RESEARCH ARTICLE
JIVA 19 (1) April 2021

JIVA

Received: 28.03.2021
Accepted: 10.04.2021

EFFECT OF CONTROLLED GLUTARALDEHYDE CROSSLINKING ON *IN VITRO* STABILITY OF DECELLULARISED BOVINE PERICARDIAL SCAFFOLDS

Suvaneeth P.[1,2*], Nair N. D.[1], UmashankarP. R.[3], VijayanN.[1], Martin K.D.J.[1] and Mammen J.A.[1]

[1]College of Veterinary and Animal Sciences, Mannuthy; [2]Faculty of Veterinary and Animal Sciences, Mirzapur, Banaras Hindu University;[3] SCTIMST, Trivandrum
**Corresponding author:dr.suvaneeth@gmail.com*

ABSTRACT

Immunogenicity prediction of decellularised pericardial scaffolds by residual DNA content analysis and the effect of glutaraldehyde crosslinking on tissue holding capacity and bioresorption of decellularised bovine pericardial scaffolds using *in vitro* enzymatic degradation properties are undertaken in this study. Residual DNA content analysis confirmed that different decellularisation protocols effectively altered predictive immunogenicity values in pericardial scaffolds. Controlled crosslinking with 0.25% and 0.5% glutaraldehyde for 10 minutes significantly altered the biodegradation using collagenase enzyme.

Keywords : Bovine pericardium, Collagenase susceptibility, Crosslinking, Decellularisation,

INTRODUCTION

Biomaterials are non-viable materials used in medical devices to interact with a biological system in a beneficial way. A lot of scaffolds of biological origin are used for inducing regeneration, but the prospects are limited. Bovine pericardium has been well identified as a better scaffold for biological application, owing to its collagenous nature and strength. Initially the focus was laid upon improving the tissue holding capacity of the scaffold and hence glutaraldehyde was used as a crosslinking agent to improve the strength of the scaffold. The present study was aimed at the comparison of the *in vitro* responses to controlled glutaraldehyde crosslinked bovine pericardial scaffolds, especially focussing on tissue holding and bioresorbability properties

MATERIALS AND METHODS

Bovine pericardia were collected from freshly slaughtered cattle brought for slaughter in Department of Livestock Products Technology, College of Veterinary and Animal Science, Mannuthy and Corporation abattoir, Kuriachira and were stored in -20°C until processed.

Detergent based decellularisation (DXCL) was achieved by a biodetergent, 1% deoxycholic acid in PBS (pH 7.4) for 24 hrs. DXCL was then crosslinked using 0.25% (2GDX) and 0.5% (5GDX) glutaraldehyde solution for 10 minutes. The scaffolds were then washed with four changes of sterile autoclaved PBS (pH 7.4) for 6 hrs each and then stored in 70% ethanol until further use. The effectiveness of decellularisation was determined by demonstrating presence or absence of nuclear remnants using routine Hematoxylin and Eosin 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 efficacy of decellularisation and residual DNA content was compared to untreated/fresh bovine pericardium (UBP) and enzymatically decellularised bovine pericardial scaffold (EDCL) collected from Sree Chitra Tirunal Institute of Medical Sciences and Technology, Trivandrum.

Digestion assay using *in vitro* bacterial collagenase susceptibility was conducted to evaluate the biostability of the processed scaffold. Approximately 50 mg wet weight (in triplicates) of EDCL, DXCL, 2GDX and 5GDX were 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^{0}C with constant shaking. The samples were then added with collagenase enzyme from *Clostridium histiolyticum* (Sigma-Aldrich) 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^{0}C for 24 hrs, 72 hrs and 96 hrs. At the end of incubation, the samples were centrifuged at 12000 rpm for 20 minutes at 4^{0}C and the remaining tissue samples were blotted in filter paper for 5 minutes to dry them. They were weighed using Afcoset Electronic Weighing

Table. 1. Residual DNA content in different modules

Material	Residual DNA (µg per mL)
EDCL	7.2 ± 3.2 ^{a}
DXCL	8.6 ± 2.7 ^{a}
2DXCL	8.5 ± 2.8 ^{a}
5DXCL	8.6 ± 1.9 ^{a}
UBP	94 ± 2.2 ^{b}

(Mean ± SD) Means bearing different superscripts in a column differ significantly p<0.05

Table. 2. Residual weights of scaffolds after collagenase digestion

Sample	Residual weight (g)		
	24h	72h	96h
EDCL	0.33 ± 0.231^a	-	-
DXCL	12.6 ± 0.1^b	0.2 ± 0.0^a	-
2GDX	12.8 ± 0.1^b	8.6 ± 0.2^b	0.8 ± 0.2^a
5GDX	47.4 ± 0.1^c	37.2 ± 0.1^c	22.1 ± 0.1^b

(Mean ± SE) Means bearing different superscripts in a column differ significantly $p<0.05$

Balance ER120A, and the weight loss was determined by paired comparison before and after treatments.

Statistical analysis was done using one-way ANOVA using IBM Statistical Package for the Social Sciences software version 20 (SPSS 20). The confidence interval was fixed at 95 per cent ($P< 0.05$).

RESULTS AND DISCUSSION

Grossly, all the processed pericardia appeared in varying degrees of off white in colour. DXCL appeared whiter than all the other treatments and 5GDX appeared to have a mild yellowish tint (Fig. 1).

Nuclear remnants could not be demonstrated in Haematoxylin and Eosin stained sections (Fig. 2) of any of the treatment groups suggesting proper decellularisation was achieved using the proprietary enzymatic protocol and biodetergent based protocols (Balasundari *et al.,* 2007; Chandrashekharan *et al.,* 2007; Galla *et al.,* 2010; Umashankar *et al.,* 2011; Thampi *et al.,* 2013; Suvaneeth *et al.,* 2016).

Fig. 1 Mildly yellowish appearance of 5GDX

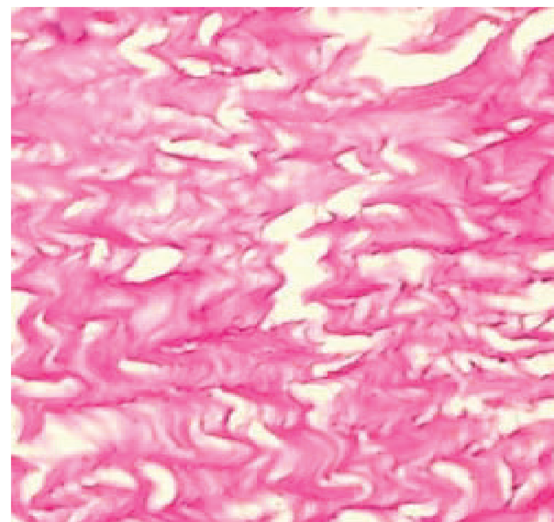

Fig. 2 DXCL showing complete absence of cellularity and normal wavy collagen (H&E X 400)

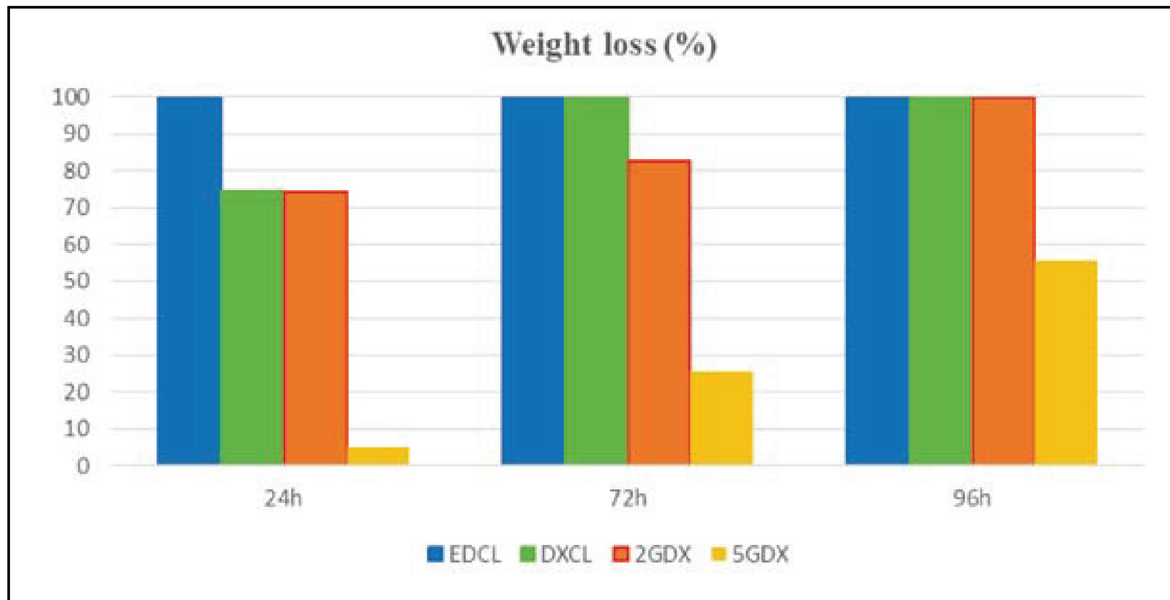

Fig.3. Weight loss of scaffolds after collagenase digestion

The residual DNA content after DNA extraction was minimal in decellularised scaffolds compared to untreated bovine pericardium. (Table 1). This indicated that adequate decellularisation was achieved, and could be considered for implantation studies. Proper decellularisation and removal of nuclear remnants ensure lower immunogenicity and thereby envisage a better host tissue response (Gilbert *et al.,* 2006; Arai and Orten, 2009).

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 predict the *in vivo* stability or bioresorption of the material (Chandran *et al.,* 2003). Accordingly, digestion assay was conducted and the residual weight of the scaffold after *in vitro* bacterial collagenase type II digestion test has been taken at 24 hrs, 72 hrs and 96 hrs. The residual weights of scaffolds varied significantly among scaffolds at all points of time (Table. 2). It was found that enzymatically decellularized scaffold underwent complete degradation within 24 hrs and deoxycholic acid treated material was completely resorbed within 72 hrs. Whereas crosslinking of DXCL using 0.25% glutaraldehyde showed a slower degradation after 24 hrs. 0.5% crosslinking with glutaraldehyde almost made the scaffold resistant to complete degradation even after 96 hrs. It can be assumed that deoxycholic acid itself can elicit a partial crosslinking rendering a slower initial

digestion in case of DXCL and controlled crosslinking using glutaraldehyde renders the scaffolds more resistant to collagenase digestion. This indicates a higher tissue holding capacity of crosslinked scaffolds and hence, they can be used for even hernia repair studies.

Based on the residual weight of the samples, the weight loss of the samples has been calculated (Fig. 3). The weight loss of EDCL was found to be more than 99 percent from 24 hrs onwards itself, suggesting a better bioresorbable nature of the scaffold. DXCL and 2GDX also got completely degraded over 72 hrs and 96 hrs respectively suggesting complete bioresorbability over a longer duration of time. Whereas 5GDX retained almost 50% of its initial weight even after 96 hrs, suggesting a severe crosslinking and a better resistance to collagenase degradation. Improved resistance to collagenase degradation is a property suitable for bioscaffolds requiring a longer *in vivo* contact without degradation. The host tissue responses in terms of inflammation and tissue holding need to be studied further using experimental models to determine the suitability for clinical trials and varied applications like hernia repair and cutaneous wound healing.

SUMMARY

The predictive immunogenicity of decellularised bovine pericardia using different decellularisation protocols were studied using residual DNA content analysis. It was found that decellularisation protocols significantly reduced nuclear remnants by the absence of visible nucleus in histological analysis and also by the significantly lower concentration of residual DNA extracted from the decellularised pericardial scaffolds. The effect of controlled glutaraldehyde crosslinking on the mechanical stability of the processed bovine pericardia has also been carried out in the present study. Crosslinking of decellularised bovine pericardial scaffold using low concentrations of glutaraldehyde solution 0.25% and 0.5% for short duration significantly improved their resistance to biodegradation using bacterial collagenase enzyme type II and hence, tend to have a better tissue holding capacity and can be used for a longer duration without complete degradation within the host. The effect of the same for host tissue responses in terms of inflammation and tissue holding need to be carried out further to determine the suitability for clinical trials.

REFERENCES

Arai, S and Orten, E.C. 2009. Immunoblot detection of soluble protein antigens from sodium dodecyl sulphate and sodium deoxycholate treated candidate bioscaffold tissue. *J Heart Valve Dis.* **18:** 439–44.

Balasundari, R., Gupta, R., Sivasubramanian, V., Chandrasekharan, R., Arumugam, S., Cherian, K.M. and Guhathakurta, S. 2007. Complete microbe free processed porcine xenografts for clinical use. *Ind. J. Thorac. Cardiovasc. Surg.* **23**: 240-245.

Chandran, K.B., Burg, K.J.L. & Shalaby, S.W. 2003. Soft Tissue Replacements. In: Park, J.B. and Bronzino, J.D. (eds.), Biomaterials – principles and applications. CRC Press, USA, pp. 250.

Chandrashekharan, R., Balasundari, R., Sivasubramanian, V., Cherian, K.M., Nayak, V. and Guhathakurta, S. 2007. Cytotoxicity and sensitization studies of processed porcine xenografts. *Ind. J. Thorac. Cardiovasc. Surg.* **23**: 246-250.

Galla, S., Mathapati, S., Nayak, V.M., Cherian, K.M. and Guhathakurta, S. 2010. Analytical study to evaluate the extracellular matrix in processed acellular xenografts. *Ind. J. Thorac. Cardiovasc. Surg.* **26**: 132–138.

Gilbert, T.W., Sellaro, T.L. and Badylak, S.F. 2006. Decellularization of tissues and organs. *Biomaterials*. **27**(19): 3675–3683.

Suvaneeth, P., Nair, N. D., Umashankar, P.R. Vijayan, N., Mammen, J.A, Divya, C and Martin, K.D.J. 2016. In vitro characterization of decellularised bovine pericardium. *J. Livestock Sci.* **7**:13-18.

Thampi, P., Nair, N. D., Lalithakunjamma, R., Vijayan, N., Venugopal, S and Ramachandra, U. 2013. Pathological Effects of Processed Bovine Pericardial Scaffolds—A Comparative in vivo Evaluation. *Artificial organs*. **37**(7):600–605

Umashankar, P.R., Arun, T. and Kumari, T.R. 2010. Short duration gluteraldehyde cross linking of decellularized bovine pericardium improves biological response. *J. Biomed. Mater. Res. A.* **97**(3): 311-320.