impair the efficacy of the xenograft in surgical implantation [30]. Furthermore, Trypsin has been deduced by Yu et al. [17] and Zou et al. [31] to be harsh for the tissue. Repeated quantification of decellularizing agents is a critical step in processing Xenograft. Optimal selection of a suitable method is also vital in this process.

Successful decellularization should maintain elasticity of extracellular matrix [ECM] similar to original tissue and not cause structural or architectural changes in the graft tissue and should be reduced cytotoxic in nature. To achieve this various protocols have been adopted, they are listed briefly in table 1 [8,17,21,28,29,31-35], which aid in selection of suitable method for processing.

Cross-linking

Grafts are cross-linked to enhance mechanical stability. Interestingly, fixation also reduces immunogenicity of the tissue as it cross-links and masks the antigenic factors [36]. In relation to this here we details different cross linking agents current used in the processing and their advantages and disadvantages. Their relative benefits typically aid in selection better cross-linker

Glutaraldehyde: Glutaraldehyde is a dialdehyde that reacts with the amino group of lysine and hydroxylysine moieties to form cross links [37]. It is the most commonly used cross-linking agent that is stable and most effective to fix tissues. In addition, it also acts as a sterilizing agent against bacteria, virus and fungi. However, glutaraldehyde has several shortcomings as discussed in table 2 [15,18-21,29,32,34,38-47]. It is unable to stabilize all components of extracellular matrix of tissues, especially elastin and glycosaminoglycans [GAGs] [48]. Moreover, the residual unbound aldehyde groups can trap host plasma calcium contributing to tissue calcification [49]. The inflammatory response initiates activation of macrophages, which in turn triggers expression of matrix metalloprotein-9 [MMP-9] and tenascin-C [TN-C]. TN-C elevates alkaline phosphatase expression leading to calcification of heart valves [37,49]. In spite of these drawbacks, it is preferred due to long-term tissue stabilization and properties of preserving and sterilizing graft tissue.

Genipin: It is a naturally occurring cross-linker that is derived from geniposide, a compound isolated from fruit Gardenia jasminoides. It is less cytotoxic than glutaraldehyde. Genipin reacts spontaneously with amino acids or proteins to form dark blue pigments, thereby the aesthetic appearance is compromised [32].

1-ethyl-3-[3-dimethylaminopropyl] carbodiimide [EDC]: EDC catalyses the reaction of carboxylic acid with amine groups to form amide bonds via an O-acylisourea intermediate. The addition of N-hydroxysuccinimide [NHS] to EDC increases cross-linking efficiency [39]. Storage of the cross linked tissues in 25 mmol/L EDC reduced calcification in comparison to storage in glutaraldehyde [37].

S.No	Chemical/ Method	Concentration	Xenograft tissues	Outcome	References
1	Sodium Dodecyl sulfate [SDS]	0.1% SDS	Bovine pericardium	Complete decellularization, intact collagen ECM maintained	[28]
		0.10% in Tris buffer with nuclease for 48 h	Porcine aorta	Complete decellularization, ECM structure, hyperelastic mechanical integrity maintained.	[31]
		0.25% for 24 h at RT with osmotic shock	Bovine pericardium	Complete decellularization, intact collagen ECM, degree of cross-linking maintained, no protein denaturation	[33,35]
		0.5% in HEPES pH 7.4, for 24 h at 25°C	Bovine pericardium	Complete decellularization, T_d not affected, stress at break and thickness increased	[34]
		0.5% in PBS for 24 h at 37°C	Bovine pericardium	Collagen fibers became edematous, fragmented, and disorganized, nondetectable presence of carbohydrate antigen α-Gal, and T-antigen	[29]
2	Triton X [TX]	1.0% in HEPES pH 7.4 for 24 h at 25°C	Bovine pericardium	Mean thickness reduced, increase in T_d Mechanical strength of graft maintained	[34]
		1.0% with 0.2% EDTA and nuclease in 10 mM Tris buffer at 37°C	Porcine aorta	Complete decellularization, ECM structure maintained, thickness reduced, hyperelastic mechanics integrity maintained	[31]
		0.9% in PBS for 48 h at 37°C	Bovine pericardium	Collagen fibers were fragmented, but organized. Nondetectable presence of carbohydrate antigen, α-Gal, and T-antigen	[29]
3	SDS+Triton X	0.25% SDS pH 8 for 24 h followed by 0.5% Triton X-100 for 24 h	Bovine pericardium	Completely decellularized, significant reduction of DNA in the treated graft	[21]
4	Triton X+Sodium deoxycholate [DCA]	0.5% triton X and 0.5% DCA in PBS	Bovine pericardium	Complete decellularization, ECM architecture and glucosaminoglycan content maintained, nondetectable presence of carbohydrate antigen, α-Gal, and T-antigen	[29]
		0.25% Triton X and 0.25% DCA at 37°C	Porcine aortic valve leaflets	Complete decellularization, no structural change or fractures	[17]
5	DCA+ Nucleases	1% sodium deoxycholate for more than 40 h followed by nuclease treatment	Bovine pericardium and porcine pulmonary artery	Complete decellularization, no elastic fiber distortion, configuration of collagen type I and type IV maintained, fibronectin and laminin structure unaffected, GAG content retained.	[35]
6	Nuclease [DNase+RNase]	40 µ/ml DNase and RNase for 30 min	Porcine cornea	complete decellularization	[8]
7	DCA+Trypsin	0.05% trypsin for 12 h [with 0.02% EDTA] and 1% DCA for 24 h [with RNAse and DNAse] at 37°C	Porcine aortic valve leaflets	complete decellularization, structural changes observed [fibers loosely arranged], presence of small amount of cellular debris	[17]
8	Trypsin	0.5% trypsin with 0.2% EDTA and nuclease in hypotonic Tris buffer for 48 h at 37°C	Porcine aorta	Complete decellularization, ECM structure distorted and fragmented, showing breakage of crosslink's and fibers	[31]
9	Freeze-thaw cycles	3 cycles: -196°C liquid nitrogen for 30 min, thawing at 37°C for 30 min	Porcine cornea	complete decellularization, minimal damage to ECM	[8]

Table 1: Detergents and chemicals involved in decellularization.
RT: Room Temperature; Td: Thermal denaturation temperature; EDTA: Ethylenediaminetetraacetic acid

Epoxide crosslinker: Alkyl polyepoxides have been used to crosslink tissues. However tissue penetration is low as the water solubility is less and require organic solvents. Polar polyepoxide compounds like triglycidyl amine are water soluble and should therefore penetrate tissues easily and be biocompatible [46].

Neomycin Sulphate: Cross linking of grafts with glutaraldehyde compromises the architecture of GAG and elastin, as it stabilizes only the collagen component. These components are lost during processing or storage of the tissue. To preserve the GAG content, Raghavan et al. [50] devised a protocol using Neomycin sulphate, a hyaluronidase inhibitor, preventing enzyme-mediated GAG degradation.

Dye mediated photooxidation: It does not require any harsh chemicals. In the presence of a suitable photosensitizer [Methylene blue, methylene green and rose Bengal dyes, certain amino acids can be oxidized by irradiation with visible light. Therefore, dye-mediated photooxidation treated grafts holds promise as a long term implantable biomaterial [47].

Several methods of cross-linking have been explored to overcome the drawback of glutaraldehyde. In certain studies glutaraldehyde was replaced with silk fibroin and chitosan (1:1) cross-linkage with irradiation (electron beam). It reduces the risk of calcification and cytotoxicity. However it continues to be the most widely used agent to cross-link the tissues. It is highly recommended to develop a suitable method without using harsh chemicals. The details of cross-linking agents are detailed in table 2.

Detoxification

Formation of calcium-containing mineral deposits could result in cusp or vessel stiffness, loss of pliability, and blockage of the valve and could be attributed in part, to the toxic effect of cross-linkers [51]. In addition, the phosphorus group in phospholipids can bind to Calcium in extracellular fluid and initiate calcium phosphate crystal formation.

Neutralization of residual aldehyde groups: Pre-treatment of tissues with glutaraldehyde releases residual aldehyde groups due to polymerization. These residual aldehyde moieties enhance rate calcification of the xenograft and are cytotoxic [18,29]. Compounds like α-amino oleic acid is highly hydrophobic and caps the residual aldehyde groups in glutaraldehyde-cross linked tissues, reducing calcification

S.No	Chemical/ Method	Concentration	Xenograft tissues	Outcome	Disadvantages	Reference
A	Glutaraldehyde [GA]	0.2% in PBS in MES at RT	Porcine aortic root	Cross-linked graft	Broad calcium bands	[38]
		0.25% in 0.05M HEPES, pH 7.4 for 3 days at RT	Acellular Bovine Pericardium	Effective crosslinking, good mechanical strength, resistant to enzyme digestion	Colour change to dark yellow, Crosslink restrict impermeation of space fillers. Cytotoxicity high	[21]
		0.5% for 3 days at RT	Bovine Pericardium	Good cross-linking	High levels of inflammatory cells observed, increases risk of calcification	[32]
		0.5% in PBS pH 7.4 for 14 days at RT	Bovine Pericardium	Stable bonds formed indicated by decrease in lysine and histidine content	Residual free aldehyde groups of GA are cytotoxic and can result in tissue calcification	[18]
		0.5% in HEPES pH 7.4 for 2 days at RT	Acellular Bovine Pericardium	Stable crosslink of tissue	Cytotoxicity highest with GA-fixed tissue, high calcification noted and collagenous fiber degeneration observed	[39]
		0.6% in 50 mM HEPES pH 7.4 for 5 days	Acellular Bovine Pericardium	Crosslinked tissue	Generation of free aldehyde, resists enzymatic degradation	[29]
		0.6% in HEPES pH7.4 overnight at 22°C followed by 0.2% GA for 2 days	Bovine Pericardium	Stable crosslink of graft	Rigid and fragile graft, macrophage infiltration, expression of MMP-9 and TN-C	[19]
		0.6% in HEPES pH7.4 overnight at 22°C followed by 0.2% GA treatment for 2 days	Porcine aorta	Resistant to collagenase digestion indicating stable crosslink with aortic collagen	Elastase-mediated degradation is high, as GA does not stably bind to elastin	[40,41]
		0.6% in 50mM HEPES pH 7.4 for 24 h followed by 0.2% GA in HEPES for 6 days	Porcine aortic valve cusps and aortic wall	Stability of collagen	Significant loss of GAGs, hence increase in depth of buckling.	[42]
		0.625% in HBS for 24 h at 25°C	Acellular Bovine Pericardium	High crosslinking index, low residual amino group content	-	[34]
		0.625% in PBS pH 7.4 for 7 days at 4°C	Bovine Pericardium	Crosslinked tissue	Calcification observed in the graft tissue	[20]
		0.625% in PBS pH 7.4 for 10 days at 4°C	Bovine Pericardium	Cross-linking of tissue on reaction with amino acid residues of collagen	GA polymerizes and unreacted aldehyde groups present as remnants	[15]
		0.3125%, 0.625%, 1.2% increasing time of exposure 5, 10, 20, 30 min	Porcine pericardium	Crosslinked tissue	Higher calcification observed with increasing concentration; Increasing treatment time *decreases* calcification	[43]
		0.3125%, 0.625%, 1.2% increasing time of exposure 5, 10, 20, 30 min	Bovine Pericardium	Crosslinked tissue	Higher calcification observed with increasing concentration; Increasing treatment time *increases* calcification	[43]
		0.2% GA comparison with 0.6% GA	Bovine Pericardium	0.2% concentration exhibited less calcification, inflammatory and antibody response than 0.6% in short duration exposure.	High concentration of GA has a toxic effect compared to lower concentration for short duration exposure	[44]
B	Genipin	0.4% for 5 days at RT	Bovine Pericardium, Acellular Bovine Pericardium	Reduced calcification and lesser inflammatory cells compared to GA-crosslinked Pericardium	Formation of dark blue pigments on graft; Cross-linking potential is inferior to GA	[32]
		0.3% in 0.01 M PBS pH7.4 for 3 days at RT	Acellular Bovine Pericardium	Effective crosslinking of graft tissue with improved mechanical strength, Least cytotoxic	Dark blue colour on reaction. Less resistance to enzyme digestion compared to GA	[21]
C	1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide [EDC]	0.1 M for 24 h at 25°C	Acellular Bovine Pericardium	Lower strain at break using TX as decellularizing agent	Cross-linking index lower than that with GA	[34]
		30 mM EDC and 6 mM NHS in 0.05 M MES pH 5.5 for 3 days at RT	Acellular Bovine Pericardium	Effective crosslinking and mechanical strength. When combined with space filler like Jeffamine, resistance to enzyme digestion improves.	Whitish colour on treatment. More cytotoxic than GA crosslinking. No much effect on calcification mitigation, susceptible to pronase digestion	[21]

		0.05 M EDC and 0.01 M NHS pH 5.5 for 2 days at RT	Acellular Bovine Pericardium	Calcific deposits and inflammatory cell infiltration reduced. Good preservation of collagen architecture. Cross-link efficiency found to be similar to GA cross-linking	-	[39]
		60 mmol/L Jeffamine in MES for 30 min at RT; then exposed to 0.3 mol/L EDC and 0.1 mol/L NHS agitating for 2.5 h at RT, pH 5	GA-crosslinked Porcine aortic wall	Crosslinked tissue, Calcification level reduced and the subsequent storage in EDC reduced calcification further	Individually EDC could not reduce calcification	[38,45]
D	Polyepoxide [Triglycidyl amine:TGA]	100 mM TGA in pH 7.4 borate mannitol buffer and its neutralization with 100 mM thiosulfate	Porcine aortic valve cusps and aortic wall	Porcine aortic valve cusp was Biocompatible, resistant to calcification	Porcine aortic wall did not show desired results. TGA preparation did not completely prevent long-term calcification	[46]
E	Neomycin trisulphate [NEO]	1 mM NEO in MES [pH 5.5] for 1 h+ 30 mM EDC, 6 mM NHS in MES for 24 h+ 0.6% GA in HEPES [pH 7.4] for 24 h+0.2% GA in HEPES [pH 7.4] for 5 days	Porcine aortic valve cusps and aortic wall	Better stability against elastase and GAG degrading enzymes than GA treated tissues, better collagen stability. Depth of buckling was lower than GA-fixed graft	-	[42]
F	Dye mediated photooxidation	Grafts immersed in photoactive dye and exposed to broad-wavelength light source	Porcine aorta	Biostable, expressed low immunogenic response, non-inflammatory and resistant to calcification, can stabilize protein solution	Mild calcification in rat model, however in juvenile sheep model no significant calcification was observed	[47]
G	Ultraviolet irradiation	15W UV lamp [254 nm] for 24 h at 4°C	Acellular Bovine Pericardium	Less inflammatory cell infiltration and calcification levels, better mechanical properties and cross-links comparable to GA, Retains pliability of tissue	Susceptible to pronase digestion, toxic to porcine fibroblast cells, collagen fiber degradation observed, less thermal stability	[39]

Table 2: Chemicals and molecules used for cross linking of grafts
RT: Room temperature; EDTA: Ethylene diamine tetraacetic acid; GA: Glutaraldehyde; NHS: N-hydroxy succinimide; GAG: Glucosaminoglycan

by ~20% compared to glutaraldehyde treatment. AOA also acts as a detergent, extracting phospholipids reducing calcification sites in the cell membranes [37]. Commercial valves like Medtronic (Minneapolis, MN, USA) employ α-amino oleic acid as an anti-calcification agent for its valve (AOA®). Heparin detoxicates the aldehyde groups of glutaraldehyde [52]. No react® glutaraldehyde of Shelhigh Inc., is a heparin based detoxification method, wherein the glutaraldehyde-treated grafts are impregnated with surfactants [53]. However heparin demonstrated deleterious effects [54] and thus can be avoided. Sodium bisulfite, being a reductive agent, forms α-hydroxyl sodium sulfonate on reaction with aldehyde [55]. Citric acid, an organic acid with four hydroxyl groups, salifies amino groups and neutralizes free aldehyde group [56,57]. It also interacts with Schiff base entities generated on aldehyde-amino group reaction inducing hydrophilicity on surface of the collagen fiber. It was also deduced that citric acid detoxification improved endothelial progenitor cell adhesion and proliferation [29].

Various amino acids have been used to detoxify the residual aldehyde remaining after glutaraldehyde treatment. This could be due to the reaction between the amine groups of amino acid and the free aldehyde groups. The treatment conditions such as concentration, pH and reaction time influences the efficiency of anti-calcification [29]. Amino compounds when used alone as anti-calcificant displayed calcification in tissue, but when used in combination with organic solvents decreased calcification to low amounts [58].

GAGs and non-collagenous proteins seem to be involved mainly in the regulation of calcification processes. Hyaluronic acid (HA), a GAG, is a component of the ECM. It is biodegradable, non-toxic and is not immunogenic. The anti-calcification property of GAG was confirmed of which HA reacts with the free aldehyde groups to form stable hydrazone bond. HA can also be used as a hydrogel on pericardium, by cross-linking the hydrazide bonds for a continuous anti-calcification effect [41,59]. In addition, space is created when the tissues are decellularized, which increases the possibility of calcium being deposited. This can be resolved by treating the tissues with space fillers before fixation [21]. The space fillers can be used synergistically with other anti-calcification techniques for a more positive effect.

Lyophilization is a freeze drying technique to store samples like tissues, preserving their architecture and viability. This process reduces the amount of residual aldehyde. Lyophilization is a favorable means of preservation of pericardium [60].

Phospholipid removal: The presence of phosphorus as phospholipids, in the cell membrane can lead to calcification. The calcium ions from plasma, reacts with phosphorus to form calcium phosphate deposits [28]. Hence the removal of phospholipids could reduce the incidence of calcification. Alcohol can reduce calcification by dissolving the cell membrane and disorder the acyl chains of phospholipids in the graft tissue [29]. Ethanol is used as anti-calcificant by St. Jude Medical, Minneapolis, MN, USA (Epic® valves), and along with Tween-80, it is used by Edwards Life sciences Corporation, Santa Ana, CA, USA for its product XenoLogiX® Long chain alcohol (LCA) has structure resembling the phospholipids. LCA would replace the phospholipids and decreases chance of calcification [60]. Short chain alcohol (SCA) used in combination with LCA would improve solubility in aqueous solutions, reduce micelle formation, and improve penetration into thick tissues [36].

S.No	Chemical/ Method	Concentration	Xenograft tissues	Outcome in animal experiments	Reference
a] Neutralization of residual aldehyde groups					
1	Alpha amino oleic acid [AOA]	Porcine aortic wall tissue from Medtronic Freestyle Valve incorporating AOA	GA cross-linked Porcine aorta	Surface calcium bands less condensed, scattered calcification sites. AOA is more efficient in reducing calcification when followed by EDC crosslink	[38]
		Porcine aortic wall tissue from Medtronic Freestyle Valve incorporating AOA	Jeffamine/EDC cross-linked Porcine aorta	Dramatic decrease in calcification.	[38]
2	Heparin	Initial exposure to Chitosan, 0.1% heparin pH 7.4, for 7 days at 4°C followed by 0.1M sodium borohydrate [$NaBH_4$], pH 8.8 at RT for 24 h	GA crosslinked bovine pericardium	Significant reduction in calcification, but change in mechanical function was noticed	[52]
		Shelhigh aortic stentless conduit	GA crosslinked bovine pericardium with incorporated porcine valve	Disintegration of graft and rupture of the aortic root.	[54]
3	Sodium bisulfate	40% at 4°C for 24 h	GA and Genipin cross-linked Bovine Pericardium	Degree of cross-linking and tissue strength maintained, prevents calcification,	[32]
4	Citric acid	10% citric acid in PBS for 30 min	Acellular GA fixed Bovine Pericardium	Free aldehyde detoxified to 79.50 ± 3.63% on reaction with cysteine, higher tensile strength	[29]
		5% & 10% for 30 min	GA crosslinked Bovine Pericardium	10% citric acid showed better reduction in free aldehyde concentration	[56]
		3.8%, pH 7.4 for 48 h at 37°C	GA crosslinked Bovine Pericardium	Calcium levels minimized compared to control	[64]
	Amino acids				
5	Arginine [Arg]	2% in PBS pH 11 at varying exposure time as 2 h, 6 h and 2 days	GA cross-linked Bovine Pericardium	Arg acts as filling agent and makes graft impermeable to plasma calcium, reducing calcification. Increased resistance to collagenase digestion. High durability, low protein adsorption and platelet adhesion, enhanced blood compatibility	[15]
		0.1 M L-Arg, pH 11 for 48 h at 37°C followed by 0.1 M $NaBH_4$ for 48 h at RT	GA cross-linked Bovine Pericardium	Increases tensile strength and thickness of graft. The thermal stability is maintained. No effect on calcification was observed.	[65]
6	Glycine	0.2 M in PBS, pH 7.4 at 4°C for 24 h	Acellular GA fixed Bovine Pericardium	Micro-structure of graft not altered, degree of cross-linking and tissue strength unaffected	[32]
		0.1 M in acetic acid buffered solution [pH 4.5] for 48 h at 37°C; then in PBS buffered 0.1M Sodium Borohydrate solution pH 7.4 for 24 h	GA cross-linked Bovine Pericardium	Structure, degree of crosslinking and tissue strength not affected, nearly complete inhibition of calcification	[19]
7	L-Glutamic acid	0.2% in 0.1 M PBS for 24 h 20°C	GA cross-linked Bovine Pericardium	Pronounced inflammatory response and calcification.	[28]
		0.2% in 0.1 M PBS for 24 h 20°C	Acellular GA cross-linked Bovine Pericardium	Reduces inflammatory reaction and calcification	[28]
		0.025 mol/L in borate buffer adjusted to alkaline pH	GA cross-linked Bovine Pericardium	Natural structure of collagen and elastin becomes fragmented with increasing processing time	[66]
8	L-Lysine (Lys)	25, 50 & 100 mM at 37°C, 2 days	0.2% & 0.7% GA cross-linked Porcine aorta	Decreased calcification.	[37]
		0.1 M for 48 h at 37°C	GA cross-linked, lysine treated Bovine Pericardium	Significant reduction in calcification	[67]
		0.1 M Lys, pH 7.6 for 48 h at 37°C followed by 0.1 M $NaBH_4$ for 48 h at RT	GA cross-linked Bovine Pericardium	Calcium deposits observed in the fibrous layer of pericardium. Tensile strength of treated graft is improved	[65]
9	Urazole	0.1 M in PBS for 1 week at RT	GA cross-linked Bovine Pericardium	Calcification observed, but on combination with alcohol significant reduction in calcium levels	[58]
10	Hyaluronic acid (HA)	HA-adipic dihydrazide (ADH) synthesis using EDC, pH 4.8 for 2 h.	GA cross-linked Bovine Pericardium	Decrease in calcification by 84.5% as HA sequesters calcium ions preventing hydroxyapatite nucleation	[59]

11	**Space Fillers**				
a	Jeffamine (polypropylene glycol-bis-aminopropyl ether)	0.06 M in 0.25 M MES pH 5 for 24 h at RT	Acellular Bovine Pericardium	Tissues become thicker. Jeffamine increases cytotoxicity.	[21]
b	Polyethylene glycol	50% in 0.01 M PBS pH 7.4 for 24 h at RT	Acellular Bovine Pericardium	Tissues become thicker. Good anti-calcification effect.	[21]
c	Polyacrylamide	30 g acrylamide/ bisacrylamide in 37:1 ratio and 4% DMPA in 0.01 M PBS pH 7.4 for 24 h at RT	Acellular crosslinked Bovine Pericardium	Tissues become thicker. Strength increases on space filling with polyacrylamide	[21]
12	Sodium borohydrate [$NaBH_4$]	0.1 M Sodium borohydrate	GA crosslinked bovine pericardium	Calcification levels are reduced. Neutralizes surface free aldehyde group created on Glut fixation to hydroxyl groups.	[52]
		0.08 M $NaBH_4$ for 24 h at RT in Glycine buffer and carbonate buffer individually (pH 7.5)	Porcine aortic valve cusps	Not much effective in reducing calcification. Has limited half life and is pH sensitive. Combined with alcohol treatment improved anti-calcification property and was most effective in carbonate buffer	[68]
		0.08 M $NaBH_4$ for 24 h in carbonate buffer (pH 10.3)	GA fixed Porcine aortic valve cusp	Significant calcium deposits observed	[22]
13	Sodium cyanoborohydride	0.08 M Sodium cyanoborohydride for 24 h at RT in 0.2 M HEPES, 0.1 M NaOH, 0.15 M glycine (pH7.5)	Porcine aortic valve cusps	Rare and irregular pattern of calcification observed in combination with alcohol.	[68]
14	Titanium coating	Plasma deposition of titanium	GA fixed Bovine Pericardium	Titanium exposure decreased free aldehyde concentration generated by GA treatment	[56]
15	Aldehyde dehydrogenase	1 ml solution for 24 h	GA fixed Bovine Pericardium	Displayed good reduction in free aldehyde concentration	[56]
16	Sequential treatment [Citric acid, Aldehyde dehydrogenase, Titanium coating]	Same reaction conditions as mentioned individually	GA crosslinked Bovine Pericardium	A combination of the three methods mitigated free aldehyde concentration. 94.1% endothelial cell survival in these grafts	[56]
17	Lyophilization		Acellular EDC crosslinked Bovine Pericardium	reduces free aldehyde content	[30]
			Bovine pericardium	Stiffness of valve leaflets increased. Opening and closing of valve altered, increase in gradient of conduit compared to untreated graft	[69]
			GA fixed bovine pericardium	Decreased inflammatory response after six months of implantation, did not increase chance of thrombus formation	[70]
			GA fixed bovine pericardium	Decreases cytotoxicity, mechanical property maintained.	[60]
b] Phospholipid removal					
18	Organic solvent	80% ethanol for 24 h at pH 7.4	GA cross-linked Porcine aortic valve cusp	Calcification levels reduced with occasional occurrence in central region of graft. Optimal results when combined with a reducing agent like sodium borohydrate	[68]
		80% ethanol in HEPES (pH 7.4) for 24 h	GA cross-linked Porcine aortic valve cusp	Reduction in calcium levels, propensity to buckling is higher	[22]
		80% ethanol for 24 h+0.1 M $NaBH_4$ for 48 h	GA cross-linked Bovine Pericardium	Anti-calcification properties observed.	[65]
		75% ethanol+5% octanol 2 days at RT	GA & Genipin fixed Bovine Pericardium	Reduction in calcium depots	[32]
		77.5% ethanol+2.5% octanol/octanediol for 48 h	GA cross-linked/ urazole treated Bovine Pericardium	Calcification significantly reduced and tissue architecture displayed no deterioration. Combination with amino compound like urazole/glutamic acid gave better results	[58]
		75% ethanol+5% octanediol, 2 days at RT	GA and Genipin cross-linked Bovine Pericardium	Excellent anti-calcification	[32]
		40% ethanol+5% octanediol in HEPES for 68 h	Bovine pericardium and porcine valve	Reduces calcification potential and phospholipid content, and biocompatibility. Elastin and collagen fiber content maintained	[71,72]
		70% ethanol+10% isopropanol, 2 days at RT	GA and Genipin cross-linked Bovine Pericardium	Excellent anti-calcification	[32]

19	Organic solvent+Glycine [Synergistic approach]	65% ethanol+5% octanol; 0.1 M glycine at pH 4.5	GA cross-linked Bovine Pericardium	No structural changes, Low level of calcification noticed after a short duration of implantation; could be beneficial in long-term	[18]
20	Alcohol [ethanol+octanol]+Glycine	75% ethanol, 5% octanol 2 days at RT; 0.1 M glycine pH 7.4 for 24 h RT	Acellular Bovine Pericardium	Mitigates calcification and cytotoxicity by extracting phospholipids using alcohol and detoxifying the free aldehyde remnants using glycine	[39]
21	Tannic acid	0.3% in GA in Na_2HPO_4 buffered saline pH5.5 for 4 days at 22°C	GA cross-linked Bovine Pericardium	Reduction in calcification [trivial levels of calcium detected], reduced MMP-9 and TN-C expression and macrophage infiltration	[19]
		0.3% in GA in Na_2HPO_4 buffered saline pH5.5 for 4 days at 22°C	GA cross-linked porcine aorta	Stabilizes elastin, preventing elastase-mediated degradation and elastin-oriented calcification	[40]
c] Crystallization inhibitors					
22	Phytate	0.25, 0.5, and 1.0 mg/l; incubated with graft for 96 h at 37°C	GA cross-linked Bovine Pericardium	Potent calcification inhibitory effect. Drastic decrease in calcification levels even at 0.25 mg/l. It is less toxic and inhibits hydroxyapatite crystallization	[20]
23	Pyrophosphate	1 mg/l & 0.5 mg/l; incubated with graft for 96 h at 37°C	GA cross-linked Bovine Pericardium	1 mg/l concentration showed less calcification and 0.5 mg/l displayed similar calcification compared to control of GA treated sample	[20]
24	Etidronate	1 mg/l & 0.5 mg/l; incubated with graft for 96 h at 37°C	GA cross-linked Bovine Pericardium	1 mg/l concentration showed less calcification and 0.5 mg/l had no effect on calcification inhibition	[20]
25	2-mercaptoethylidene-1,1-bisphosphonic acid (MABP)	20 mM MABP in borate mannitol buffer [pH 7.4] overnight incubation	Polyepoxide crosslinked Porcine aortic valve cusps and aortic wall	Complete inhibition of calcification	[46]
26	EDTA	11% EDTA for a period of 48 h	GA cross-linked Bovine Pericardium	Calcium levels lower than control group	[64]
		100 µg/ml in PBS at 4°C for 24 h followed by GA fixation, then second exposure to 100 µg/ml in PBS at RT for 24 h	GA cross-linked Bovine Pericardium	Diminished calcium crystal formation and decreases aldehyde-initiated calcification. Reduces calcification but does not prevent it	[73]
d] Other methods					
27	Chitosan	0.125% chitosan pH 6 at 4°C for 72 h	GA fixed bovine pericardium	Calcification levels were not mitigated and deformation in mechanical behavior was observed	[52]
28	Diethyl ether	70% ether [pH 3] continuous stirring at RT for 4 h	GA fixed bovine pericardium	Good anti-calcification effect, levels of calcium ions dropped to 3.69 µg/mg from 194.5 µg/mg of control	[34]
29	Thermal treatment	50°C for 2 days	GA fixed bovine pericardium	Inhibits calcification, no mechanical deformation	[34]
30	Sequential treatment (chitosan, heparin and others)	Same reaction conditions as mentioned individually	GA crosslinked bovine pericardium	Efficient, showing drastic reduction in calcium ion levels. Mechanical behaviour changed on treatment, but considered adequate	[34]
31	Cysteine (Cys)	0.25-2.0 w/v cysteine for 24 h at 25°C	Acellular GA crosslinked Bovine Pericardium	Cys utilizes residual aldehyde and decreases calcification. Cys might also exchange nitric oxide with S-nitrosothiols [donors] *in vivo* and this might reduce platelet adhesion.	[34]
		0.25-2.0 w/v cysteine for 24 h at 25°C	Acellular EDC fixed Bovine Pericardium	Incorporation of cysteine was lower	[34]
32	Nitric Oxide	NO donor DETA-NONOate 5-1s00 µM, sodium nitroprusside [SNP] 3 µM	Porcine Aortic Valve Cells	Inhibit TGF-β1 mediated calcification by increase in cGMP concentration.	[62]

Table 3: Detoxification of cross linked grafts
RT: Room temperature; GA: Glutaraldehyde; EDC: 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide

Tannic acid [TA] is a hydrolysable polyphenol. Glutaraldehyde fixation leads to elastin degradation, decreasing the mechanical stability. TA forms multiple bonds with elastin and collagen. Preservation of elastin content inhibits elastin-oriented calcification of glutaraldehyde-treated tissue [46].

Crystallization inhibitors: Crystallization inhibitors prevent development of calcium crystals by binding to the crystal nucleus. Pyrophosphate, a naturally occurring polyphosphate present in serum and urine was used to prevent calcification by binding to hydroxyapatite from the 1960's [61]. However pyrophosphate inhibits calcification only when injected, as it hydrolyzes on oral administration and is inactivated. Hence bisphosphonate, a stable analogue of pyrophosphate and a calcium-binding compound was synthesized [61]. It is known as crystal poison as it binds to hydroxyapatite crystals and inhibits further crystal growth [59]. Ethylenediaminetetraacetic acid (EDTA)

is a chelating agent which can be used to minimize calcification by sequestering metal ions like Ca^{2+} and reducing the dimensions of the crystal. EDTA also inhibits matrix metalloproteinase. Nevertheless it was deduced that none of these methods prevent the process of crystal formation, but only minimizes it.

Nitric oxide and other methods: Nitric oxide donors inhibit TGF-β1 mediated calcification by increase in intracellular cGMP and decreased superoxide concentration. TGF-β1 is a cytokine which plays a role in aortic valve stenosis and could induce the formation of cell aggregates leading to calcific nodules in aortic valve cell cultures *in vitro* [62]. Nitric oxide reduces the thrombogenicity of target tissue by cGMP dependent and independent methods, acting in the form of S-nitrosothiols [like cysteine] [63]. Ether and chitosan also enable detoxification of graft tissue. The details are tabulated in table 3 [15,18-22,28-30,32,34,37-40,46,52,54,56,58-60,64-73].

Novel approach: incorporating endothelial cells and/or nanotechnology

The novel approaches that may help improve this field to serve the human race can be

- Incorporation of nanoparticles to increase the efficacy and aid in the healing of chronic wounds and ulcers.
- Addition of anti coagulants in valve xenografts with sustained/controlled release.
- Addition of anti cancer drugs in sites of tumour removal seeding of endothelial cells and collagen in the decellularised tissue can improve the durability and bio compatibility of xenografts. These tissues are found to be non-cytotoxic, non-immunogenic, non-calcific and had an improved resistance to thrombogenicity [74]. This approach would decrease the complications arising from compatibility issues and can be applied to generate allogenic/autogenic grafts by culturing the endothelial cells on biodegradable polymers [75].
- Coating xenografts with immune suppressive agents may help to prevent rejection. Clinical research into these methods is necessary for clinical and medical application.

Barriers in xenotransplantation

a) Immunological barriers: Humans have Xenoregulated Natural Antibodies that lead to hyperacute rejections when they receive xenograft from discordant species. This rejection is mounted on the basis that humans lack α-1,3 galactosyl transferase enzyme. The target for most human non-Gal xenoantibodies is the sialic acid N-glycolylneuraminic acid (Neu5Gc) synthesised by the CMAH (cytidine monophospho-N-acetylneuraminic acid hydroxylase) gene, which is inactive in humans [76]. It can be overcome by use of genetically engineered knockout transgenic animals. The risk of transplant rejection can be estimated by measuring the panel reactive antibody levels. Cases of dysregulated coagulation episodes have also been reported. Insertional mutagenesis is a serious entity that needs to be explored in depth.

b) Biological barriers: Humans are posed to the risk of xenozoonosis following transplant. The porcine endogenous retroviruses (PERV), Swine influenza virus, Japanese Encephalitis virus, Hepatitis E virus, Nipah virus, Bubaline herpes virus, Capripoxvirus and bovine arbovius pose a serious threat of viral xenozoonosis. Furthermore, transmissions of prion diseases such as Cruezfeld Jacobsons disease, bacterial and nematode infections have also been a potential barrier [77]. Harvesting organs from Specific pathogen free animals may control the issues of xenozoonosis.

Conclusion

This detailed review illustrates the step wise methods in making a xenograft, the challenges met with and possible methods to overcome them. The mechanical strength of the processed xenograft is equivalent to or higher than the homograft. The anti-calcification treatment of the graft reduces the calcifying potential of the xenograft to levels comparable to homograft. Various detoxification protocols neutralize the toxic residues that remain after the chemical treatment, thus making it safe for human use. The barriers need to be taken into mind and dealt with accordingly. The prospects of making a xenograft more compatible and less toxic can be explored further, to make it an ideal one. Slaughtering of animals for graft purpose can be minimized by the use of other alternates such as homografts, prosthetic and biodegradable synthetic grafts.

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