Is gamma irradiation as a secondary sterilization procedure required for decellularized xenogenic tissue material?

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Biological tissues for clinical use typically require gamma irradiation to achieve targeted sterility assurance level (SAL). Gamma radiation produces deleterious changes to physical and surface properties of tissues. In this study, we evaluate the requirement of gamma irradiation as a secondary sterilization procedure by comparing it with non-irradiated chemically treated xenograft tissues. Sixty four bovine pericardia (BP) were decellularized and subjected to nonconventional (glutaraldehyde free) cross-linking. Xenograft samples were screened for bacterial and fungal contaminations both at pre- and post-processing stages, after cross-linking and preservation. Microbial evaluations performed revealed that the xenografts were rendered 'microbe free' by subjecting to a new multistaged decellularization technique and cross-linking. Five of these cross-linked tissues were subjected to gamma irradiation as recommended by IAEC and were tested for surface and mechanical properties to understand the ultrastructure, surface and bulk properties. Surface tension and thrombogenicity parameters were also evaluated. Gamma-irradiated specimen showed reduced physical and mechanical properties of these xenogenic tissues significantly along with biological property. Validation and analysis led us to conclude that this microbe-free decellularization method and subsequent processing for xenogenic tissues is a viable alternative for clinical usage without the deleterious secondary sterilization using gamma irradiation.

Keywords: Bovine pericardium, decellularized xenograft, gamma irradiation, thrombogenicity.

THE ability of ionizing radiation to kill microorganisms was established in the past century. Radiation sterilization of biological tissues is a useful tool for reducing bioburden after processing it for clinical use. Gamma irradiation technique is based on the penetrating ability and the ease of delivery of the required dose[1] and it is commonly used as a safe and effective means of secondary sterilization. The minimum acceptable dose of gamma rays for sterilization of biomedical devices is 25 kGy (ref. 2).

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In India, the first tissue bank was started in 1988 in the Tata Memorial Hospital (TMH), as part of International Atomic Energy Agency project to promote the use of ionizing radiation for sterilization of biological tissues[3]. Frozen human skin (stored at –80°C), lyophilized human bone and amniotic membranes were gamma irradiated before being preserved in chemical preservatives or in cryo-preservation. They have also worked on development of xenografts such as lyophilized pig skin and demineralized grounded bovine bone. These tissues were sterilized by gamma irradiation in order to get 10^{-6} sterility assurance level (SAL)[4]. Of late, the tissue engineering endeavour has taken a big leap and the usefulness of xenogenic tissues has been well appreciated by the clinical groups all over the world.

The use of xenografts for tissue engineering and to make it suitable for clinical use requires chemical and physical pre-treatment aimed at removing the immunogenicity of the material, strengthening and sterilizing the tissue and subsequently preserving the tissue for a better shelf life. Bovine pericardium (BP) has been used as a potent clinical biomaterial for any anatomical gap repair in cardiovascular surgeries such as, vascular graft repair and tubular pericardium acting as a blood vessel, closing defect in the heart chambers or patching the vascular tree, pericardial sac closure after open-heart surgery effectively to maintain an anatomical correction and making a heart valve, apart from its many other uses in other sectors of surgical practices.

Bovine parietal pericardium is made up mainly of flattened and overlapping bundles of collagen fibres (basically Type I collagen) oriented in various directions at random[5] (which has got high thrombogenicity) and mesothelial cells with some amount of elastin fibres. In the present study, we evaluate the requirement of gamma irradiation for secondary sterilization by comparing non-irradiated processed xenograft tissues (Group A) and irradiated processed tissues (Group B). The biomaterial is derived from buffalo pericardium and was indigenously processed. Processing involved decellularization (to make it non antigenic), antimicrobial treatment and crosslinking (to strengthen). Subsequently, anticalcium treatment and anti-thrombogenic processes were carried out. As

decellularization ensures complete removal of cellular materials and it is done in a way where the ultrastructure remains intact along with the presence of cell adhesion molecules i.e. the scaffold of the tissue remains intact thereby reducing the probability of tissue rejection after implantation.

Further sterilization of the tissue by gamma irradiation may be detrimental for mechanical and other surface properties[6,7]. In this study, we have focused on analysing changes in surface characteristics of the tissue and overall collagen character and thrombogenicity along with biocompatibility, as they are designed for use in cardiovascular applications such as patches, vascular graft and heart valve materials. We also evaluated the possible bacterial and fungal contamination before and after processing of the bovine pericardium in accordance with our laboratory protocols, to provide a better biomaterial for clinical use. Approvals from appropriate research and ethics committees were obtained prior to undertaking this study.

Materials and methods

Seventy bovine pericardia from the apical region of the bovine heart were harvested from specific pathogen-free animals (Certified abattoir, Medak, Hyderabad, India) soon after the animals were slaughtered. The pericardia were collected in sterile HBSS (Hank Balanced Salt Solution, Sigma) with a cocktail of antibiotics[8] and stored in a refrigerator at 4–6°C as a quarantine procedure until we obtained confirmation of microbiological sterility including RT-PCR for PERV, which was given within 72 h of harvesting. Along with pericardia, approximately 5 ml of the respective animal blood was collected and tested for microbial analysis, including Brucella (abortus and melitensis) and Leptospira broad spectrum.

Serological evaluation from bovine blood serum

Diagnosis of Brucella infection is mainly based on the detection of antibodies in serum by serological tests (Remel-stained Brucella suspensions). Stained Brucella suspensions are used to detect, identify and quantify specific antibodies to Brucella abortus and B. melitensis in sera. Lepto Tek Dri-dot assay (LeptoTek DriDot, Biomerieu X-bv, Netherlands) was used for detection of Leptospira.

Microbial evaluation of the xenogenic pericardial tissue

A small portion of the pericardium was taken before it was processed. This was further cut into smaller pieces and were inoculated into the broth based media and LJ media (Lowenstein–Jensen media) for isolation and culture of Mycobacterium species. The broth-based media used were: (1) Nutrient broth for aerobes; (2) Thioglycholate broth and Robertson's cooked meat media (RCM) for Gram positive and negative anaerobes and facultative anaerobes; (3) Sabouraud's dextrose broth (SDB) for culturing yeasts, moulds and aciduric microorganisms in 37°C; (4) Sabouraud's dextrose broth with antibiotics – SDBA (Gentamycin 80 μg/ml and Chloramphenicol 200 μg/ml) – two tubes, one at room temperature and other at 37°C. SDBA favours the growth of fungus but inhibits bacterial growth.

Any turbidity in the tubes was followed up by Gram's staining and subculture on appropriate plates such as Blood agar for isolation of fastidious organisms and to detect haemolytic activity, MacConkey agar for Gram negative organisms, Sabouraud's dextrose agar for fungus and yeast-like organisms. The organisms grown in the above plates were subjected to biochemical tests for identification.

After the pre-sterility check, selected (completely microbe free) bovine pericardia were subjected to dissection with sterile precautions in clean room under Class II laminar flow unit. The bovine pericardial sacs were separated and freed from fat and fascia for further processing and preservation.

Processing of xenogenic tissue

The processing of the bovine pericardium involved complete decellularization, followed by cross-linking and anti-thrombosis and anti-calcium treatment. The details of the process have been described in a previous article[8] and summarized here.

Complete decellularization was achieved by detergent and enzymatic digestion in an alkaline medium. After sequential washing in non-ionic water, crosslinking was initiated using non-glutaraldehyde treatment. Subsequently, this tissue was treated in solutions of heparin sodium, formalin and glutamic acid for varying durations depending on the thickness of the tissue.

Tissue was then subjected to heparin sodium of similar concentration during the previous process with 70% ethyl alcohol for a week after which microbial surveillance was done again. Fresh solution of above mixture was made to preserve the tissue with or without gamma radiation sterilization. A gamma dosage of 2.5 MRad (25 KGy) was used for sterilization of the specimen. Ethyl alcohol was used to prevent calcification of the biomaterial *in vivo* and heparin used to prevent initial thrombogenicity *in vivo*.

Analysis of surface and mechanical properties of the processed tissues

The two groups of tissues, groups A and B, were taken for different microscopical analysis (Confocal laser scanning microscopy (CM), transmission electron microscopy

(TEM) and environmental scanning electron microscopy (ESEM)), followed by mechanical testing and finally thrombogenicity studies *in vitro*. A collagenase degradation study was also performed in these two groups of BP by bacterial collagenase. All the laboratory procedures followed were according to the protocols standardized and optimized in our laboratory[6–8]. Mechanical studies, thrombogenicity studies and TEM were conducted in a CE approved laboratory (Sri Chitra Thirunal Institute of Medical Sciences and Technology, Thiruvananthapuram, India); ESEM conducted in Indian Institute of Technology, Madras, India and Laser Confocal Microscopy was conducted in IGCAR, Kalpakam, India.

Mechanical tests: Tensile tests were performed on testing machine (Instron series IX), at a cross head speed of 10 mm/min. Dum-bell shaped samples were prepared from the tissue using ISO 527-2:1993(E) specimen type 5B die and immersed in water until the time of test. Each group had seven samples tested. Exclusion criteria used were breakage at different sites and premature break.

Confocal laser microscopy: Confocal scanning laser microscopy (CSLM) required drying of tissue then stained with protein fluorescence. For confocal microscopy two different specimens were subjected to fluorescence staining and then subjected to examination. They were visualized under microscope with Argon laser waves with a wavelength of 488 nm. The original mesothelial surface side of the two groups was studied under CSLM without fluorescence.

Transmission electron microscopy: Transmission electron microscope study of the same materials was undertaken (groups A and B). Ultrathin sections (50–70 nm) were cut using diamond knife, stained with uranyl acetate and lead citrate and observed in TEM (H-600).

Environmental scanning electron microscopy: ESEM study was done under 500 and 1000 magnifications. Figures 1 and 2 show the various ESEM images.

Results

Six of the bovine pericardium tissues were rejected before being subjected to processing. Four of them were rejected due to microbiological contamination (Lepto Dridot test positive) and two for shape deformities (high thickness, irregular shape and sizing of the tissue). All other samples cleared the microbiological tests before and after processing as they qualified microbiological surveillance for bacterial and fungal contaminations after cross-linking and after preservation. A week after preservation, small bits of the tissues from the decellularized bovine pericardia were subjected to microbial testing as performed before processing. All the broth based media were kept for one month and checked for any organisms/contamination. None of the samples had shown any microbiological growth throughout the processing or after preservation. Hence, this primary sterilization procedure for xenogenic tissue was referred to as 'microbe-free' processing. The uniqueness of the procedure revealed in H&E staining and also by agarose gel electrophoresis total acellularity (Figure 3). Table 1 gives a summary of CM, TEM and ESEM findings. This confirms the extent of crosslinking in the processed bovine pericardium which is further supported by the mechanical strength studies.

An interesting observation was made from CM without fluorescence staining of the tissues. The group A tissue appeared to be smoother on the luminal surface and very sparse light penetration deficiency was noticed (green luminescence) only up to 3.54 μm. Whereas in the gamma irradiated group B, BP had shown pitting of the surface with light penetration giving a sieve-like appearance (green luminescence) with a depth of penetration of light 19.08 μm (Figure 4).

TEM studies showed organized ultrastructure of BP collagen fibres in group A but after treatment with gamma ray, the fibres appeared to be disorganized as in Figure 5. This study exposed damages to the collagen fibril at places in group B. The ESEM picture revealed the fragmentation of surface collagen of the gamma-treated group B where the pericardium showed a granular appearance (Figure 2 *b*).

The mechanical testing of tensile strength, elongation percentage at break and Young's modulus were the highest in case of group A when compared to group B as

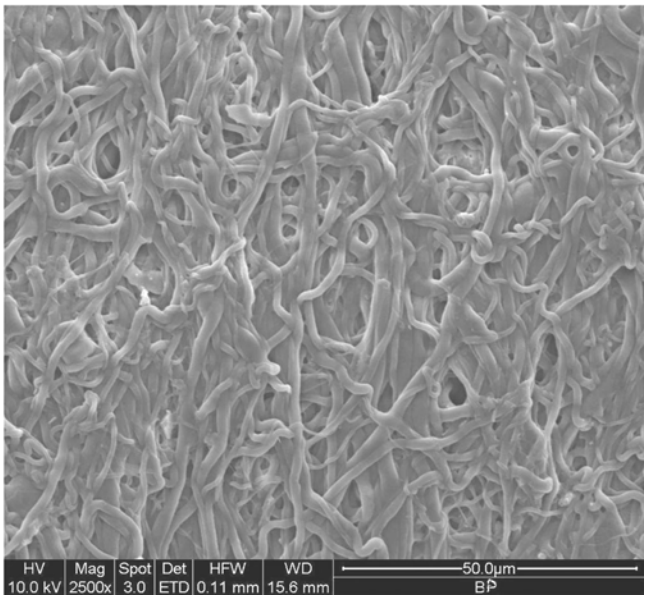

Figure 1. Group A processed BP with effective cross-linking was seen under SEM at 5000×, showing homogeneous surface orientation of the collagen fibres.

Table 1. Findings of various microscopical, physical and thrombogenic studies of the two groups of processed bovine pericardium (group A and group B). It is observed that the heparin cross-linked BP (non-irradiated) (group A) has better properties under all tests

Properties	Group A	Group B
Microsopic		
Confocal laser microscopy ARG ion laser wavelength 488 nm	Homogeneous (Figure 4 *a*) appearance with less light penetration, few deficiency with green luminescence	Sieve-like appearance of the material with plenty of deficiency in the material with green luminescence of light penetration, thickness of 19.08 mμ (Figure 4 *b*)
Transmission electron microscopy (10000X)	Collagen fibril integrated in organized way (Figure 5 *a*)	Disorganized collagen fibrils with large deficiencies (Figure 5 *b*)
Environmental scanning electron microscopy [10,000× (a and b) and 500× (a′ and b′)]	Tissue density is more and acellular surface with organized lie of the fibrils. Figure 2a, a′	Fragmented matrix with granular surface Figure 2b, b′
Physical		
Tensile strength (MPa)	18.29 ± 4.46	13.46 ± 4.31
Elongation at break (%)	122 ± 40	114 ± 19
Young's modulus (MPa)	49.7 ± 14.6	33.0 ± 08.9
Thrombogenic		
Platelet reduction (%)	6.67	35.52
Leucocyte reduction (%)	3.39	5.00
Fibrinogen reduction (%)	12.43	28.13
Clotting time acceleration	–2.69 min	–40.05 min

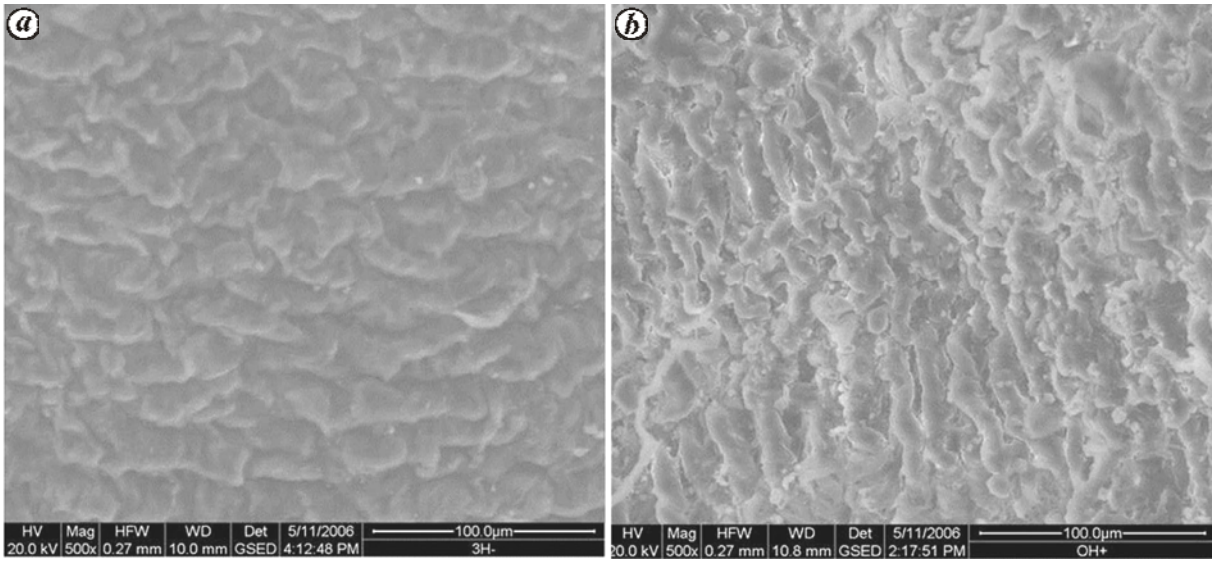

Figure 2. Bovine pericardium under scanning electron microscope at 10,000× and 500× magnification. ***a***, Homogenous surface appearance without any fragmentation in the surface fibres; ***b***, Granular, fragmented and embrittled surface in the gamma irradiated group B specimen.

shown in Table 1. Collagenase degradation study for 20 h in bacterial collagenase did not show any change in any of these tissues and both the groups retained their weight after the treatment. Subsequently, thrombogenicity studies were performed and its summary is shown in Table 1. The lowest haemolysis was observed in group A but overall haemolysis was not significant in any of these tissues. The overall thrombogenicity study revealed group A had the best nonthrombogenic property, whereas the thrombogenicity increased significantly *in vitro* when the group B material was used. The platelet percentage reduction went up to >35; fibrinogen reduction percentage was >28 and accelerated clotting time became 40 (ref. 7).

Discussion

Radiation sterilization has extensive applications in sterilization of medical devices and biological tissues. Isotope

sources used are either machine generated accelerated electrons or gamma rays from Cobalt-60. Gamma irradiation is the most popular sterilization technique used when materials are sensitive to high temperature of autoclaving, but compatible with ionizing radiation, such as biological tissues where protein denaturation occurs at high temperature. The suitable dose validated to sterilize medical devices for a defined period of time is 25 kGy.

The bactericidal effect of gamma ray depends on oxidation level of biological tissue. It is a simple, rapid and efficacious method of sterilization requiring high capital expenditure. This sterilization is known to cause physical changes, including embrittlement, discolouration, odour generation, stiffening, softening, increase or decrease in melting temperature and decreases in molecular weight[9]. These changes are due to chain scission, low-molecular weight fragments and unsaturated bonds releasing gaseous vapours.

Mechanical properties such as tensile strength, elastic modulus, impact strength, shear strength and elongation can also be affected. It had been reported that physical properties in medical grade ultra high molecular weight polymers used in joint reconstruction surgeries were modified when treated with gamma ray, and which was due to the change in crystalline property as chain scission continued[10]. The evidence of embrittlement is obvious in TEM and confocal microscopic pictures of our material. Cross-linking can increase tensile strength initially in polymers while impact strength decreases. Collagen digestion with bacterial collagen did not reduce weight of the two groups of pericardium, indicating collagenase resistance of the tissues. Enzymatic digestion *in vivo* had most probably been protected by gamma ray that adds to the advantage of the method of sterilization[11]. This may be due to further crosslinking of the tissue by ionizing radiation which made the tissue stable though brittle[12,13]. However this benefit was achieved at the cost of the significantly decreased mechanical property (Table 1) in group B material.

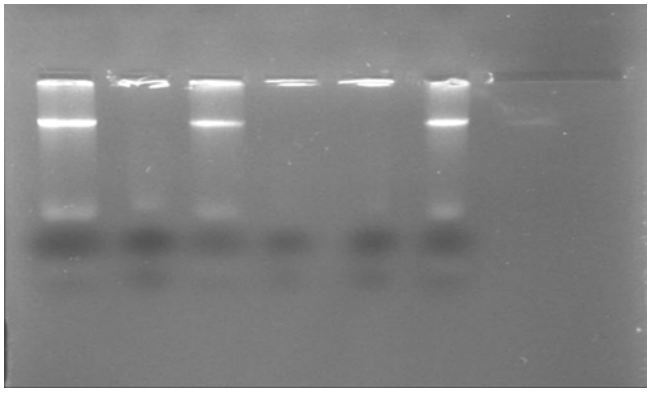

Figure 3. An agarose gel electrophoresis showing absence of genomic DNA after the processing of xenograft (1,3,6 – cellular; 2,4,5– decellular).

Thrombogenicity is a surface property of biomaterials and is important, especially when it is designed for use in the vascular system where it is in direct contact with blood. Clot formation and subsequent occlusion of the vascular system by thrombosis or embolization of thrombus in the distal flowing area of circulation are due to the high thrombogenicity of the biomaterial. In addition, thrombus or platelet aggregation can produce calcification of the biomaterial. Group A (non-irradiated) material appeared to be highly compatible in this respect in comparison to the reference values[7], but being a xenogenic material, inclination is towards gamma ray sterilization to safeguard the controversy of sterility management. Following the stipulation for anticipated bio-burden would be the conventional practice of further gamma irradiation if we accept the older practice. However if a material can be proved to be microbe free without undergoing the irradiation process, necessity of this physically destructive process needs to be questioned. The sufficient conditions to prove this would be practically implausible. On the positive perspective, gamma irradiation has one important role other than sterilization; it increases the tissue's resistance towards *in vivo* enzymatic digestion[11]. In this study, a single dose of 25 kGy (2.5 MRad) gamma irradiation was used for sterilization in group B. Although this is a high dose of radiation, this is the norm decided by IAEC.

A dose-response relationship was demonstrated, with the greatest effect seen at higher doses (>0.014 kGy)[14–16]. Intra-arterial beta irradiation at a dose of 0.018 kGy had been demonstrated to be effective in hyper-cholesterolemic coronary artery restenosis in rabbits[14]. Hehrlein *et al.*[15] showed that a radioactive stent can inhibit neointimal proliferation after implantation in non-diseased rabbit iliac arteries. At 4 and 12 weeks after stent implantation, there was almost complete inhibition of neointimal proliferation in their subjects. Though these are the positive properties of dose-dependent gamma irradiation on the cardiovascular implants, the surface damage is evident in the biological material due to oxidation, which is detrimental for thrombogenicity and for mechanical sturdiness. So the dilemma remains whether to irradiate or not.

Considering the positive effects and safety issues of irradiation, a compromise can be drawn in this dilemma. As platelet aggregation (or thrombogenicity) is a major cause of concern, conventional anti-platelet (or anti-coagulation) therapy in the initial stages of implantation of gamma-irradiated xenograft can be initiated. Typically these patients could be on the anti-platelet therapy for a period of 3–6 months. In current surgical practice for biological prosthesis, such as bovine pericardial valve implantation, the patients are required to have oral anti coagulants for 3–6 months after implantation[17]. This kind of surface damage whether can be rectified with mere medication needs to be experimentally proven. It is

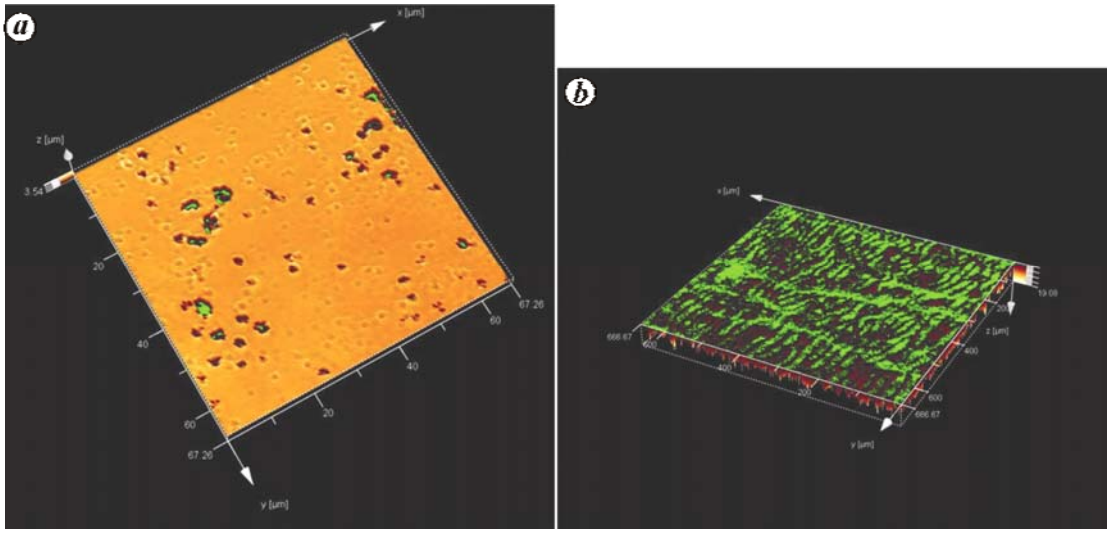

Figure 4. Confocal microscopy of heparin cross-linked bovine pericardium showing luminal surface appearance and light penetration. ***a***, Chemically processing (group A) tissue showing smooth surface with few pittings (seen as green luminescence). Highly crosslinked as the depth of light penetration is 3.54 μm. ***b***, Gamma-irradiated (group B) tissue with shallow sieve-like pits. Multiple deficiencies (green hue) seen due to embrittlement. The light penetration thickness is 19.08 μm suggesting reduced crosslinking than group A.

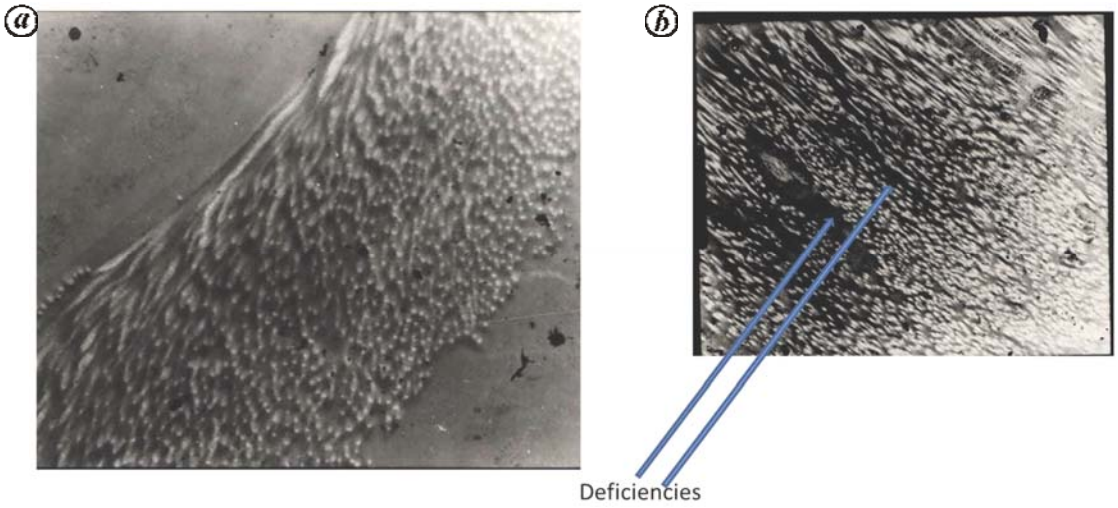

Figure 5. Bovine pericardium under transmission electron microscope at 10000× magnification. ***a***, Highly organized collagen fibril in group A; ***b***, Breach in the organized fibrils of the gamma-irradiated group B specimen.

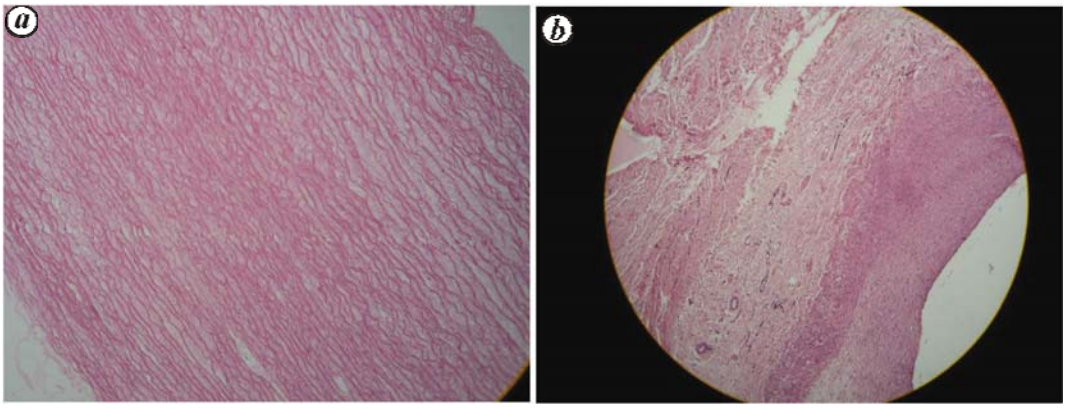

Figure 6. Histological studies using H&E staining when viewed through optical microscope at 10× magnification. ***a***, Acellular bovine pericardium after processing. ***b***, Group A pericardia explanted after six months in sheep as a jugular vein tubular interposition graft. Note the autologous cell seeded and cellular layers formed in the scaffold.

expected that the deficiencies on the surface (such as the sieve-like appearance) will endothelialize during this period and hence lifelong anti-coagulation therapy will not be necessary. But whether that can increase the strength of the tissue in the long run is yet to be proved as well.

Mechanical strength of BP in group B had reduced to 13.46 MPa, which was significantly lower than group A (18.4 MPa), but this value is still higher than the native bovine pericardium mechanical strength (8.24 MPa)[18]. Its use as a vascular graft or patch material may be permissible in low pressure area of circulation but its use still remains questionable in high pressure system, i.e. systemic side of blood circulation, due to considerable reduction in material property tensile strength. Considering this and taking into account the results obtained from this study, the question arises if it is possible to proceed without gamma irradiation as a secondary sterilization process. Secondary sterilization has been termed so because the decellularization procedure and the crosslinking procedure have the primary sterilization goal as they are removing cells and cellular elements where organisms will also succumb if present. Before the non-conventional crosslinking procedure, a step we adopt where we are using formalin 4% and 10% at stages during xenogenic tissue processing with an idea to protect the material from viral affection and then detoxifying the aldehyde with glutamic acid treatment as the part of the procedure. The antiviral effect of the formalin is well known. Finally 70% if ethyl alcohol was added as one of the ingredients in preservation. Subsequently, the material had been tested *in vitro* for cytotoxicity and guineapig hypersensitization studies were performed. Both of them provided favourable outcomes for the material. Animal experiments with regulatory permission also had proved the efficacy of the material which was microbe-free without gamma ray sterilization[7].

During earlier days, irradiation of the biological tissue was considered probably because the tissues were cellular, such as bone bank, skin bank, etc. Present day study and practice of tissue engineering is to make the natural biomaterial completely acellular. In that context probably we have to rethink about gamma irradiation mandate, when knowingly the quality of the scaffold decreases in terms of physical properties especially in the vascular system which is under continuous wear and tear due to lifelong flow pulsatility.

The acellular non-conventionally cross-linked xenograft tissue (group A) had been proved as a good substrate for cell growth (Figure 6) during *in vivo* experiments, thereby strengthening the material over time. If a lower dosage of irradiation can be determined, tissue damage in group B can probably be minimized. Perhaps in future one could achieve SAL by means of fragmented smaller daily doses of less than 1 MRad of gamma irradiation in lieu of a large single dose currently being used. This is likely to reduce the harmful effects on the biomaterial to be sterilized[11]. Thus the benefits of radiation can be gained without compromising on structural integrity of the implant material.

The microbiological surveillance data suggest that the bovine pericardium is fully sterilized and is totally 'microbe free' after processing of tissue by these new methods[6]. This primary sterilization procedure also maintains the integrity of the processed tissue without damaging the surface, making it suitable for clinical use. Though it is imperative to protect biological tissue damage from radiation sterilization, by and large, one common view was that the use of the ionizing radiation for the sterilization/decontamination of tissues in the preliminary stage of processing method could be done, so that by modifying further processing, the tissue damage could be prevented. But as radiation crosslinks the tissues, complete xenogenic cell removal would have been an impossible task. It was recognized that existing methods for sterilizing tissues are proving inadequate in many instances. Infections have been transmitted from the graft to the recipient and in USA, the Centre for Disease Control and other regulatory bodies are concerned for the development of a reliable end sterilization method which does not damage the functionality of the final tissue[19].

Sterilization doses of radiation for medical devices were derived from basic and applied research on microbiology and also from experiences. Since sterilization is a probabilistic event and not absolute, it is difficult to express it in mathematical context, as completeness is not achieved. Sterilization index is considered in terms of destruction rate, regarded as D value. According to D-value, the medical device sterilization dose by gamma radiation had been standardized to 2.5 MRads. The dose level had been fixed irrespective of batch size in order to achieve probability of contaminant survivors below 10^{-6}, from the original level of contamination[20]. Whether the above suggestion holds good in this context is a matter of further research.

Conclusion

Gamma irradiation damages the cross-linked collagen in processed bovine pericardium. This results in higher thrombogenicity and lower mechanical property and poor structural integrity. The present new, complete, microbe-free decellularization methods and subsequent processing protocols is a plausible alternative to the deleterious gamma irradiation, a secondary sterilization process, to achieve the required SAL.

1. Roberto, S. B., Use of radiation in biomaterials science. *Nucl. Inst. Meth. B*, 2001, **191(**1–4), 752–757.
2. Hafeez, Y. M. *et al.*, Effect of freeze-drying and gamma irradiation on biomechanical properties of bovine pericardium. *Cell Tissue Bank*, 2005, **6**, 85–89.

3. Lobo, G. A., Tissue banking in India: Gamma-irradiated allografts. *Cell Tissue Bank.*, 2003, **4**, 203–211.
4. Sterilization of health care products – Radiation – Part 2: Establishing the sterilization dose, ISO 11137-2: 2013.
5. Tokuhiro, I., Victor, J. F., Michael, J., Steven, W. B., Boyce, B. A. and William, C. R., Structure of bovine parietal pericardium and of unimplanted Ionescu–Shiley pericardial valvular bioprostheses. *J. Thorac. Cardiovasc. Surg.*, 1981, **81**, 747–757.
6. Balasundari, R. *et al.*, Post processing complete microbe free porcine xenograft suitable for clinical use. *Indian J. Thorac. Cardiovasc. Surg.*, 2007, **23**, 240–245.
7. Guhathakurta, S. *et al.*, Thrombogenicity studies of three different variants of processed bovine pericardium. *IRBM*, 2008, **29**(4), 223–230.
8. Guhathakurta, S. *et al.*, Technique to process xenogenic tissues for cardio vascular implantation. *Curr. Sci.*, 2006, **91**(8), 1068–1073.
9. Simmons, A., Sterilization of medical devices business briefing. Graduate school of Biomedical Engineering, The University of New South Wales, Business briefing: Medical device manufacturing and technology, 2004.
10. Rimnac, C. M., Klein, R. W., Betts, F. and Wright, T. M., Post-irradiation aging of ultra-high molecular weight polyethylene. *J. Bone Joint Surg.*, 1994, **76**(7), 1052–1056.
11. Cheung, D. T., Perelman, N., Tong, D. and Nimne, M. E., The effect of gamma-irradiation on collagen molecules, isolated alpha chains, and cross-linked native fibres. *J. Biomed. Mater. Res.*, 1990, **24**(5), 581–589.
12. Hill, D. J. T., Lewis, D. A., O'Donnell, J. H. and Whittaker, A. K., The crosslinking mechanism in gamma irradiation of polyarylsulfone: evidence for Y-links. *Polym. Adv. Technol.*, 1998, **9**(1), 45–51.
13. Oral, E. and Muratoglu, O. K., Radiation cross-linking in ultra-high molecular weight polyethylene for orthopaedic applications. *Nucl. Instrum. Methods Phys. Res. B*, 2007, **265**(1), 18–22.
14. Verin, V. *et al.*, Intraarterial beta irradiation prevents neointimal hyperplasia in a hypercholesterolemic rabbit restenosis model. *Circulation*, 1995, **92**, 2284–2290.
15. Hehrlein, C., Zimmermann, M., Metz, J., Fehsenfeld, P. and von Hodenberg, E., Radioactive coronary stent implantation inhibits neointimal proliferation in non-atherosclerotic rabbits. *Circulation*, 1993, **88**(s1), 1–651.
16. Hehrlein, C., Riessen, R., Gollan, T., Dsnges, K., Fehsenfeld, P. and von Hodenberg, E., Arterial wall cell distribution after implantation of radioactive stents. *Circulation*, 1994, **90**(s1), 1–597.
17. Stein, P. D., Alpert, J. S., Dalen, J. E., Horstkotte, D. and Turpie, A. G., Antithrombotic therapy in patients with mechanical and biological prosthetic heart valves. *Chest*, 1998, **114**, 602s–610s.
18. Forti, F. L., Goissis, G. and Plepis, A. M. G., Modifications on collagen structures promoted by 1,4-dioxane improve thermal and biological properties of bovine pericardium as a biomaterial. *J. Biomat. Appl.*, 2006, **20**, 267–285.
19. Kennedy, J. F., Phillips, G. O. and Williams, P. A., *Sterilisation of Tissues Using Ionising Radiations*, ISBN-10: 0849337976/ISBN-13: 978-0849337970, CRC Press, 2005.
20. Mukherjee, R. N., *Radiation: A Means of Sterilization, Radiation Biology*, Division of Life Sciences, IAEA; http://www.iaea.org/Publications/Magazines/Bulletin/Bull176/17605882837.pdf

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