In-vitro characterization of differently processed decellularised bovine pericardium

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Abstract

The efficacy of two different *in vitro* protocols for the decellularisation of bovine pericardium has been evaluated in this experiment. The pericardia were decellularised using a non-detergent based proprietary enzymatic protocol and a biodetergent (deoxycholic acid) based new protocol. The *in vitro* characterisation to estimate the *in vivo* stability was done using histological characterisation, residual DNA analysis, uniaxial tensile testing, contact angle measurement and collagenase susceptibility. The results indicated that the newr detergent based protocol was equally effective in inducing decellularisation and produce similar *in vitro* characteristics as in enzymatically processed pericardium, and hence can be used for *in vivo* evaluation and experimental studies.

Keywords: pericardium, bovine, decellularisation, *in vitro* characterisation

Introduction

Biomaterials constitute parts of medical implants, extracorporeal devices, and disposables that have been utilized in medicine, surgery, dentistry, and veterinary medicine as well as in every aspect of patient health care (Anderson et al., 1996; Badylak, 2007; Williams, 1987). Biomaterials produced by different protocols envisage that, the materials so developed must be biocompatible to minimise the risk of patients treated. Most of the adverse reactions of bioprostheses are caused by cellular components and attempts have been made to reduce the effects of cellular components (Badylak, 2007; Badylaket al., 2009; Badylak and Gilbert, 2008; Reideret al., 2006). Bovine pericardium is a suitable choice as a biomaterial owing to its collagenous nature and the reduced immunogenicity due to lower cellular contents. One of the methods employed to effect reduced reaction was cross linking with glutaraldehyde. However it has its limitations as it resulted in a chronic inflammatory response, bioprosthetic calcification thus severely limiting its use (Umashankaret al., 2010). Further investigation resulted in the development of decellularised animal tissues. A host of responses ranging from constructive remodelling (regeneration) to undesirable scar formation were observed in the healing produced by decellularised tissue obtained by different methods. So a comparison of differently processed materials with respect to the host response is required to spot out a suitable protocol and scaffold for biological application. Hence in this study, enzymatic and detergent based decellularisation protocols of bovine pericardium are compared and an attempt is made to analyse the effect of the treatment protocols on the biomechanical properties of the bioscaffold.

Materials and methods

The experiment and the histochemical characterisation were carried out jointly in the Department of Pathology, College of Veterinary and Animal Sciences, Mannuthy and Biomedical Technology Wing, SreeChitra Tirunal Institute of Medical Sciences and Technology, Trivandrum.

Bovine Pericardium

Bovine pericardium was collected from freshly slaughtered BSE free cattle of less than four years of age, brought for slaughter in Department of Livestock Products Technology, College of Veterinary and Animal Science, Mannuthy and Corporation Abattior, Kuriachira, Thrissur, as per availability, and were immediately transported in ice to the Department of Veterinary Pathology for further procedures.

Decellularisation

The choice of decellularisation protocols selected was enzymatic and detergent based. Enzymatic decellularisation of bovine pericardium (EDCL) was achieved using a proprietary enzymatic protocol devised and patented by SCTIMST, Trivandrum. Detergent based decellularisation (DXCL) was carried out by taking 60 x 60 mm sized samples (n=45) of bovine pericardium and subjecting three samples to different treatments using 0.25, 0.5, 1.0, 1.5 and 2.0% solution of deoxycholic acid in PBS (pH 7.4) for different time periods of 8, 24 and 72 hrs each. The decellularised tissue (Plate 1) was treated with 70% isopropyl alcohol for sterilization.

The effectiveness of decellularisation was determined by demonstrating absence of nuclear remnants using routine HE staining as well as by spectrophotometric estimation of the concentration of residual DNA in 100mg of the processed tissue using Mammalian Genomic DNA miniprep kit (Sigma-Aldrich). The extractable DNA was demonstrated using 1% agarose gel electrophoresis.

Figure 1

Figure 2

Fig.1. Enzymatically processed decellularised bovine pericardium (EDCL)
Fig..2. Decellularised bovine pericardium – deoxycholic acid treatment process (DXCL)

Collagenase susceptibility

Approximately 50 mg wet weight (in triplicates) of EDCL and DXCL had been taken randomly. To the weighed samples, 0.5ml of 0.1M Tris chloride (pH 7.4) containing 0.005M Calcium Chloride and 0.05 mg/mL Sodium Azide was added, and incubated for one hour at 37^0C with constant shaking. The samples were then added with collagenase enzyme from *Clostridium histiolyticum* prepared in 0.1M Tris Chloride (pH 7.4) so as to make the final concentration 2U/mg of tissue. The vials were incubated at 37^0C for 24h and 72 h. At the end of incubation, the samples were centrifuged at 12000 rpm for 20 minutes at 4^0C and the remaining tissue samples were blotted in filter paper for 5 minutes to dry them. They were weighed using Afcoset Electronic Weighing Balance ER120A, and the weight loss was determined by paired comparison before and after treatments.

Uniaxial tensile strength

Five strips of 10 X 60 mm were cut randomly from decellularised bovine pericardium. The thickness of the material at five different points was taken and the mean was calculated. Tensile strength was evaluated by subjecting to uniaxial tensile testing using Instron 3345 tensile testing machine, USA.

Contact angle

Three pieces of 10 x 10 mm size has been cut from decellularised bovine pericardium. The samples were lyophilized and the angle of contact of a small sessile drop of distilled water placed on the material surface was measured using Goniometer GII, Kerno Instruments Inc, USA. The angle was measured at two edges and the readings were repeated thrice per sample to improve accuracy.

Results

Decellularisation

Nuclear remnants could not be demonstrated in Haematoxylin and Eosin stained sections of enzymatically decellularised bovine pericardium (Plate 3&4). The results of detergent based decellularisation are represented below (Table. 1).

The residual DNA content after DNA extraction yielded 7.2 ± 3.2 µg per mL in EDCL. The DNA content in deoxycholic acid treated bovine pericardium is listed in Table 2.

Table. 1. Presence/Absence of nuclear remnants in different treatment protocols- H&E based analysis

Time (hrs)	Concentration (%)				
	0.25	0.5	1.0	1.5	2.0
8	+	+	+	-	-
24	+	+	-	-	-
48	+	+	-	-	-

Table 2. Residual DNA content in different modules

Time (h)	Residual DNA (µg per mL)				
	0.25	0.5	1.0	1.5	2.0
8	92.7 ± 2.8	91.5 ± 3.1	52.8 ± 3.1	46.2 ± 3.2	45.9 ± 2.1
24	57.4 ± 2.2	52.6 ± 1.9	8.6 ± 2.7	7.9 ± 2.8	8.2 ± 1.9
48	52.8 ± 2.3	52.1 ± 1.9	8.2 ± 2.6	7.5 ± 2.2	7.8 ± 2.1

Collagenase susceptibility

The residual weight of the scaffold after *in vitro* bacterial collagenase type II digestion test has been taken at 24h, 72h and 7 days. The weight loss has been calculated from the initial weight of the scaffold taken.

The residual weight of DXCL after collagenase digestion at 24h was 12.6 mg, at 72h 0.2 mg and negligible at 7 days as measured using an electronic weighing balance. The residual weight of EDCL after collagenase digestion at 24h was 0.33 mg, and negligible at 72h and 7 days (Table.3). Based on the residual weight of the samples, the weight loss of the samples has been calculated (Table.4). The weight loss of EDCL was found to be more than 99 percent from 24h onwards itself. DXCL showed 75 percent weight loss at 24h and 99 percent from 72h onwards.

Table 3. Residual weights of EDCL and DXCL after collagenase digestion

Sample	Weight loss (%)		
	24h	72h	7days
EDCL	0.33± 0.231[a]	-	-
DXCL	12.6± 0.1[b]	0.2 ± 0.0	-

Means bearing different superscripts in a column differ significantly $p<0.05$

Table 4. Weight loss of decellularised pericardium after collagenase digestion

Sample	Weight loss (%)		
	24h	72h	7days
EDCL	>99	>99	>99
DXCL	74.8	>99	>99

Uniaxial tensile strength

The ultimate tensile strength observed for DXCL was 15.821 MPa and Elastic modulus observed was 42.410 MPa and for EDCL it was 13.73 MPa and 45.28 MPa respectively (Table 5).

Table 5. Uniaxial tensile testing of decellularised bovine pericrdia

Sample	Load at Max. Load (kN)	Stress at Max. Load (MPa)	% Strain at Auto. Break (%)	Modulus (AutYoung) (MPa)
EDCL	0.033 ± 0.018[a]	13.73 ± 9.05[a]	70.28 ± 14.76[a]	45.28 ± 27.47[a]
DXCL	0.037 ± 0.009[a]	15.821 ± 3.347[a]	96.534 ± 15.638[a]	42.410 ± 15.714[a]

Means bearing different superscripts in a column differ significantly $p<0.05$

Contact angle

Contact angle formed by a sessile drop of distilled water on DXCL was 52.67 ± 4.26^0 and on EDCL was 29.0 ± 0.57^0.

Discussion

Decellularisation of biomaterials can be effected using physical, chemical and biological agents (Gilbert et al., 2006; Crapo et al., 2011). A successful decellularisation can be effected by a combination protocol involving the use of these agents within a stipulated time schedule. Accordingly, a variety of processing protocols have been employed in decellularisation of biomaterials. The success of effective decellularisation varied from process to process. The processing protocols significantly alter the extracellular matrix (ECM) histoarchitecture and hence the processed biomaterials differed in biomechanical properties and *in vivo* responses. Yet, evaluations on efficacy of different processing protocols on the scaffold properties are rarely studied.

The present study therefore was aimed at the comparison of two different processing protocols on the biomechanical properties and the *in vitro* responses to the processed scaffold. The mechanical strength, *in vitro* collagenase resistance and surface hydrophilicity testing were used as a measure to evaluate the *in vivo* stability of scaffold prior to in vivo studies.

Freshly collected bovine pericardia have been decellularised using a biodetergent (Deoxycholic acid) based protocol and an enzymatic treatment based protocol. The enzymatic process is a proprietary protocol devised at Biomedical Technology Wing of SreeChithraTirunal Institute of Medical Sciences and Technology, Trivandrum (Umashankaret al., 2010). Balasundariet al. (2007), Chandrashekharanet al. (2007) and Galla et al. (2010) reported one percent solution of Sodium salt of deoxycholic acid is observed to be effective in decellularisation of porcine xenografts. Hence when chosen deoxycholic acid, trials with a combination of different conentrations and time were carried out and found out the least concentration and time combination eliciting a satisfactory decellularisation. Accordingly, one percent solution of deoxycholic acid for 24 hrs has been identified as the best agent for decellularisation in this study. The decellularisation of fresh bovine pericardium obtained for the current study has been achieved through the application of a newer protocol employing one percent deoxycholic acid combined with a set of physical and chemical processes from those described by Gilbert et al. (2006) and Crapo et al. (2011).

The effectiveness of the decellularisation protocols were analysed by histological observation of the processed bovine pericardia using haematoxylin and eosin staining; and analysis of residual DNA content. The histology revealed normal wavy collagen with absence of cellularity. The observations were in accordance with those made by Gilbert et al., 2010, Reing et al., 2010, Umashankar et al., 2010 and Thampi, 2011. The residual

DNA content gave satisfactory decellularisation with a minimum 1% concentration of deoxycholic acid for a minimum duration of 24h.

The effect of processing protocols on the mechanical stability of the processed bovine pericardia used for the present study has been carried out. Deoxycholic acid treated bovine pericardia showed a higher ultimate tensile strength compared to the enzymatic process in uniaxial tensile testing suggesting a better tissue holding capacity of the material processed with deoxycholic acid.

Contact angle measurement was another parameter analysed for the characterisation of the material as it correlated well with the hydrophilicity or hydrophobicity of the material, and is hence an important tool in analysing the nutrient diffusion and tissue ingrowth to the material (Chandran et al., 2003) and an indirect assessment of the partial crosslinking elicited in the material (Umashankar et al., 2010). The observations showed that enzymatically processed pericardium was more hydrophilic compared to deoxycholic acid treatment. This indicated chances of an improved *in vivo* response in EDCL.

In vitro collagenase susceptibility using bacterial Collagenase type II enzyme was conducted as described by Umashankar et al. (2010). This could be used to assess the partial crosslinking (Umashankar et al., 2010) of the biomaterial and as an aid to understand the *in vivo* stability or bioresorption of the material (Chandran et al., 2003). Digestion assay conducted in EDCL showed complete degradation within 24h whereas DXCL has completely resorbed within 72h. This supported that the increased tensile strength of deoxycholic acid treated material which may be attributed to the partial crosslinking brought about by the detergent. The *in vitro* characterisation suggested that both enzymatic decellularision protocol and deoxycholic acid treatment protocol produced completely biodegradable and replaceable biomaterials which can be used for *in vivo* treatment and experimental purposes.

Conclusion

In the present experiment, efficacy of two different decellularisation protocols on bovine pericardium based on *in vitro* characterisation. Bovine pericardium was decellularised using a detergent based and an enzyme based protocol. Efficacy of decellularisation was then analysed by routine histological examination using H&E and residual DNA analysis. The decellularised bovine pericardia were then subjected to a variety of *in vitro* tests such as tensile testing, contact angle measurement and collagenase resistance test to characterise them. Routine histopathological examination using H&E revealed a normal wavy collagenous matrix with absence of cellularity in both treatments. The newer protocol treated pericardia showed a higher tensile strength and *in vitro* collagenase resistance compared to the enzymatic process, suggesting partial crosslinking of the pericardium by the detergent, a better tissue holding capacity and biostability. A higher contact angle was also seen in the pericardium which was decellularised by the detergent showing moderate hydrophilicity, suggesting a decreased tissue ingrowth response than the enzymatic process. The results of the current study indicated that the newer detergent based protocol was equally effective in inducing decellularisation and provided similar *in vitro* characteristics as in enzymatically processed pericardium, and hence can be used for *in vivo* evaluation and experimental studies.

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References

1) Anderson JM, Gristine AG, Hanson SR. 1996. Host reactions to biomaterials and their evaluation. In:Ratner, BD, Hoffman AS, Schoen FJ, Lemons JE (Eds.), Biomaterials Science; An Introduction to Materials in Medicine, Academic Press, San Diego, pp. 165-214.
2) Badylak SF. 2007. The extracellular matrix as a biologic scaffold material. Biomaterials 28: 3587-3593.
3) Badylak SF, Gilbert TW. 2008. Immune response to biologic scaffold materials. SeminImmunol20: 109-116.
4) Badylak SF, Freytes DO, Gilbert TW. 2009. Extracellular matrix as a biological scaffold material: structure and function. Acta Biomater 5: 1-13.
5) Balasundari R, Gupta R, Sivasubramanian V, Chandrasekharan R, Arumugam S, Cherian KM, Guhathakurta S. 2007. Complete microbe free processed porcine xenografts for clinical use. Ind J Thorac Cardiovasc Surg 23: 240-245.
6) Chandran KB, Burg KJL, Shalaby, SW. 2003. Soft Tissue Replacements. In: Park JB, Bronzino JD (Eds) Biomaterials – principles and applications. CRC Press, USA. pp. 250.

7) Chandrashekharan R, Balasundari R, Sivasubramanian V, Cherian KM, Nayak V, Guhathakurta S. 2007. Cytotoxicity and sensitization studies of processed porcine xenografts. Ind J Thorac Cardiovasc Surg 23: 246-250.
8) Crapo PM, Gilbert TW, Badylak SF. 2011. An overview of tissue and whole organ decellularization processes. Biomaterials 32: 3233-3243.
9) Galla S, Mathapati S, Nayak VM, Cherian KM, Guhathakurta S. 2010. Analytical study to evaluate the extracellular matrix in processed acellular xenografts. Ind J Thorac Cardiovasc Surg 26:132-138.
10) Gilbert TW, Freund JM, Badylak, SF. 2010. Quantification of DNA in biologic scaffold materials. Biomaterials 152: 135-139.
11) Gilbert TW, Sellaro TL, Badylak, SF. 2006. Decellularization of tissues and organs. Biomaterials 27: 3675–3683.
12) Reing JE, Brown BN, Daly KA, Freund JM, Gilbert TW, Hsiong SX, Huber A, Kullas KE, Tottey S, Wolf MT, Badylak, SF. 2010. The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds. Biomaterials 31: 8626-8633.
13) Rieder E, Nigisch N, Dekan B, Kasimir MT, Muhlbacher F, Wolner E, Simon P, Weigel G. 2006. Granulocyte-based immune response against decellularized or glutaraldehyde cross-linked vascular tissue. Biomaterials 27: 5634-5642.
14) Thampi P. 2011. Inflammatory and immune responses of decellularised bovine pericardial implants in rats. M.V.Sc thesis. Kerala Veterinary and Animal Sciences University, Pookode, Wayanad. p.63.
15) Umashankar PR, Arun T, Kumari TR. 2010. Short duration gluteraldehyde cross linking of decellularized bovine pericardium improves biological response. J Biomed Mater Res A 97: 311-320.
16) Williams DF. 1987. Definitions in biomaterials. Elsevier, Amsterdam, The Netherlands.