Cytotoxicity and sensitization studies of processed porcine xenografts

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Abstract

Back ground: **The unlimited requirement of valved graft material and nonavailability of homogratfs has made it necessary to resort to xenogenic sources. Immunogenicity of xenografts plays a vital role in the graft biocompatibility. Immunogenic reaction may lead to calcification and finally graft failure.**

Methods: **To render the processed xenografts non-cytotoxic, the xenografts were processed at different stages with chemicals, while keeping a keen check on their toxic properties. Tests were done at various levels in vitro and in vivo to evaluate the cytotoxicity of the processed xenografts. In vitro cytotoxicity studies of the test samples were conducted on Bagg Albino clone mice strain (BALB/C3T3 cell line), using direct and indirect contact method. In vivo toxicity studies were conducted by administering the test sample extracts into Swiss albino male mice. Sensitization studies were performed on Guinea pigs. Test extracts were administered to the animals over the period of induction phase; topical induction phase and challenge phase.**

Results: **The processed xenograft tissues when subjected to in vitro cytotoxicity test with BALB/C3T3 cell line and the in vivo tests did not show any cytotoxicity.**

Conclusion: **The studies proved that the processing methodology rendered the xenografts non-cytotoxic and safe for clinical use.** ***(Ind J Thorac Cardiovasc Surg 2007; 23: 246-250)***

Key words: Graft, Immunogenicity, Decellularisation.

Introduction

The use of valved grafts from xenogenic sources plays a vital role in the field of cardiothoracic surgery[1]. Xenogenic bioprosthetic valves consist of chemically cross linked intact porcine aortic valves[2]. These bioprosthetic valves display better hemodynamics than mechanical valves, and have an advantage of not requiring life-long anticoagulation therapy. But, unfortunately, these tissue-based valves tend to have shorter life due to complications with calcification and immunogenicity[3-5]. The porcine xenografts can cause immunogenic reactions in the human host. Hence, they have to be processed such that they are decellularized[6] and cross-linked to aid in the removal of the immunogenic epitopes.

Several methods of treating natural tissues are being explored to produce a complete acellular tissue matrix by specifically targeting the removal of cellular components that are believed to promote calcification and also give rise to a residual immunological response. Various cell extraction procedures [e.g. detergents, enzymes, sonication] have been pursued as a means to create completely acellular tissues for use as biomaterial implants[7,8]. The decellularised tissue though said to be devoid of cells, is never completely acellular. Hence, residual cellular components and lipids within processed tissues may promote undesired effects such as calcification and immunological recognition[9, 10].

The tissue once decellularised is subjected to crosslinking using varied chemicals. These chemicals are cytotoxic, so it is important to check their toxic limit.

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Glutaraldehyde (GA) is the most commonly used crosslinker in natural tissue treatment. GA crosslinking accomplishes the task of reducing immunogenicity of the graft, but several problems such as, altered mechanical properties leading to early mechanical failure; calcification, cytotoxicity and incomplete suppression of immunological recognition in the host, are encountered[11].

However, because the fundamental mechanisms underlying cytotoxicity, calcification and immunological recognition are not completely understood, the development of new approaches to create an ideal natural biomaterial is an even larger challenge[12].

Materials and Methods

We decellularised xenografts using 1% Sodium deoxycholate (DCA) for more than 40 hours. Further enzymatic digestion was preferred with the help of nucleases. Next, the decellularised tissue was subjected to cross-linking using formaldehyde at stages for more than 40 hours. The tissue was then made to undergo anti-thrombotic and anti-calcification treatment as per the patent protocol of the laboratory.

Invitro cytotoxicity studies:

The in vitro cytotoxicity of processed porcine pulmonary valve was tested using BALB/C3T3 mouse fibroblast cell line. The cell line was incubated in Dulbeccos modified Eagles Medium (DMEM, Source-PAN laboratories,USA) with 10% fetal bovine serum (FBS, Source-PAN laboratories,USA) at 37°C in humidified atmosphere of 5% CO_2 for 3 days before the start of the experiment for sub culturing.

Treatment procedure: Equal quantities of cells, 4.4 x10^5 were seeded in each cell culture flask.

Preparation of test substance: 0.2 g of porcine tissue per ml of extraction medium was mashed well and incubated in 37°C for 72 hours. Extracts were prepared using physiological saline (0.9% - Extract 1) and vegetable oil (Extract 2). The prepared extracts were filtered using 0.2 mm cellulose acetate filter paper and used for animal experiment.

Indirect contact method: The concentration of each extract, one as such (100%) and two dilutions of extracts (25% and 50%) adjusted using DMEM, were added to the above culture and incubated for 24 hours at 37°C in humidified 5 % CO_2.

Direct contact method: The 1 sq cm bit of porcine xenograft was allowed to be in direct contact with the cells by incubating with DMEM and10% FBS at 37°C with 5% CO_2 for 24 hours. After incubation period the test material was removed from each well.

The cell culture flasks (both direct and indirect contact) along with negative control (cell culture flask without test material) and positive control (cell culture treated with 50% phenol) were incubated for 3 days.

After 3 days of incubation, the medium was separated and the cells were analyzed for qualitative evaluation. The cells were washed with phosphate buffered saline, and were detached by trypsinization followed by centrifugation at 800 g for 10 minutes. The supernatant was removed and 500 µl of culture medium was added to make a cell suspension. The cell viability check was done with the help of Trypan blue dye exclusion method. Cell populations were expressed as total cells/ml. The total cell protein was estimated using Bradford assay

Systemic toxicity studies:

Swiss albino male mice of body weight 17-21 grams were used for this experiment. Animals were randomly distributed into 4 groups (5 animals each) as Group1-Group 4. Animals of each group were housed in standard clean polypropylene cages, air-conditioned room with a temperature of 22±3°C, relative humidity of 55±10 % with 12 hours light and 12 hours dark cycle. Animals were provided adlibitum feed (Amrut brand pellet feed) and water through out the test period.

Sensitization studies:

Guinea pigs were used for this study. Extracts were prepared in saline (0.9 % NaCl solution – Extract 1) and vegetable oil (Extract 2) by incubating 0.2 g of test sample at 37°C for 72 hours. In the induction phase, the animals were injected intradermaly with three pairs of injections (FCA -Freund's complete adjuvant, Extract, FCA+ Extract).

After seven days in the topical induction phase, filter paper soaked in solvent was placed over the injected site and secured by occlusive dressing. Since the intradermal injections did not cause irritation, the test animal skins were pretreated with 10% sodium dodecyl sulfate 24 hours prior to topical induction. The control animals were treated in the same way using the solvent alone.

After fourteen days in the challenge phase, the test and control group of animals were challenged with the extract. The extract was administered by topical application to one flank (which was not treated during

induction) of each animal using appropriate patches soaked in the extracts. The patch was secured by occlusive dressing, which was removed after 24 hours.

Results

Under these conditions of study, the processed porcine pulmonary valved conduit did not induce cytotoxicity in BALB/C3T3 cell line in vitro. The results of indirect and direct contact between cells and the test sample, the cell viability count and mean protein value show that the processed porcine xenograft was non cytotoxic (see Table 1 and 2).

Table 1. Mean viable cell count and cell death

		Cells (x10 ^{5} / ml)					
		Physiological saline		Vegetable oil		Direct contact	
Treatment	Initial concentration	Live cell after treatment	Cell death	Live cell after treatment	Cell death	Live cell after treatment	Cell death
Test concentration – 25 %	4.4	3.9	0.5	4.0	0.4	4.0	0.4
Test concentration – 50%	4.4	3.9	0.5	3.9	0.5	nil	nil
Test Concentration - 100 %	4.4	3.8	0.6	3.7	0.7	Nil	Nil
Negative control	4.4	4.0	0.4	4.1	0.3	4.0	0.4
Positive control	4.4	0.1	4.3	0.2	4.2	0.1	4.3

The above values are the mean of triplicate tests

Table 2. Mean protein value

	Physiological saline		Vegetable oil		Direct contact	
Treatment	Protein concentration µg/ml	Percentage growth inhibition	Protein concentration µg/ml	Percentage growth inhibition	Protein concentration µg/ml	Percentage growth inhibition
Test concentration- 25 %	1390	Nil	1440	Nil	1480	Nil
Test concentration- 50 %	1370	Nil	1460	Nil	-	-
Test concentration- 100 %	1390	Nil	1450	Nil	-	-
Negative control	1370	-	1460	-	1460	-
Positive control	100	-	100	-	100	-

The above values are the mean of triplicate tests

The systemic toxicity (in vivo) studies, showed no abnormal clinical signs in the test animal. There was no loss of body weight or mortality observed during the experiment.

In sensitization studies using guinea pigs, no dermal sensitization or abnormal clinical signs were observed in the test animals. The skin reactions were scored as per Magnusson and Kligman grading scale at 24 and 48 hours after patch removal. All test animals showed no visible change as per the grading scale. Hence, the processed porcine pulmonary conduit proved to be non-cytotoxic under both in vitro and in vivo experimental conditions.

Discussion

Various research groups around the world are actively investigating cardiovascular prosthesis of biological origin. The need for such xenogenic bioprosthesis and the potential role of natural tissues in cardiovascular applications such as cardiac valves and vascular grafts is ever increasing. Upon implantation, unprocessed natural biomaterials are subjected to chemical and enzymatic degradation, seriously decreasing life of prosthetic grafts. This setback has been the motivation for various decelllularisation processes, which have been developed to stabilize the tissue while attempting to maintain its natural mechanical properties. Also, residual cellular components in a bioprosthetic material have been associated with undesired effects, such as immunological recognition and calcification.

The use of xenograft tissues as part of bioprosthetic vascular devices such as heart and vascular grafts has long been the focus of research[13]. The use of natural biomaterials have typically required chemical or physical pre-treatment aimed at (a) preserving the tissue by enhancing the resistance of the material to enzymatic or chemical degradation (b) reducing immunogenicity and (c) sterilizing the tissue. Further cross-linking techniques have been explored to stabilize collagen-based structures of tissue while maintaining its mechanical integrity and natural compliance. In addition to cross-linking, decellularisation approaches may reduce host immune response to bio-prosthesis and generate natural biomaterial for use[12].

The chemicals used at the various steps of processing must be checked for their cytotoxic properties. Decellularised tissues are cross-linked using chemicals. GA treated tissues elicit a cytotoxic T-cell and a humoral cell response when implanted into rats[14]. But cellular debris and extra cellular matrix present in GA treated tissues are thought to contribute to such immune response[15]. Thus immunogenicity can only be partially attenuated by GA. As noted by Carpentier et al[16], cells are the source of immunogenicity, which is followed

by immunological reaction and calcification. This immunological reaction cannot be totally held responsible for calcification, degeneration[16,17], and early graft failure. There could be an immune response if there was any trace of cell remnants after the decellularization and cross linking process. Toxic cross-linking chemicals may be released from the cross-linked tissue despite rinsing due to cross-link degradation or leaching out of GA and its derivatives in the long run[18,19] producing cytotoxic effects[12,20,21]. Alternate storage solutions are being investigated, which have partially reduced the cytotoxic effects[20] (Table 3). However, regardless of the storage solution and prosthetic rinsing, the degradation of the cross-links[22] and the continual release of cytotoxic chemicals appear to contribute to prolonged toxic effects of treated tissues[23,24]. Thus, more comprehensive approaches to reduce the effects of toxic chemicals are still being pursued.

Table 3. SD of viable cell count and cell death

		Cells (x10^5 / ml)					
		Physiological saline		Vegetable oil		Direct contact	
Treatment	Initial concentration	Live cell after treatment	Cell death	Live cell after treatment	Cell death	Live cell after treatment	Cell death
Test concentration – 25 %	4.4	0.06	0.06	0.06	0.06	0.06	0.06
Test concentration – 50%	4.4	0.06	0.06	0.06	0.06	Nil	Nil
Test Concentration - 100 %	4.4	0.06	0.06	0.06	0.06	Nil	Nil
Negative control	4.4	0.06	0.06	0.06	0.06	0.06	0.06
Positive control	4.4	0.06	0.06	0.06	0.06	0.06	0.06

The above values are the mean of triplicate tests

The patent protocol utilizes chemicals at various stages of processing while keeping a keen check on their toxic properties and limits. Novel modifications in processing methodologies have been designed by us, such that the natural biomaterial is devoid of its native adverse qualities, while retaining its mechanical properties and tissue architecture.

The porcine xenografts processed in our laboratory were tested for their cytotoxic properties. They were subjected to tests with BALB C3T3 cell line by direct and indirect contact method. Cell viability count in either tests were similar to negative control, proving the non-cytotoxic nature of the processed grafts. Then systemic toxicity studies and sensitization studies carried out in animals did not show any cytotoxic effects. Thus the protocol rendered the processed porcine xenografts non cytotoxic. Histopathological studies were not conducted in this study but in the earlier experiments,the processed porcine xenograft was exposed to human peripheral blood cells. Tetrazolium(MTT) proliferation assay kit showed that the cells are viable and proliferating on the seeded porcine graft. (Fig.1) The cell viability and presence of dividing cells prove that the processed grafts are perfectly non cytotoxic.

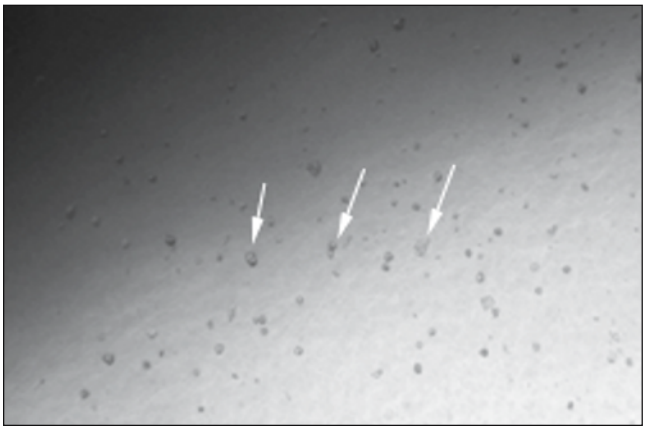

Fig. 1. Figure shows presence of dividing cells (white arrows) proving that the processed grafts are perfectly non cytotoxic.

Conclusion

A novel chemical modification of the biological tissues has been developed in the laboratory. The treated tissue samples were evaluated in vitro and in vivo to investigate the effect of modification. The patent protocol rendered the processed porcine pulmonary conduit non-cytotoxic with negative sensitization and systemic toxicity.

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