STUDIES ON IMMUNOGENICITY OF DECELLULARISED TISSUE AND ITS EFFECT ON *IN-VIVO* TISSUE REGENERATION

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Ph.D THESIS

2011

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram

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A THESIS PRESENTED BY

P.R. UMASHANKAR

TO

THE SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY,
TRIVANDRUM
Thiruvananthapuram

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF
DOCTOR OF PHILOSOPHY

2011

DECLARATION

I, P.R. Umashankar, hereby declare that I had personally carried out the work depicted in the thesis entitled **'Studies on immunogenicity of decellularised tissue and its effect on *in-vivo* tissue regeneration'** under the direct supervision of Dr. T.V. Kumary, Scientist-G, Division of Implant Biology, Biomedical Technology Wing, Sree Chitra Tirunal Institute of Medical Sciences and Technology, Trivandrum. External help sought are acknowledged. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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Date: 26-7-2011

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CERTIFICATE

This is to certify that Dr. P.R. Umashankar in the Division of In-vivo models and Testing, of this Institute has fulfilled the requirements prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

The thesis entitled **'Studies on immunogenicity of decellularised tissue and its effect on *in-vivo* tissue regeneration'** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date. Clearance was obtained from Institutional Animal Ethics Committee for carrying out the study.

Dr. T.V. Kumary

Date: 26-7-2011

The thesis entitled

'Studies on immunogenicity of decellularised tissue and its effect on *in-vivo* tissue regeneration'

Submitted by

Dr.P.R.Umashankar

for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
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ACKNOWLEDGEMENTS

I sincerely thank my research supervisor, Dr. TV Kumary, for her patience, support, advice and guidance throughout the research work. I also wish to thank her for all her efforts in correcting this thesis amidst her other responsibilities. I thank members of my doctoral advisory committee, Dr. G.S. Bhuvaneshwar, Head BMT Wing and Dr. T. Anoop Kumar Scientist F for all the support, suggestions, ideas and comments at each stage of the work.

I remember Dr. K. Mohandas, our former Director, who has inspired me to undertake such an endeavour. I am grateful to Prof. K. Radhakrishnan, Director of our Institute for all the support provided during the research work. I am thankful to The Dean, Associate Dean, Registrar and Deputy Registrar for their academic assistance.

I am indebted to Department of Biotechnology for funding the project from which this research work could be accomplished. I am thankful to Dr. Krishna Manohar, former Prof. of Paediatric Cardiac Surgery, SCTIMST and Dr. Wolfgang Goetz MD, PhD (Cardiac Surgery), Transcatheter Technologies, GmbH, Regensbrug, Germany, whose constant inspiration, advice and timely help by providing valuable literature has propelled me to do this arduous task. I cannot forget Mr. Arun T, Dr. Sajeesh. S, Mr. Prem Mohan, Dr. Sachin J Shenoy, Dr. Lekshmy, Dr. Sabareeswaran and all the staff of my division for their valuable help during this research work. I thank Prof. C.C. Kartha, RGCB from whom I have learned much. I am thankful to my

senior colleagues Er. Muraleedharan C.V, Er Nagesh D.S, Dr. Maya Nandakumar, Dr. Mohanan, P.V, Dr. Lissy K.K, Dr. Kalyana Krishan, Dr. K. Sreenivasan, Dr. Prabha D Nair, Dr. Sharma CP, Dr. Mira Mohanty and Dr. H.K Varma for all the support.

This page will not be complete unless I acknowledge the support of my wife Sunitha, daughter Keeku and son Chankaran for sharing my frustrations and often bearing with my absence during the last few years. I am reminded of my aged parents Shri. Ramachandra Iyer and Smt. Lalitha, who have wondered 'what happened to their son' for the last few years, for he is silent most of the time.

Finally I submit myself to the will of the 'God', who makes my path and also is my fellow traveller.....

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ABBREVIATIONS

%	:	Percentage
μ	:	Micron
μl	:	Micro liter
0.2DCL	:	Minimally glutaraldehyde crosslinked decellularised bovine pericardium
0.6DCL	:	Moderately glutaraldehyde crosslinked decellularised bovine pericardium
^{0}C	:	Degree Celsius
ASTM	:	American Society of Testing and Materials
DMSO	:	Dimethyl sulphoxide
ECM	:	Extracellular matrix
EDCL	:	Enzyme decellularised bovine pericardium
EDTA	:	Ethylene Diamine Tetra Acetic Acid
ELISA	:	Enzyme-linked immunosorbent assay
eNOS	:	Endothelial nitric oxide synthase
ESEM	:	Environmental scanning electron microscopy
FBS	:	Fetal Bovine Serum
FCS	:	Fetal Calf Serum
FDA	:	Food and Drug Administration
GlutBP	:	Fully glutaraldehyde crosslinked non-decellularised bovine pericardium
h	:	Hours
HCl	:	Hydrochloric acid

HE	:	Hematoxylin and Eosin stain
HRP	:	Horseradish peroxidase
IHC	:	Immuno-histochemistry
IL	:	Interleukin
ISO	:	International Organization for Standardization
KD	:	Kilo Dalton
mg	:	Milligram
ml	:	Milli liter
mM	:	Milli molar
MTT	:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl		Sodium Chloride
NaOH	:	Sodium hydroxide
NIH		National Institute of Health
nm	:	Nano meter
OD	:	Optical Density
PAGE		Polyacrylamide gel electrophoresis
PAMP	:	Pathogen Associated Molecular Pattern
PBS	:	Phosphate Buffer Saline
PMA	:	Phorbol 12-myristate 13- acetate
RPMI	:	Rosewell Park Memorial Institute
RT	:	Room Temperature
SC	:	Subcutaneous

SDS	:	Sodium Dodecyl Sulphate
TE	:	Tris EDTA
TGF	:	Transforming growth factor
TMB	:	3,3',5,5' tetramethylbenzidine
TNF	:	Tumor necrosis factor
UTHSCSA	:	The University of Texas Health Science Center at San Antonio
VEGF	:	Vascular endothelial growth factor

SYNOPSIS

The research work presented here attempts to find out the effect of immune response, both innate and acquired, elicited by decellularised xenograft on induced regeneration in adult animal models. Adult mammals respond spontaneously to a severe injury by scar formation instead of regeneration. In contrast, foetal healing response leads to regeneration of the injured organ which is the structural and functional recovery of the organ at the original anatomical site. It was observed that with the evolution of adaptive immune system, appearance of new cell types and new uses of cell signaling cascades have changed the response of appendages and other organs to injury. Scarless skin wound healing occurs through out embryonic development and during fetal stages equivalent to the early second trimester of human gestation, after which there is a transition to the typical scarring repair seen with adult skin. The basis of scarless repair is poorly understood, but considerable evidence points to a central role for components of the immune system. The acute inflammatory response is minimal in fetal skin regeneration but during the transition to adult type scarring, there is a gradual increase in the level of inflammation and specific changes in the types of immune cells elicited in the wound with proportionately more monocytes/ macrophages infiltration occurring in comparison to neutrophils in scarless healing. The above observations clearly indicate a possible role for immune response in the process of regeneration or scarless healing.

Decellularised xenograft is an excellent scaffold which can be used for the purpose of induced regeneration. Decellularised tissue produced from different sources and by different methods is known to produce a host of healing response ranging from scarring

to more desirable regenerative response, the reasons of which is not fully elaborated. Apart from inflammatory effect of residual detergents, immunogenicity of decellularised xenograft is known and it is reported that the immune response against incompletely decellularised xenograft was seen much more prominent compared to isografts or even allografts. It was also reported that certain decellularised tissue products has produced induced regeneration, while certain other similar products failed to induce such a response. The effect of immune response on the above observation is not reported so far. It was also observed that during regeneration M2 phenotype of macrophages and promotion of Th2 pathway was prevalent.

In this study, it is thus hypothesized that the immune response, innate or acquired elicited against the decellularised scaffold may have an effect on the induced regeneration. The present research work attempted to record the effect of immune response on the regenerative response elicited by decellularised xenograft such as bovine pericardium in an adult animal model of regeneration. In order to achieve this, our study had the following objectives.

1. To characterize decellularised bovine pericardium (BP) and to chemically modify it to induce variation in *in-vivo* response (Phase 1).
2. To assess induction of variation in *in-vivo* response between differently treated decellularised bovine pericardium on account of chemical modification. (Phase 2).

3. To assess and compare innate and acquired immune response between different treatment groups and to analyze Th1/Th2 and M1/M2 preference exhibited by them (Phase 3).
4. To study regeneration in different treatment groups using adult animal models of regeneration and to correlate chemical modification, the difference in immune response, Th1/Th2, M1/M2 preferences with the observed 'Induced regeneration' (Phase 3).

In the Phase 1 of the study, decellularisation was achieved using a proprietary non-detergent based method which was developed to avoid inflammatory effect of residual detergents. Decellularisation was confirmed by demonstrating absence of nuclear remnants using routine HE staining and nuclear stain such as Hoechst 33258. It was further characterized by estimating residual DNA and extractable protein. Environmental scanning electron microscopy was done to study the surface and Movats Pentachrome staining of sections was done to study the structure. Decellularised tissue was chemically cross-linked with glutaraldehyde at different levels to yield the following treatment groups, 1. EDCL: unmodified decellularised bovine pericardium, 2. 0.2DCL: mildly cross linked decellularised bovine pericardium, 3. 0.6DCL: moderately crosslinked decellularised bovine pericardium and 4. GlutBP: fully cross linked normal bovine pericardium. Glutaraldehyde cross-linking was confirmed by studying Collagenase resistance (Collagenase type2), Shrinkage temperature, Thermogravimetry and Contact angle measurement. Preliminary screening for basic biocompatibility using

tests such as sterility testing, endotoxin testing and cytotoxicity testing of samples and their extracts were done to qualify them for further studies.

In the phase 2 of the study, the *in vivo* response against different treatment groups was studied by subcutaneously implanting samples into Wistar rats. Here, antibody response (indirect ELISA), delayed hypersensitivity response, calcification response and tissue response were recorded at the end of 60 days implantation.

In phase 3 of the study, both innate and acquired immune response were studied using *in vitro* and *in vivo* methods. *In vitro* macrophage activation potential of different treatment groups was studied by estimating the release of cytokines such as TNF alpha, IL1beta, IL6 and IL10 on macrophage transformed THP-1 (human acute monocytic leukemia cell lines) using specific ELISA kit. This was followed by *in vivo* evaluation of inflammatory response of the different treatment groups by implanting samples in to the abdominal wall of adult Wistar rats and the type and intensity of the inflammation observed was semi-quantitatively recorded at 21 and 90 days. Acquired immune response was studied by assessing humoral immune response against bovine pericardial proteins and cell mediated immune response by using Lymphocyte transformation test. Antibody response (IgG, IgM and IgA) against fresh bovine pericardial proteins was assessed using indirect ELISA. In the Lymphocyte transformation test, presence of antigen (fresh bovine pericardial protein) sensitized lymphocyte were detected by isolating them from homogenized spleen in animals implanted with different treatment groups and their proliferation response in the presence of fresh bovine pericardial protein was studied using MTT assay at the end of

7 days incubation. Th1/Th2 preference was delineated in different treatment groups by studying the type of IgG response, IgG1(Th2 response) or IgG2 (Th1 response) elicited in different treatment groups at 21 and 90 days using indirect ELISA. M2/M1 preference shown by different treatment groups was identified by looking in to the predominance of M2 (CD163+) or M1 (CD80+) phenotype of macrophages observed in the tissue sections as demonstrated by immunohistochemistry following abdominal wall implantation at 21 days and 90 days in adult Wistar rats. Regeneration was studied in rat abdominal wall regeneration model at 21 and 90 days. For rat abdominal regeneration model, a full thickness abdominal defect was made and the regenerative response at 21 days and 90 days elicited by the different treatment groups were studied using HE staining as well as special stains to identify different structural components of abdominal wall regenerate. The observations were recorded and compared between treatment groups. The difference in healing response in another tissue site and another species was studied through aortic implantations in pig model for 6 months duration and the regenerative response was studied by Hematoxylin-eosin stain, Massons Trichrome and immuno-labeling of alpha actin and ENOS.

The data was subjected to appropriate statistical tests and statistically significant difference between means was assumed whenever p value was less than 0.05.

In the Phase 1, decellularisation could be done effectively and chemical modification yielded different bovine pericardium such as uncross-linked decellularised bovine pericardium (EDCL), minimally cross-linked decellularised bovine pericardium (0.2DCL), moderately crosslinked decellularised bovine pericardium (0.6DCL) and

fully cross-linked non-decellularised bovine pericardium (GlutBP). They differed in characteristics such as collagenase resistance, shrinkage temperature, contact angle, thermal degradation confirming the intended chemical modification. On biocompatibility screening, all the samples passed sterility test and were endotoxin free. With respect to cytotoxicity, only GlutBP samples were moderately cytotoxic and rest were non-cytotoxic.

In the Phase2 studies, different treatment groups varied in their *in vivo* response further confirming modulation of in vivo response consequent to chemical modification. In the Phase 3, different groups were further studied for their potential to activate innate and acquired immune response. Fully cross-linked bovine pericardium (GlutBP) activated macrophages indicating stimulation of innate immune response. Significant acute inflammatory response was also noticed indicating a similar trend in *in vivo.* Mildly cross-linked and uncross-linked decellularised bovine pericardia did not activate macrophages. In the *in vivo* situation, uncross-linked decellularised bovine pericardium showed more plasma cell response compared to mildly cross-linked decellularised bovine pericardium. Otherwise both uncross-linked and mildly cross-linked decellularised bovine pericardium showed similar picture of chronic inflammation consisting of macrophages and lymphocytes. With respect to acquired immune response, bovine pericardial protein sensitized lymphocytes were noticed at both 21 and 90 days in uncross-linked decellularised bovine pericardium implanted animals. Similarly antibody response against bovine pericardial protein was high and sustaining, indicating sustained stimulation of acquired immune response in this group. In comparison

sensitized lymphocytes could not be detected in mildly cross-linked decellularised bovine pericardium implanted animals whereas sensitized lymphocytes could only be noticed at 21 days and un-detectable at 90 days in fully cross-linked bovine pericardium implanted animals. Similarly, antibody response was also high at 21 days and was significantly reduced at 90 days in these groups. In subcutaneously implanted animals, a preference of IgG1 over IgG2 was noted prominently in un-crosslinked decellularised bovine pericardium implanted animals, indicating a preference for Th2 response which may favor a regenerative response. Mildly cross-linked decellularised pericardium implanted animals showed a moderate Th2 preference and minimum Th2 response was seen in fully cross linked bovine pericardium group. IgG1/IgG2 levels in abdominal muscle implanted animals were not at all predictive and fully cross linked bovine pericardium implanted animals showed maximum response. A predominance of CD163+ macrophages (M2 phenotype) was seen maximum at 21 days in uncross-linked decellularised bovine pericardium implanted animals, which was reduced at 90 days, nevertheless a trend for M2 predominance continued even at this period. Interestingly, mildly cross linked group showed predominance for M2 phenotype at 21 days and this trend was revered at 90 days. Fully cross-linked bovine pericardium showed a predominance of M1 phenotype of macrophages indicating a sustained inflammatory response not favoring regeneration. At 21 days, in rat abdominal regeneration model, indications of regeneration could not be observed in all the groups, although extension of muscle bands could be seen at the peritoneal side through the mesenteric attachments in all the groups. At 90days, regeneration could be appreciated in un-crosslinked

decellularised bovine pericardium group alone, which was not observed in either mildly cross-linked decellularised bovine pericardium or fully cross-linked bovine pericardium. No aortic wall regeneration could be observed in pig aortic model at 6 months in all the groups, although a difference in healing response could be appreciated in this model. Additional experiment on cell adhesion study revealed that even by mildly crosslinking decellularised bovine pericardium, cell adhesion property was lost.

From the above findings, it is observed that innate immune response may have an effect on induced regeneration since fully cross-linked bovine pericardium showed activation of innate immune response at both *in vitro* and *in vivo.* This group also did not show regeneration. Another confounding factor is lack of degradation and acellularity of graft explants on account of chemical cross-linking. This might have also affected regeneration. Interestingly mildly cross-linked decellularised bovine pericardium which did not activate innate immune response both *in vitro* and *in vivo* also did not show regeneration. Hence the effect of innate immune response on induced on regeneration is ambiguous. Even mildest chemical crosslinking is reversing the trend for regeneration in the case of decellularised bovine pericardium. In the case of acquired immune response neither antibody response nor cell mediated response is adversely affecting the induced regeneration. IgG1/IgG2 response indicating a Th2/Th1 preference is indicative only in subcutaneously implanted samples and not in abdominally implanted ones. M1/M2 response is indicative of regeneration at both sites. Induced regeneration occurs around 3 months in adult rat abdominal wall regeneration model and in pig aorta, regeneration fails to happen even at the end of 6 months.

In conclusion, un-crosslinked decellularised bovine pericardium induced regeneration in rat abdominal implantation model in 3 months. This group did not stimulate macrophages *in vitro*, but produced an inflammatory response *in vivo,* with a predominance of M2 macrophage phenotype. Mildly crosslinked decellularised bovine pericardium which showed a similar *in vitro* and *in vivo* response, did not produce any induced regeneration. Hence it is concluded that Innate immune response have an ambiguous effect on induced regeneration. Acquired immune response seems to have no effect on induced regeneration, as un-crosslinked decellularised bovine pericardium stimulated both humoral and cell mediated immune response un-ambiguously and yet produced induced regeneration. On the other hand, mildly crosslinked decellularised bovine pericardium was the least immunogenic and yet induced regeneration could not be observed in this group. It is also important to note the type of antibody produced (IgG1 or IgG2) is also relevant while correlating with induced regeneration. With respect to predictive value of Th1/Th2 or M1/M2 response, it was observed that Th2 preference is indicative only in subcutaneously implanted animals and not in abdominal muscle implanted ones. M1/M2 response is indicative of regeneration in both subcutaneous and abdominal muscle implanted animals. It is also important to note that even mildest chemical crosslinking is reversing the trend for regeneration in the case of decellularised bovine pericardium. Mildest crosslinking is also affecting the cell adhesion properties of decellularised bovine pericardium.

Through this study it was shown that for evaluation of a biological scaffold, it is important to consider the type of innate or acquired immune response rather than the

intensity. This study had also demonstrated that, it is equally important to demonstrate *in vivo* regeneration capacity of a biological scaffold to accept it as a tissue engineering material.

As future studies, understanding the basic mechanism of the effect of M1/M2 polarization or Th1/Th2 preference on induced regeneration will enable design and development of biological scaffold, or even synthetic scaffolds which will promote 'induced regeneration'.

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Introduction and Review of Literature

CHAPTER 1

INTRODUCTION & REVIEW OF LITERATURE

1.1 Introduction

In medical science, six approaches are typically used to treat a disease or defect at organ scale. They are transplantation, autografting, implantation of a permanent prosthesis, use of stem cells, *in-vitro* synthesis of organs and 'induced regeneration'. Induced regeneration is the recovery of physiological structure and function of non-regenerative tissues in an organ by use of external means such as ECM analogues, which are used with or without growth factors or with or without cells (Yannas, 2001). In adult mammals only induced regeneration seems possible as they respond spontaneously to a severe injury by scar formation instead of regeneration. (Goss, 1992). In contrast, fetal healing response leads to regeneration of the injured organ which is the structural and functional recovery of the organ at the original anatomical site. It is hypothesized that with the evolution of adaptive immune system, appearance of new cell types and new uses of cell signaling cascades have changed the response of appendages and other organs to injury while providing better defenses against pathogens. (Harry *et al.,* 2003). Healing of full thickness incisional skin wounds without scarring has been documented in embryonic and early fetal stages of birds and several mammalian species (Grag &

Longaker, 2000; McCallion & Ferguson, 1996). With very little inflammation such wounds undergo rapid epithelial closure, followed by development of a normal pattern of reticular collagen and capillaries in the dermis. Scarless skin wound healing occurs through out embryonic development and during fetal stages equivalent to the early second trimester of human gestation, after which there is a transition to the typical scarring repair seen with adult skin. The basis of scarless repair is poorly understood, but considerable evidence points to a central role for components of the immune system. The period of scar free skin healing precedes development of platelet forming cells and most other cells of immune system. The acute inflammatory response is minimal in fetal skin regeneration but during the transition to adult type scarring, there is a gradual increase in the level of inflammation and specific changes in the types of immune cells elicited in the wound with proportionately more monocytes/ macrophages infiltration occurs in comparison to neutrophils resulting in scarless healing (Chin *et al.,* 2000). The dispensability of the fetal environment for scarless wound healing is also shown by regeneration of fetal skin in organ culture following incision injury and after transplantation to adult nude mice (Chin *et al.,* 2000).

Apart from the above observations, the innate immune response that serves to eliminate infections is proposed to be active in restoring the structural and functional integrity of injured organs (Alessandri *et al.,* 2004; Frantz *et al.,* 2005; Martin, 1997; Saltzman, 1999). Toll like receptors (TLR) are an emerging family of pattern recognizing receptors (PRR) that recognize pathogen associated molecular patterns (PAMP) and regulate the activation of both innate and adaptive immunity (Zhang &

Schluesener, 2006). Deficiency in certain TLRs recognized in mice has been shown to impair liver and lung regeneration in them (Campbell *et al.*, 2006; Jiang *et al.*, 2005; O'Neill, 2005; Seki *et al.*, 2005).

The above observations clearly indicate a possible role for immune response in the process of regeneration or scarless healing.

Decellularised xenograft is an excellent ECM scaffold which can be used for the purpose of induced regeneration. Use of decellularised xenograft is an approach where acellular tissue matrices are produced by selective removal of cellular components that are believed to promote immunogenicity and calcification. These acellular matrices promote induced regeneration through remodeling of the prosthesis by neo-vascularization, re-cellularisation and laying of new extracellular matrix by the host (Yannas, 2001). Native matrix architecture of decellularised tissue may provide structural and chemical cues for cell interactions (*Badylak et al.,* 2009). Decellularised tissue can be remodeled by cellular enzymes present in human host which makes them an ideal regenerative matrix (Gilbert *et al.,* 2006). Although clinically used decellularised tissue still represents a relatively new biomaterial whose biological performance is still uncertain and not well understood (Kasimir *et al.,* 2003). Decellularised tissue produced from different sources and by different methods is known to produce a host of healing response ranging from scarring to more desirable regenerative response. (Badylak & Gilbert, 2008). Immunogenicity of decellularised xenograft is known and it is reported that the immune response against incompletely decellularised xenograft was seen much more prominent compared to isografts or even

allografts. (O'Brien,1984; Rossini *et al.,* 1999). Even fully decellularised xenograft is reported to elicit specific acquired immunity *in vivo* as demonstrated by immunoblot analysis (O'Brien, 1984). The residual immunogenicity of decellularised xenograft was reported as tissue over growth, inflammatory cell infiltration and incidence of aneurismal dilation by Hilbert *et al.,* 2004 through comparing various reported decellularisation protocols in long term sheep implantation model. Despite many studies, it is still not possible to truly predict biocompatibility of one decellularised material over another (Badylak *et al.,* 2009). Host response as reported by Valentin *et al.,* 2006 for five different commercially available biological scaffolds showed that the biological scaffold composed of ECM differed markedly in the elicited host inflammatory and remodeling response. This study highlighted that a more detailed investigation of host immune response, the ECM constituents that affect the response and the effect of these factors upon scaffold remodeling and outcomes is warranted (Badylak & Glibert, 2008). The above observations clearly indicates that the decellularised stimulates immune response and the range of healing response so far observed in them can be due to their difference in their immune characteristics.

1.2 Review of Literature

1.2.1 Decellularised tissue

Xenogeneic and allogeneic cellular antigens are recognized as foreign by the host organism and therefore elicit an inflammatory response or rejection response. However components of extracellular matrix are generally conserved among species and

are well tolerated even by xenogenic recipients (Bernad *et al.,* 1983a; Bernad *et al.*, 1983b; Constantinou & Jimencz, 1991; Exposito *et al.,* 1992). Methods are developed to produce completely acellular matrices from allogenic or xenogenic tissues by specifically removing the cellular components leaving a material composed of extracellular matrix components (Crapo *et al.,* 2011; Schmidt & Baier, 2000). Decellularised tissue has a complex mixture of structural and functional proteins that constitutes the extracellular matrix. (Crapo *et al.,* 2011; Gilbert *et al.,* 2006). The extracellular matrix is a complex mixture of structural and functional proteins, glycoproteins, and proteoglycans arranged in a unique, tissue specific three dimensional ultra structure serving as structural support and a reservoir of growth factors and cytokines (Badylak, 2002; Crapo *et al.,* 2011). The ECM increase local concentration of these factors and present them efficiently to resident cell surface receptors as well as protect them from degradation and modulate their synthesis. (Bonewald, 1999; Kagami *et al.,* 1998; Roberts *et al.,* 1998; Sjaastad & Nelson, 1997). Collagen is identified as the most abundant protein within mammalian ECM and is mainly constituted by collagen type I which is also widely used for therapeutic applications (Vanderrest & Garrone 1991). Fibronectin is second only to collagen in ECM and exists in soluble and tissue forms. It has ligands for adhesion of many cell types (Miyamoto *et al.,* 1998; Schwarzbauer, 1991). Laminin is another adhesion molecule primarily seen in basement membrane ECMs (Schwarzbauer, 1999). Laminin is very important for vascularisation of scaffolds since it has a prominent role in formation and maintenance of vascular structure due to its favorable interaction with endothelial cells (Ponce *et al.,*

1999; Werb *et al.,* 1999). Glycosaminoglycans are another important components of ECM and play important role in binding of growth factors and cytokines, water retention and the gel properties of ECM. The heparin binding properties of numerous cell surface receptors and of many growth factors such as VEGF make the heparin rich GAGs in ECM extremely important (Hodde *et al.*, 1996). The list of growth factors present within ECM although in small quantities includes VEGF, bFGF, EGF, TGFbeta, KGF, HGF and PDGF (Badylak, 2002). The most commonly utilized method of decellularisation of tissues involves a combination of physical and chemical methods. Physical methods used are snap freezing (Gulati AK, 1988; Jackson *et al.,* 1987; Jackson *et al.,* 1990; Roberts *et al.,* 1991;) and mechanical force or agitation (Dahl *et al.,* 2003; Freytes *et al.,* 2004; Lin *et al.,* 2004; Schenke-Layland *et al.,* 2003). Chemical methods consists of either alkaline or acid treatment (Dc Filippo *et al.,* 2002; Falke *et al.,* 2003; Probst *et al.,* 1997; Schenke-Layland *et al.,* 2003; Yoo *et al.,* 1998), non-ionic detergents such as Triton X100 (Cartmell & Dunn, 2000; Chen *et al.,* 1999; Dahl *et al.,* 2003; Grauss *et al.,* 2003; Gulati AK, 1988; Lin *et al.,* 2004; Woods & Gratzer, 2005) ionic detergents such as sodium dodecyl sulphate, sodium deoxycholate and Triton X200 (Chen, 2004; Hudson *et al.,* 2004a; Hudson *et al.,* 2004b; Ketchedjian *et al.,* 2005; Lin *et al.,* 2004; Reider *et al.,* 2004). Zwitterionic detergents such as CHAPS (Dahl *et al.,* 2003), Sulfobetaine 10 and 16 and Tri(nbutyl)phosphate, hypotonic/ hypertonic solutions (Dahl *et al.,* 2003), EDTA/ EGTA (Bader *et al.,* 1998; Gamba *et al.,* 2002; Mc Fetridge *et al.,* 2004; Teebken *et al.,* 2000) and enzymes such as trypsin and endonucleases (Courtman *et al.,* 1994; Dahl *et al.,* 2003; Rieder *et al.*, 2004; Woods &

Gratzer, 2005). Decellularisation is verified by standard histological staining with Hematoxylin and Eosin as the first line of inspection to determine if nuclear structures can be observed (Gilbert *et al.,* 2006). Inspection for the presence of DNA can be performed by staining the specimen with Hoechst 33258 which is a fluorescent molecule that bind to AT clusters in the minor groove of DNA (Kakkar & Grover, 2005). Based on findings of the studies in which an *in vivo* constructive remodeling response has been observed and adverse cell and host responses have been avoided, the following minimal criteria for presence of DNA following decellularization is arrived such as <50ng dsDNA per mg ECM dry weight, <200bp DNA fragment length, lack of visible nuclear material in tissue sections stained with 4',6-diamino-2-phenylindole or Hematoxylin & Eosin (HE) (Crapo *et al.,* 2011).

The effectiveness of decellularisation and the alterations to the ECM vary depending on the source of tissue, the composition of the tissue, the tissue density and other factors (Gilbert *et al.,* 2006). Decellularised tissue such as pericardium (Courtman *et al.,* 1994), heart valves (Bader *et al.,* 1998; Schenke- Layland *et al.*, 2003), blood vessels (Conklin *et al.*, 2002; Uchimura *et al.*, 2003), skin (Chen *et al.,* 2004), nerves (Hudson *et al.,* 2004; Kim *et al.,* 2004), skeletal muscle (Borschel *et al* 2004), tendons (Carmell & Dunn, 2000), ligaments (Woods and Gratzer, 2005), small intestinal submucosa (Badylak *et al.,* 1989; Badylak *et al.,* 1995; Kropp *et al.,* 1995) , urinary bladder(Chen *et al.,* 1995; Freytes *et al.,* 2004; Gilbert *et al.,* 2005) and liver (Lin *et al.,* 2004) have been studied for tissue engineering application. Biological scaffolds derived from decellularised tissue are successfully used in human clinical applications.

(Badylak, 2004; Chen *et al.,* 1999; Dellgren *et al.,* 1999; Harper, 2001; Kolker *et al.,* 2005; Lee, 2004; Metcalf *et al.,* 2002; Wainwright, 1995).

1.2.2 Glutaraldehyde crosslinking and its confirmation

Glutaraldehyde can introduce stable crosslinks into collagens fibers in comparison to other aldehydes (Carpentier & Dubost, 1972; Nimni, 1968). Heat of denaturation with associated shrinkage and enzymatic degradation studies of collagenous tissue indicated the level of glutaraldehyde crosslinking (Nimni *et al.,* 1971; Nimni, 1975; Strawich *et al.,* 1975). Thermogravimetric analysis (Shamis *et al.,* 2009) and contact angle measurement (Ghanbaria *et al.,* 2010) also indicated chemical crosslinking. The specific chemistry of glutaraldehyde fixation of collagen is not fully understood. It is assumed that inter-intra molecular covalent bonds are formed in two ways, formation of Schiff bases by reaction of an aldehydes group with an amino group of lysine or hydroxylysine or an aldol condensation between two adjacent aldehydes (Jayakrishan & Jameela, 1996). Besides amino groups, glutaraldehyde can also interact with carboxy, imido and other groups of protein (Anderson, 1967; Blauer *et al.,* 1975; Bowers & Cater 1964). Fixation time and concentration of glutaraldehyde used are important. Slower time dependent crosslinking of glutaraldehyde occurs at lower concentration and at higher concentration; crosslinking occurs only at surface of the collagen fibers due to rapid polymerization and impairment of glutaraldehyde molecule access to the interstitium of the larger collagen fibers by steric hindrance (Nimni *et al.,* 1987). The concentration of glutaraldehyde employed for processing tissue ranges from 0.2 to 0.6% (Jayakrishnan & Jameela, 1996). Short duration crosslinking of bovine

pericardium was shown to have advantages such as lesser calcification and is used clinically for cardiac surgery (Chachque *et al.,* 1988; Liao *et al.,* 1995). The degree of crosslinking determines the degradation rate of chemically crosslinked acellular tissue and as a consequence it can significantly affect its regeneration pattern (Liang *et al.,* 2004). Highly crosslinked matrices are more stable to degradation and therefore less likely to be remodeled as part of tissue morphogenesis. However modification of the crosslinking process to produce a lower crosslinking rate can produce "growable" materials that are easily infiltrated with cells, degraded and remodeled as part of the wound healing response. (Noishiki *et al.*, 1989). Alternatively, acellular tissues that are not crosslinked in any form, and thus are susceptible to remodeling in the body, may prove to be ideal biomaterial for cell repopulation and new tissue growth. (Badylak *et al.,* 1998; Courtman *et al.,* 1994). ECM materials that are thoroughly decellularised, sterilized and not modified by chemical crosslinking agents or other processing methods that produce unnatural crosslinks may induce constructive remodeling because of bioinductive properties, mechanical and material properties, the host tissue response to naturally occurring ECM and the degradation properties of the material (Badylak, 2007). ECM scaffold that resist or retard the degradation process elicit a chronic inflammatory response and host fibrous connective tissue deposition (Valentin *et al.,* 2006).

1.2.3 Host tissue response to decellularised tissue

The source of biological scaffold, decellularisation method and methods of terminal sterilization vary widely and each of these variables affects host response to the ECM (Gilbert *et al.,* 2006; Reing *et al.,* 2010). Residual detergents in the

decellularised scaffold can also potentially cause adverse tissue reaction (Cebotari *et al.,* 2010). Host response as reported (Valentin *et al.,* 2006) for five different commercially available biological scaffolds such as GraftJacket™, Restore™, CuffPatch™, TissueMend™, and Permacol™ is presented in the table below .

Scaffold	**Tissue &Process**	**Host response**
GraftJacket™,	Human dermis, proprietary cryogenic processing	Elicited most intense acute cell response, which was not predictive of an adverse remodeling outcome. Cellular response was predominantly mononuclear response. Multinucleate giant cell was observed. The device was replaced with fibrous connective tissue and a persistent low grade chronic inflammatory response.
Restore™,	Porcine SIS, minimally processed	Elicited most intense acute cell response, which was not predictive of an adverse remodeling outcome. Cellular response was predominantly mononuclear response. Biological scaffold was replaced with a mixture of muscle cells and organized connective tissue.
CuffPatch™,	Porcine SIS, carbodiimide	Multinucleate giant cell was observed. Cellular response was predominantly neutrophilic

	crosslinked	response throughout the entire study. Device showed accumulation of dense collagenous tissue , a persistent foreign body response and relatively slower remodeling.
TissueMend™,	Fetal bovine skin , proprietary process	Cellular response was predominantly mononuclear response, low grade chronic inflammation, minimal scaffold degradation and fibrous encapsulation.
Permacol™	Porcine dermis, crosslinked with isocyanate	Cellular response was predominantly mononuclear response Multinucleate giant cell was observed. low grade chronic inflammation, minimal scaffold degradation and fibrous encapsulation.

Table: 1-1: Host response to different commercially available biological scaffolds

The above study highlighted that a more detailed investigations of host immune response, the ECM constituents that affect the response and the effect of these factors upon scaffold remodeling and outcomes are warranted (Badylak & Glibert, 2008).

Studies on another product, decellularised porcine Matrix P valve revealed a foreign body-type reaction accompanied by severe fibrosis and massive neointima formation around decellularised porcine valve wall. There was low re-cellularisation of decellularised matrix and neovascularisation was observed only in the neointima and scar tissue. Inflammatory infiltrates, composed mainly of T cells, B cells, and plasma

cells, as well as the presence of dendritic cells, macrophages, and mast cells were detected in the tissue surrounding the porcine matrix. In the fibrous tissue, over-expression of connective tissue growth factor was observed. The above observation was attributed to incomplete decellularization of porcine matrix, which may have contributed to increased immunogenicity of these conduits (Cicha *et al.,* 2010). The immunogenicity of un-cross linked decellularised xenograft was observed as tissue over growth, inflammatory cell infiltration and incidence of aneurismal dilation (Hilbert *et al., 2004)* through comparing various reported decellularisation protocols in long term sheep implantation model. Positive results found in short term animal trials of an un-cross linked decellularised xenograft valve had culminated in catastrophic failure during clinical trial (Simon *et al.,* 2003). Angiogenesis, abundant host cell infiltration, mitogenesis and deposition and organization of new host ECM are the desirable events during the remodeling of ECM scaffolds (Badylak, 2007). Component growth factors such as such as VEGF, bFGF and TGF-β are released during scaffold degradation and exert their biological effects as they are dissociated from their binding proteins and activated (Hodde & Hiles, 2001; Hodd *et al.,* 2001; Hodde *et al.,* 2002a; Hodde *et al.,* 2002b; McDevitt *et al.,* 2003; Voytik-Harbin *et al.,* 1997). Cryptic peptides such as Endostatin, Angiostatin, Anastellin fragment III1C, Canstatin, Restin, Tumstatin, ABT 510 released by the degradation process initiate and sustain the recruitment of circulating bone marrow derived cells that actively participate in long-term tissue remodeling (Badylak *et al.,* 2001; Zantop *et al.,* 2006). A better understanding of the relationship between the classic indicators of inflammation, such as cellular infiltration,

angiogenesis, hyperemia and tissue swelling and the same process that are involved in constructive remodeling of tissue is warranted (Badylak et al., 2007).

1.2.4 Immune response to decellularised tissue

Unfixed decellularised tissue such as bovine pericardium is increasingly used for tissue engineering application (Crapo *et al.,* 2011; Erdbrugger *et al.,* 2006; Griffiths *et al.,* 2008; Stock & Schenke-Layland, 2006). It is observed that the apparent elimination of intact cells based on light microscopic examination does not assure adequate removal of xenoantigens or mitigation of immune response. (Gonclaves *et al.*, 2005; Kasimir *et al.,* 2006; Simon *et al.,* 2003; Vesely *et al.,* 1995). The presence of Gal epitope on the surface of vascular endothelium is the primary cause of rejection in xenogeneic organ transplants. (Collins *et al.,* 1994; Cooper *et al.,* 1993; Galili *et al.,* 1985; Oriol *et al.,* 1993). Humans produce large amounts of anti gal antibodies (1% of circulating IgG) including IgG, IgM and IgA as a result of constant exposure to intestinal bacteria that carry Gal epitope (Galili *et al.,* 1984; Gabrielli *et al.,* 1991; Goldberg *et al.,* 1995; Koren *et al.,* 1992). The presence of Gal epitope in biologic scaffolds is reported on porcine bioprosthetic heart valves (Konakci *et al.,* 2005), porcine anterior cruciate ligament and cartilage (Stone *et al.,* 1998; Stone *et al.,* 2007a; Stone *et al.,* 2007b), and porcine SIS ECM (McPherson *et al.,* 2000). Immunomodulatory effect of Gal epitope does not adversely affect *in vivo* remodeling of xenogeneic ECM (Daly *et al.,* 2009). An immunologic response to the xenoantigens other than alpha gal (non gal antigens) has also been reported (Baumann *et al.,* 2007; Stone *et al.,* 2007; Zhu & Hurst, 2002). Residual DNA in bioscaffolds is directly correlated to adverse host reactions (Nagata *et*

al., 2010; *Zheng et al.,* 2005). Despite the universal presence of DNA remnants in commercially available ECM devices, the clinical efficacy of these devices for their intended application has been largely positive and hence it appears unlikely that the remaining DNA fragments contribute to any adverse host response or a cause for concern (Badylak & Gilbert, 2008). The immune response against in-completely decellularised xenografts was seen much more prominent compared to isografts or even allografts (Allaire *et al.,* 1994; Allaire *et al.,* 1997). Decellularised un-crosslinked xenograft is reported to elicit antibody response as demonstrated by immunoblot analysis (Arai & Orten, 2009). The fundamental concept of decellularisation now focuses on identification and removal of antigenic proteins (Goncalves *et al.,* 2005; Kasimir *et al.,* 2005). Decellularised porcine valves were shown to have considerable amount of residual proteins with different molecular weights and has significantly higher potential for inciting monocyte migratory response (Reider *et al.,* 2005). Complete elimination of cells and remnants could not be achieved with different decellularisation techniques and decellularised porcine heart valve matrix has the potential to attract inflammatory cells and to induce platelet activation (Kasimir *et al.,* 2006). Migration of granulocytes could not be abolished by decellularisation or glutaraldehyde fixation in case of porcine pulmonary roots (Rieder *et al.,* 2006). Incubation of decellularised porcine tissue with human plasma lead to adsorption of IgG, activation of classical complement pathway and adherence of activated polymorphonuclear leukocytes (Bastan *et al.,* 2008). Decellularisation has been shown to reduce early non-specific inflammatory response in case of human allografts in

comparison to xenografts (Simon *et al.,* 2006). Few controlled studies have been reported that evaluate and characterize the host immune response to most non-autologous ECM scaffold materials except xenogeneic small intestinal submucosa (Badylak *et al.,* 2007).

1.2.5 Evaluation of immune response

The potential responses of the immune system to a biomaterial can be irritation/acute inflammation, chronic inflammation, immune-suppression, immune-stimulation, hypersensitivity and autoimmunity. The immunotoxicity of biomaterials can be carried out using *in vivo* and *in vitro* assays, wherein in vivo assays can be more useful as they mimic the complexity of intact immune system. Two kinds of assays are distinguished in the immunotoxicity testing, functional and non-functional. Non-functional assays have a descriptive character in that they measure such as morphological or qualitative forms, alterations in the extent of lymphoid tissue, the number of cells and level of immunoglobulin. Functional assays determine activities of cells or organs, such as proliferative response of lymphocytes to mitogens or specific antigens, cytotoxic activity and specific antibody formation (ISO 10993-20:2006). The table below provides examples of the specific types of tests that might be used to study the immunologic response as suggested by FDA (CDRH 635, 1999).

IMMUNE RESPONSES	FUNCTIONAL ASSAYS	SOLUBLE MEDIATORS	PHENOTYPING	OTHER**
HISTOPATHOLOGY	NA	NA	Cell surface markers	Morphology
HUMORAL RESPONSE	Immunoassays (e.g. ELISA) for antibody response to antigen plus adjuvant* Plaque-forming cells Lymphocyte proliferation Antibody-dependent cell-mediated cytotoxicity Passive cutaneous anaphylaxis Direct anaphylaxis	Complement (including C3a and C5a anaphylatoxins)*, Immune complexes	Cell surface markers	
CELLULAR RESPONSES				
T-CELLS	Guinea pig maximization test* Mouse local lymph node assay* Mouse ear swelling test Lymphocyte proliferation Mixed lymphocyte reaction	Cytokine patterns indicative of T cell subsets (e.g. Th1 and Th2)	Cell surface markers (helper and cytotoxic T-cells)	
NATURAL KILLER CELLS	Tumor cytotoxicity	NA	Cell surface markers	
MACROPHAGES	Phagocytosis* Antigen presentation	Cytokines (IL-1, TNFα, IL-6, TGFβ)	MHC markers	
GRANULOCYTES***	Degranulation Phagocytosis	Chemokines, Bioactive amines, Inflammatory cytokines, Enzymes	NA	Cytochemistry
HOST RESISTANCE	Resistance to bacteria, viruses and tumors	NA	NA	
SIGNS OF ILLNESS	NA	NA	NA	Allergy, Skin rash, Urticaria, Edema, Lymphadenopathy

Table: 1-2: Different immunological tests suggested by FDA (CDRH 635,1999)

ASTM has issued standard practice guidelines for evaluation of immune responses following introduction of foreign substances into the mammalian body that may induce formation of an immune response (ASTM F1906-98: reapproved 2003). As per this practice, immunologic testing is done using specimens including blood and organs obtained from animals implanted with test material. The testing protocol is divided into two specific areas of humoral immunity and cell mediated immunity. Test on humoral immunity includes Enzyme linked immunoassays (ELISA) techniques. Six different ELISA systems available for detection of specific antibodies, soluble antigens or cell surface antigens are Indirect ELISA, Direct competitive ELISA, Antibody-

sandwich ELISA, Double antibody-sandwich ELISA, Direct Cellular ELISA and Indirect Cellular ELISA. Of these Indirect ELISA and Double antibody-sandwich ELISA are used for antibody screening. Indirect ELISA is a relatively simple method for antibody screening or epitope mapping. Here pure or semipure antigen can be used for coating the wells. Unlike the Double antibody-sandwich ELISA, this does not require the use of preexisting specific antibodies. (Hornbeck, 1991). For testing cell mediated immunity, Lymphocyte transformation tests are used. In this test lymphocytes obtained from peripheral blood, peritoneal exudates or minced lymphoid organs of animals implanted with test material is stimulated with antigens causing release of cytokines and interleukins that induce other lymphocytes to proliferate thus amplifying the response. This proliferation is detected by adding tritiated thymidine to cultures and uptake of radioisotope is measured after 4-6h incubation. Alternatively MTT is increasingly used as a non-radioactive simple dye. Other test suggested are Leukocyte migration inhibition test and Macrophage migration inhibition test.

1.2.6 Th1/Th2 pathway

The role of Th1 and Th2 lymphocytes in cell mediated immune response has been widely studied (Strom *et al.,* 1996; Zhai *et al.,* 1999). These lymphocytes produce cytokines such as interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-β leading to macrophage activation, stimulation of complement fixing antibody isotypes (IgG2a and IgG2b in mice) and differentiation of CD8+ cells to a cytotoxic phenotype (Abbas *et al.,* 1996; Matsumiya *et al.,* 1994). Activation of this pathway is associated with both allogenic and xenogeneic transplant rejection (Chen *et al.,* 1996;

Strom *et al.,* 1996; Zhai *et al.,* 1999). Th2 lymphocytes produce IL-4, IL-5, IL-6 and IL-10 cytokines that do not activate macrophages and that lead to production of non-complement fixing antibody isotype IgG1 in mice. Activation of Th2 pathway is associated with transplant acceptance (Bach *et al.,* 1997; Chen & Field, 1995; Picotti *et al.,* 1997). Following implantation in mice, SIS ECM graft showed an antibody response restricted to Ig1 type indicating aTh2 response and the healing was found similar to that of animals implanted with syngeneic muscle tissue (Badylak & Gilbert, 2008).

Tissue cytokine and serum humoral response to SIS was shown to be consistent with a Th2 type immune response (accommodation) in to contrast to the expected Th1 (cell mediated rejection) type of response (Allman *et al.,* 2002).

1.2.7 M1/M2 polarization

A distinct phenotype polarization profile has been described for macrophage polarization (Mantovani *et al.,* 2004; Strom *et al.,* 1996; Zhai *et al.,* 1999). M1 activation represents classical activation with production of inflammatory cytokines and M2 activation has three different forms with either immunosuppressive or immunoregulatory function as depicted in Figure 1-1. (Mantovani *et al.,* 2004). Studies on host response to implanted biological scaffold suggest that macrophages differentiate towards a phenotype that is associated with either cytotoxic inflammation or constructive remodeling (Mantovani *et al.,* 2004; Mantovani *et al.,* 2005). The pro-inflammatory cytotoxic macrophage phenotype, M1 is characterized by cells that are associated with classic signs of inflammation whereas, the anti-inflammatory

macrophage M2, promotes immunoregulation, tissue repair and constructive remodeling (Badylak and Gilbert, 2008).

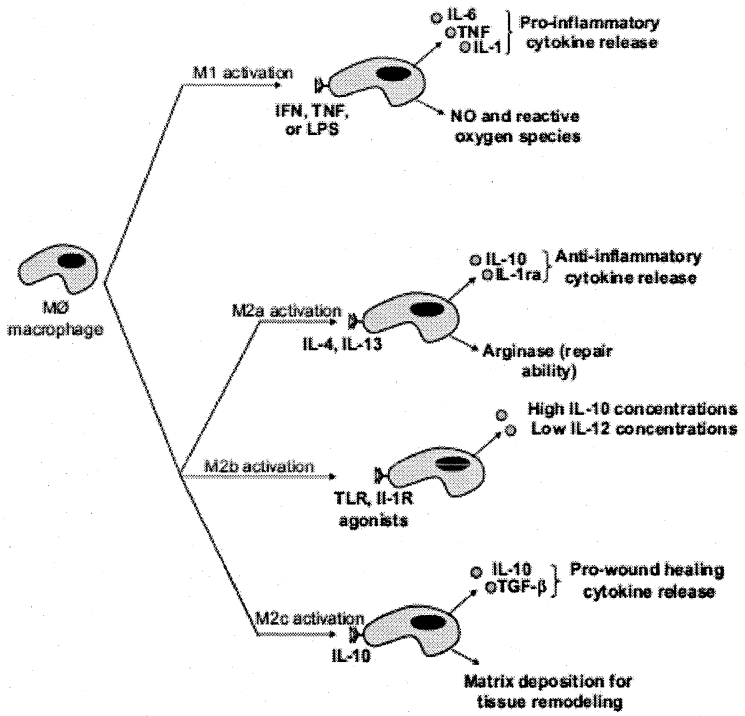


Figure 1-1: Phenotypic polarization of macrophages and its effect

The macrophages from these two pathway can be distinguished by their cell surface markers and by their cytokine and gene expression profiles (Mantovani *et al.*, 2004; Mosser, 2003; Stout *et al.,* 2005). M1 macrophages are characterized by CD68+ and CD80+ cell surface markers in rat, the production of large amount of nitric oxide and other reactive oxygen species and copious amounts of pro-inflammatory cytokines such as IL-12 and TNF-α. Conversely M2 macrophages produce high levels of IL-10 and TGF-β expression, produce large amounts of arginase inhibit release of pro-inflammatory cytokines, scavenge debris, promote angiogenesis and recruit cells

involved in constructive tissue remodeling. M2 macrophages express CD163 surface markers in rat (Badylak & Gilbert, 2008). It was noted that macrophages respond differently to ECM scaffold materials depending upon the ECM source and processing methods. Chemical crosslinking of SIS-ECM with carbodiimide resulted in a switch from an M2 dominant profile to an M1 dominant profile. Interestingly autologous tissue graft showed an M2 response early followed by a duality of M1 and M2 response which may have been a consequence of pro-inflammatory cytokines produced as a product of cell death occurring within autologous tissue graft (Badylak *et al.,* 2008). In a study in rat abdominal defect model for 28 days, it was noted that acellular graft showed a predominantly M2 type of response and resulted in constructive remodeling, while those containing a cellular component, including those with autologous cellular component elicited a predominantly M1 type response and resulted in deposition of dense connective tissue and scarring (Brown *et al.,* 2009).

1.2.8 Induced regeneration models

The differences and similarities in regenerative capabilities and mechanisms among diverse animal species are delineated now (Alvarado & Tsonis, 2006). However adult mammals generally do not regenerate organs that they may have lost from disease or trauma. Convincing evidence of regeneration is based on observation of definitive recovery of both structure and function in an anatomically well defined defect. When it is induced regeneration, there must be evidence that the mass of the regenerate which forms in the absence of template (spontaneous regeneration) is negligibly small relative to that which is induced in its presence. (Yannas, 1997). Scaffold induced regeneration

has been extensively studied in full thickness skin loss using guinea pig model which could be extended to humans (Murphy *et al.,* 1990). Regeneration of the dermis was demonstrated on the basis of conventional histological and ultrastructural studies. The new integument was structurally and functionally competent but was totally lacking in hair follicles and other skin adnexa (Yannas, 1997). The ECM analogue which produced regeneration had a collagen/GAG ratio of 98/2 w/w, average pore diameter between 20 to 120μm, sufficiently high crosslink density to resist degradation by collagenases over about 10 days following grafting. The ECM scaffold which showed high activity in promoting dermal regeneration also delayed significantly the onset of wound contraction (Yannas *et al.,* 1989). A rat model of abdominal defect model for evaluation of muscular tissue remodeling is well described (Brown *et al.,* 2009; Valentin *et al.,* 2010; Watchko *et al.,* 1992). The abdominal defect created varied from a partial thickness one, where a 1.5x1.5cm section of external and internal oblique layers of the ventro-lateral was excised keeping the underlying transverse fascia and peritoneum (Valentin *et al.,* 2010). Another model is described in which a 2 -2.5 cm full-thickness defect consisting of muscles, fascia and peritoneum was created in abdominal wall and the patch was implanted in the defect (Dufrane *et al.,* 2008; Shi *et al.,* 2011). In these studies structural regeneration was demonstrated using routine Heamtoxylin and Eosin staining as well as by Massons Trichrome staining. Immuno-staining was done using antibodies against dystrophin, myosin (type I and II) or alpha sarcomeric actin for demonstrating skeletal muscles. New capillary formation was studied by immune labeling of von Willebrand factor. Innervation of skeletal muscle regenerate was studied by immune-

labeling of neurofilament (Dufrane *et al.*, 2008; Shi *et al.,* 2011; Valentin *et al.,* 2010). Functional regeneration was demonstrated by studying contractile force testing, fatigue resistance testing (Valentin *et al.,* 2010). Tensile testing of the explants is also done to understand the mechanical properties (Shi *et al.,* 2011). The regenerated skeletal muscle consisted of both type 1 and II muscle fibers in relative proportions very similar to native skeletal muscle. Vascular healing pattern of aortic patches was studied by Cheung *et al.,* 1999. They have studied vascular healing by Hematoxylin and Eosin staining, Massons Trichrome staining and immune-staining for von Willebrand factor and smooth muscle alpha actin. Fibrosis and tissue retraction was observed as negative findings. At 30 days they observed that the most suitable patch was difficult to distinguish from the surrounding aorta, and it was covered with alpha actin positive smooth muscle cells with a coat of von Willebrand positive endothelial cells. Newly deposited collagen in between the fibers of scaffold collagen could be observed. Pig aortic patch model for comparing the healing response between autologous and glutaraldehyde fixed pericardium was used by Haluck *et al.,* 1990. Nevertheless in all these studies structural regeneration of aorta is not described.

1.3 Current status

- Both innate and adaptive immune response have been attributed a role in regeneration in fetus. Deficiency in certain components of adaptive immune response in adult mice has been shown to impair liver and lung regeneration in them.

- Although clinically used, decellularised tissue still represents a relatively new biomaterial whose biological performance is still uncertain and not well understood.
- The biological scaffold composed of ECM differed markedly in the elicited host inflammatory and remodeling response. A more detailed investigation of host immune response, the ECM constituents that affect the response and the effect of these factors upon scaffold remodeling and outcomes is warranted.
- A better understanding of the relationship between the classic indicators of inflammation, such as cellular infiltration, angiogenesis, hyperemia and tissue swelling and the same process that are involved in constructive remodeling of tissue is warranted.

The present research work attempted to record the effect of immune response on the regenerative response elicited by decellularised xenograft such as bovine pericardium in an adult animal model of regeneration. In order to achieve this, our study had the following objectives.

1.4 Hypothesis

It is hypothesized that immune response (innate or adaptive) against decellularised biological scaffold can have effect on induced regeneration in an adult mammal.

1.5 Objectives of the study

1. To characterize decellularised bovine pericardium (BP) and to chemically modify it to induce variation in *in-vivo* response (Phase 1).
2. To assess induction of variation in *in-vivo* response between differently treated decellularised bovine pericardium on account of chemical modification. (Phase 2).
3. To assess and compare innate and acquired immune response between different treatment groups and to analyze Th1/Th2 and M1/M2 preference exhibited by them (Phase 3).
4. To study regeneration in different treatment groups using adult animal models of regeneration and to correlate chemical modification, the difference in immune response, Th1/Th2, M1/M2 preferences with the observed 'Induced regeneration' (Phase 3).

Materials and Methods

CHAPTER 2

MATERIALS AND METHODS

2.1 Phase 1: Preparation of decellularised bovine pericardial samples (EDCL, 0.2DCL, 0.6DCL and GlutBP)

2.1.1 Materials

Hematoxylin and Eosin stain (Sigma Aldrich, USA), Hoechst 33258 (Sigma Aldrich H-6024, USA), DNA X-Press kit (Himedia MB501, India), Agarose DNA grade (Himedia RM1490, India), DC protein assay kit (Bio-Rad 500-0116, USA), Silver stain protein molecular marker (Sigma Aldrich M-6539, USA), DNA molecular weight marker (Himedia MBT-049, India) DNA gel loading dye (HimediaML-015,India), Ethidium Bromide (Himedia RM-813,India), Sodium chloride (Merck 7647-14-5, India), Protenase K 20mg/ml (AMRESCO E-195, India), Sodium dodecyl sulphate (Sigma Aldrich L-3771, USA), EDTA (Nice Chemicals E10217, India), Xylene (Nice Chemicals X10137, India), Ferric chloride (Himedia RM1379, India), Sodium thiosulphate (Sigma Aldrich S 7026,USA), Acid fuchsin calcium salt (Sigma Aldrich A-3908,USA), Phosphotungstic acid (Sigma Aldrich P-4006,USA), Acetic acid (Fisher scientific, 1105,USA), Saffron (Sigma Aldrich S8381,USA).

2.1.2 Characterization of decellularised bovine pericardium

Decellularisation of bovine pericardium was achieved using a proprietary process based on a non-detergent method. Decellularisation was confirmed by demonstrating absence of nuclear remnants using routine HE staining as well as by

use of specific nuclear stain such as Hoechst 33258 on 5 micron paraffin embedded sections (n=10). Besides this, residual DNA was extracted from 100mg decellularised BP and concentration was estimated spectrophotometrically. This was followed by demonstration of extractable DNA by 1% agarose gel electrophoresis. Structural content was evaluated using Image analysis (Image Tool3) of Movat's Pentachrome stained sections. Surface examination of decellularised bovine pericardium in comparison to normal pericardium was done using Environmental scanning electron microscopy (ESEM). Residual protein was extracted from decellularised tissue and estimated and this was followed by SDS-PAGE electrophoresis.

2.1.2.1 Nuclear staining with Hoechst 33258 (Kakkar & Grover, 2005)

Five micron size paraffin sections were hydrated using graded alcohol and finally to distilled water. The sections were washed in 0.1MPBS containing 0.9% NaCl followed by staining for 15min in 0.1M PBS containing 1mg/ml Hoechst33258. The stained sections were rinsed extensively in PBS, dehydrated, cleared and mounted with aqua mount.

2.1.2.2 DNA isolation from fresh and decellularised samples (Rieder *et al.*, 2006)

Hundred mg of tissue specimens were homogenized and digested with 30µg/mg tissue proteinase K in a buffer containing 150mmol/L NaCl, 1mmol/L EDTA, 0.5% SDS(pH 10.5) at 65°C for 2h. The extracted DNA was precipitated by adding butanol/chloroform (1:5) and pelleted at 12,000g for 5 min. The DNA was

solubilized in distilled water and visualized by electrophoresis in agarose-EtBr gels. The quantity of DNA was estimated spectrophotometrically at 260nm.

2.1.2.3 Structural analysis:

2.1.2.3.1 Movat's Pentachrome staining (Russell modification)

The sections were deparaffinised by 2 minute immersion in Xylene (two changes) and hydrated to water followed staining with alcaine blue for 20 min, washed with running water for 10min and placed the sections in alkaline alcohol for 1-2h. This was followed by washing in running tap water for 10min and staining with Verhoeffs' hematoxylin solution for 15 min. The sections were rinsed in several changes of water, differentiated in 2% aqueous ferric chloride. The slides were placed in sodium thiosulphate for 1 min followed by washing in running tap water for 5min. The sections were stained in Crocein-scarlet acid fuchsin (8:2) for 30 sec. The sections were then rinsed in several changes of distilled water followed by rinsing in 0.5% acetic acid water. Differentiation was done in 5% aqueous phosphotungstic acid for 5-10min, rinsed in 0.5% acetic acid water, followed by rinsing in 100% alcohol (3 changes). The sections were now stained with Saffron for 15-20 min in sealed coplin jar, rinsed in 100% alcohol (3 changes), cleared in xylene and mounted. Nucleic acid and elastin stained black, collagen- yellow, ground substance and mucin – blue, fibrinoid and fibrin- red and muscle –red.

2.1.2.3.2 Image analysis

Digital images of the Movats Pentachrome stained sections were obtained at 1000x magnification using Olympus CX41 microscope and Jenoptik ProgRes C5 camera with ProgRes Capture Pro software ver 2.8.0, Jenoptik Optikal systems, GmbH, Jean, Germany. This was followed by image analysis using UTHSCA

ImageTool Version 3 software. As the first step, the digital images were subjected to grey conversion from color to grey scale. Image thresh-holding was done either to mask collagen or elastin. The grey level value of thresh-holding for this masking is noted. This was followed by viewing the result in histogram window and the percentage value up to this grey level is recorded as percentage of the component (elastin or collagen) under study.

2.1.2.4 Surface study using Environmental Scanning Electron Microscopy (ESEM)

The decellularised bovine pericardium was washed and fixed in 2.5% glutaraldehyde overnight. Washed in PBS, blotted dry and was observed directly under ESEM (FEI QUANTA 200, Japan) using high vacuum secondary electron detector.

2.1.2.5 Extractable protein from decellularised tissue

The residual extractable protein after decellularisation and three day wash in sterile normal saline is estimated in triplicate samples. For this approximately 100 mg (wet weight) of decellularised tissue sample was weighed and washed in PBS for 30 min with constant agitation. Washed tissue is minced into 1mm pieces and homogenized in 2ml ice cold sterile PBS at 700rpm for 20 min in pre chilled tubes. The homogenate was aspirated and transferred into fresh pre chilled centrifuge tubes and debris was sedimented by centrifugation at 10,000g at 4°C for 30min. The supernatant was collected, filter sterilized using 0.22µm filter and aliquoted into sterile tubes and stored at -20^0C. The protein content of the supernatant was estimated using Bio-Rad DC protein assay kit and expressed as mg per 100mg of

wet tissue. This was followed by SDS-PAGE for identifying the molecular weight of the extractable protein. 10μg extractable protein from fresh bovine pericardium and decellularised bovine pericardium together with molecular weight marker were subjected to electrophoresis on a 10% (wt/vol) SDS-PAGE gel overlaid with 5% (wt/vol) stacking gel. On completion of the electrophoresis, the gel was removed and the protein bands were demonstrated by routine silver staining. The gel was analyzed and plotted using ImageJ 1.44P software, NIH, USA.

2.1.2.5.1 Estimation of protein

Three to five dilutions of protein standards containing from 0.2 mg/ml to about 1.5 mg/ml protein were prepared.100 μl of standards and samples were pipetted into clean, dry test tubes. 500μl of reagent A (an alkaline copper tartrate solution) was pipetted into each test tube and vortexed.4.0 ml reagent B (a dilute Folin Reagent) was added into each test tube and vortexed immediately. Incubated for 15 minutes at room temp, absorbance was read at 750 nm within an hour. Sample concentration was calculated using the graph plotted with absorbance on 'y' axis and the standard concentration on the 'x' axis.

2.1.3 Chemical modification of decellularised tissue and its confirmation

2.1.3.1 Materials

Gluteraldehyde solution 25% (Sigma Aldrich G-5882, USA), L-Glutamic acid (Merck Cat no: 56-86-0, India), Ethanol (Merck, India), Collagenase from *Clostridium histolyticum* (Sigma Aldrich C-6885, USA), ampiclox (Megapen, India)

, calcium chloride (Himedia RM534, India), Sodium azide (Himedia RM1038, India), Tris chloride (Himedia RM613, India).

2.1.3.2 Chemical modification using glutaraldehyde crosslinking

Decellularised pericardium was washed for 3 days in normal saline containing antibiotics (Ampicillin and Cloxacillin at 1mg/ml). This was done under constant shaking in an incubator shaker set at 37^0C. The washed tissue was fixed for 10 minutes in 0.2% (0.2DCL group) and 0.6% (0.6DCL group) Glutaraldehyde solution (25%, Sigma Aldrich) in PBS at pH7.4, at 37^0C under constant agitation. The Glutaraldehyde fixed tissue was washed thoroughly in several changes of PBS to remove residual Glutaraldehyde. Further inactivation of residual Glutaraldehyde was done by treating with 8% Glutamic acid solution for 24 hours under continuous agitation in an incubator shaker. The treated tissue was thoroughly washed in at least 3 changes of sterile PBS containing antibiotics (Ampicillin-Cloxacillin at 1mg/ml), with one washing cycle lasting for 10 minutes. The final tissue was preserved in 70% ethanol (Merck) in a clean sealed container, which also acted as a sterilant. Decellularised bovine pericardium without any chemical cross-linking (EDCL) preserved in 70% ethanol was used for comparison. Fresh bovine pericardium fixed with 0.6% Glutaraldehyde (25%, Sigma Aldrich) for 7 days, prepared in PBS (pH 7.4) followed by storage in 0.2% Glutaraldehyde was used as glutaraldehyde control (GlutBP). The chemical modification yielded the following samples

- EDCL (non crosslinked decellularised bovine pericardium)
- 0.2DCL (minimally glutaraldehyde crosslinked decellularised bovine pericardium)

- 0.6DCL (moderately glutaraldehyde crosslinked decellularised bovine pericardium)
- GlutBP (fully glutaraldehyde crosslinked non-decellularised bovine pericardium).

2.1.3.3 Confirmation of glutaraldehyde crosslinking

Glutaraldehyde crosslinking was confirmed by assessment of Collagenase susceptibility, estimation of Shrinkage temperature, Contact angle measurement and Thermogravimetric analysis of minimally crosslinked sample.

2.1.3.3.1 Collagenase susceptibility

In vitro degradation study was conducted using Collagenase from *Clostridium histolyticum* (125u/mg, Sigma Aldrich). Approximately 50 mg (wet weight in triplicates) of 0.2DCL, 0.6DCL, EDCL and GlutBP samples were weighed. To the weighed samples, 0.5 ml of 0.1M Tris Chloride (pH 7.4) containing 0.005M Calcium Chloride and 0.05mg/ml Sodium Azide was added. The samples were incubated at 37 ^{0}C for 1 hr with constant shaking. Collagenase enzyme was prepared in 0.1M Tris Chloride (pH 7.4) at 37 ^{0}C and added in to the vials so as to make the final concentration to 2U/ mg of tissue. The vials were incubated at 37 ^{0}C for 24 hrs, 72 hours and 7 days, at the end of which, the samples were centrifuged at 12000rpm for 20 min at 4^{0}C and the remaining tissue samples were blotted in filter paper for 5 min to dry them. They were weighed using Afcoset Electronic Weighing Balance ER120A. The weight loss was determined by paired comparison before and after treatments.

2.1.3.3.2 Shrinkage temperature

The temperature at which the denaturation of collagen started was visualized as shrinkage and it was measured in EDCL, 0.2DCL, 0.6DCL and Glut control groups. 30x6mm sized samples (n=6) were immersed in water taken in a beaker under constant load by keeping under a glass slide. Water heated up to 90^0C was pumped into the beaker at 38-48ml/minute using a positive displacement pump (Masterflex, Cole Parmer, model 7518-60, USA). The temperature at which shrinkage is observed was recorded using a digital thermometer. (Accurad 2020, Radix, India).

2.1.3.3.3 Contact angle measurement

Contact angle measurement was employed as an indicator of surface chemical modification such as increased hydrophobicity subsequent to cross-linking. For this 1 cm^2 triplicate samples of 0.2DCL, 0.6DCL and EDCL groups were dehydrated by keeping in -20^0c for overnight followed by lyophilization. Angle of contact of a small sessile drop of distilled water placed on the material surface was measured on triplicate samples, with three measurements on each sample using Goniometer GII, Kerno Instruments Inc, USA.

2.1.3.3.4 Thermogravimetry

Thermogravimetry was done using the instrument SDT-2960, TA Instruments Inc, Delaware USA.. The temperature at which structural degradation of the minimally cross-linked test sample (0.2DCL group) started was compared with the un-cross linked decellularised bovine pericardium (EDCL group). For this triplicate 1 cm^2 samples of the above groups were dehydrated by keeping in -20^0c for overnight followed by lyophilization. The samples were studied for weight loss

during controlled heating at a range of 50 to 400^{0}C at a heating rate of 10^{0}C/ minute. Calcined alumina was used as reference material.

2.1.4 Preliminary screening of samples for biocompatibility

The test samples were subjected to Sterility testing, Endotoxin screening, Cytotoxicity testing (both Direct and Indirect) as preliminary screening for assessing its suitability for *in vivo* studies as well as to make sure that the test samples are free from microorganisms or PAMP (pathogen associated molecular pattern) such as bacterial lipopolysacharide which might affect further experiments. Cytotoxicity testing will assure that the pericardial samples are free from residual chemicals which might affect further experiments.

2.1.4.1 Materials

Thiazol blue tetrazolium bromide (MTT) (Sigma Aldrich M2128, USA), Isopropanol (SRL research cat no:092956, India), Thioglycolate medium (Sigma Aldrich B2551, USA), Trypton Soya broth (Sigma Aldrich 22091, USA).

2.1.4.2 Sterility testing (Direct transfer method USP 31/NF26<71>)

One ml of test sample (storage media) was diluted in minimum 20ml each of sterile fluid Thioglycolate medium and Trypton Soya broth and incubated at 37^{0}C for 14 days. At the end of the incubation period, the media was examined for presence of viable organism evidenced by presence of turbidity of medium and by microscopy.

2.1.4.3 Endotoxin screening (Limulus Amoebocyte Lysate Assay using Endosafe PTS Endotoxin).

This test is done to detect the bacterial endotoxin level in a test sample using Kinetic Chromogenic method. Test sample consisted of the medium in which the samples were stored. One ml of this medium was diluted in 25ml of pyrogen free sterile normal saline. 25µl of this sample was added into the four sample reservoirs of the cartridge. The instrument pump drew samples into the channels and initiated the test. Each cartridge contained precise amount of licensed LAL reagent, chromogenic substrate and control standard endotoxin. The instrument automatically drew and mixed the sample with the LAL reagent in two test sample channels. The LAL reagent and positive product control were drawn in the spike channels. The samples were incubated and then combined with the chromogenic substrate automatically. The results were obtained as readout. The spike recovery was 50% to 200% and coefficient of variance < 25%. Samples with less than 0.5EU are considered as non-pyrogenic as per USP 23 NF21<85>.

2.1.4.4 Cytotoxicity testing (Direct contact method: ISO10993-5).

Cytotoxic potential of Glut BP, EDCL , 0.2DCL and 0.6DCL groups on account of their direct contact or due to leachables was evaluated by Direct contact method (ISO 10993-5). The test samples were rinsed thrice with normal saline before cytotoxicity evaluation. Test samples, negative control (High density poly ethylene -USP), and positive control (Copper) in triplicate were placed on subconfluent monolayer of L-929 mouse fibroblast cells. After incubation of cells with test samples at $37\pm2^{0}C$ for 24±1 hours, cell culture was examined

microscopically for cellular response around the samples. Cellular responses were expressed as non-cytotoxic, mildly cytotoxic, moderately cytotoxic and severely cytotoxic.

2.1.4.5 Cytotoxicity test on extract

In-vitro MTT assay on extract of Glut BP, EDCL, 0.2DCL and 0.6DCL in physiological saline was performed to measure the effect of extract on the metabolic activity of L929 fibroblast cells. The ability of the fibroblast cells to metabolize yellow colored tetrazolium salt 3-(4,5-Dimethyl thiazol -2-yl)-2,5-diphenyltetrazolium bromide to purple colored formazan was measured as an indication of its activity. Different dilutions (50% and 25%) of extracts of the above samples, positive control (dilute phenol), negative control (high density poly ethylene (USP) and reagent control in triplicate were placed on subconfluent monolayer of L929 cells. After incubation of cells with the extract at 37 ± 2^0C for 2h, extract and control medium were replaced with 50ml MTT solution (1mg/ml in medium without supplements) wrapped with Aluminium foils and were incubated at 37 ± 2^0C for 2h. After discarding the MTT solution, 100ml of Isopropanol was added to all wells and swayed the plates. The color developed was quantified by measuring absorbance at 540nm using microplate reader. The data obtained was compared with negative control.

2.2 Phase2: Confirmation of modulation of immune response and tissue response of test samples consequent to chemical modification

To confirm whether modulation of immune response has occurred on account of chemical cross linking, different treatment groups were subcutaneously implanted into 21 day old male Wistar rats and antibody response (IgG, IgM and IgA), delayed hypersensitivity response, calcification response and tissue response was studied at the end of 60 days implantation.

2.2.1 Materials

Rabbit polyclonal to rat IgG+IgM+IgA conjugated with HRP (Abcam ab8521, UK), Nunc F96 MAXISORP immunoplates (Nunc cat: 113587,Roskilde, Denmark), Sodium carbonate (Himedia RM3951, India), Pottasium chloride (Himedia RM697, India), Tween 20 (Himedia RM154, India), Bovine Serum Albumin (Himedia RM3157, India), sodium hydrogen carbonate (Nice chemicals cat no: S11929, India), Di sodium hydrogen orthophosphate (Merck India cat no: 30157), TMB 3,3',5,5' tetramethylbenzidine (Sigma Aldrich T0440, USA). Calcium Kit OCPC method (Crest Biosystems, India). Paraffin wax (Nice chemicals P10129, India), Hematoxilin monohydrate (Merck cas no: 208-237-3, India), Eosin Yellow (Himedia RM115, India).

2.2.2 Animal implantation

Animal protocol was approved by Institutional Animal Ethics Committee. Three week old male Wistar rats were grouped into EDCL, 0.2DCL , 0.6DCL and GlutBP groups with each group consisting of 5 animals. Under general anesthesia,

animals were subcutaneously implanted under the dorsal skin with 0.5x0.5cm size, evenly placed six implants of the same treatment group. The animals were observed daily for 60 days and at the end of which they were euthanized.

2.2.3 Antibody response

The antibody response (IgG, IgM and IgA) elicited in different groups of animals implanted with 0.2DCL, 0.6DCL, EDCL and GlutBP samples were assessed using Indirect ELISA.

2.2.3.1 Indirect ELISA (Hornbeck, 2001).

For this, extractable protein of fresh bovine pericardium (prepared sterile as per section 2.1.2.5 and confirmed free of endotoxin and non-cytotoxic) was added in the 96 well ELISA immuno plate (F96 MAXISORP, NUNC) in PBSN (2 microgram protein per well) wrapped and kept overnight at 37^0C followed by washing thrice in washing buffer. The prepared ELISA plates were filled with blocking buffer for 45minutes and then washed thrice in washing buffer. Serum samples at 1in1000 dilution prepared in blocking buffer from animals belonging to the above groups (n=5) was used as primary antibody. Serum from animals which is immunized with extractable protein from fresh bovine pericardium was treated as positive control. 50 µl primary antibody was added into each well and wrapped followed by incubation at room at room temperature (RT) for 4 hours. 50µL of rabbit polyclonal to rat IgG+IgM+IgA conjugated with HRP (Abcam ab8521) at a concentration of 300ng/ml prepared in carbonate buffer was added to each well, wrapped and incubated at RT for overnight. The plates were washed for 3 times in washing buffer and 75µL of substrate was added and incubated at RT for 1 hour. The

reaction was stopped by adding 75µL 1N HCl and read at 450/620 dual filter using microplate reader Expert Plus (ASYS Hitech GmbH, Austria) and OD readings were recorded. OD readings of micro wells without secondary antibody were taken as negative control.

2.2.4 Delayed hypersensitivity response (Luo & Dorf, 1993)

For studying delayed type hypersensitivity reaction (cytotoxic T cell response) fresh bovine pericardial protein (prepared sterile as per section 2.1.2.5 and confirmed free of endotoxin and non-cytotoxic) was injected into the foot pad of Wistar rats which were subcutaneously implanted with different treatment groups and the reaction was studied by recording the increase in the foot pad thickness using Mitutoyo micrometer at 24 hour interval up to 48 hours.

2.2.5 Calcification response

Calcium estimations from explanted samples were done as per the procedure reported earlier (Vasudhev *et al* 2000). In brief, the explanted 0.2DCL, 0.6DCL, EDCL and GlutBP samples (n=15, three samples from each animal) were digested in 2ml of 2N HCl for 48 hrs at 60°C in an hot air oven. The supernatant was neutralized with 2N NaOH followed by estimation of Calcium using colorimetric method of O-Cresolphthalein complexone obtained as a standard kit. OD reading were taken at 578 nm using Shimadzu UV-1700 spectrophotometer, Japan . Calcium chloride solution of known concentration (10mg/DL) was used as standard.

2.2.6 Tissue response

The explanted 0.2DCL, 0.6DCL, EDCL and GlutBP samples (n=15, three samples from each animal) were fixed in 10% buffered formalin, embedded in

paraffin and 5 micron sections were made. The sections were stained by routine Hematoxylin and Eosin stain and a qualitative assessment was made for peri-implant necrosis, inflammatory response, peri-implant fibrosis, tissue incorporation and angiogenesis in the implanted material.

2.3 Phase 3: Study of immune response and its effect on regeneration

Based on the results of the first phase of the study, both innate and acquired/ adaptive immune responses were studied on samples such as EDCL: un-crosslinked decellularised bovine pericardium, 0.2DCL: partially cross-linked decellularised bovine pericardium and GlutBP: fully crosslinked non-decellularised bovine pericardium. For studying the *in vitro* innate immune response, cytokine release from activated macrophage on exposure to different treatment groups were estimated. Semi-quantitative assessment of inflammatory response against different treatment groups following intramuscular implantation into abdominal muscle of adult Wistar rats at 21 and 90 days will give information on *in vivo* innate response. Acquired humoral immune response was studied by assessing the antibody response in animals implanted with different treatment groups at 21 and 90 days. Lymphocyte transformation test on the lymphocytes isolated from the animals implanted with different treatment groups at 21 and 90 days, gave information on the cell mediated immune response.

2.3.1 Innate immune response (*in vitro*)

2.3.1.1 Materials

Human IL10 ELISA kit (U-Cytech Biosciences, Utrecht, The Netherlands Lot no: 16-06-2g), RPMI1640 (Sigma Aldrich R8758, USA), Fetal Bovine Serum (Gibco Cat no: 16000-036, USA), Benzylpenicilin (Sigma Aldrich, USA), streptomycin (Sigma Aldrich S9137, USA), Phorbol 12-myristate 13 acetate (Sigma AldrichP8139), Bacterial lipopolysaccharide (Sigma Aldrich L 2880, USA), Hematoxilin monohydrate (Merck cas no: 208-237-3, India), Eosin Yellow (Himedia RM115, India).

2.3.1.2 Macrophage activation

Macrophage activation was studied by looking for inflammatory cytokine release by adhered macrophages on contact with materials belonging to different groups. THP-1 (human acute monocytic leukemia cell lines) cells were grown in RPMI 1640 (Sigma) containing 10 % v/v Fetal Bovine Serum (Gibco), 160 U/ml benzylpenicillin and 100 U/ml streptomycin (Sigma). The cells were maintained at 37 °C in an atmosphere of 95% air and 5% CO2 at 90% relative humidity. The differentiation into macrophages was induced by treating THP-1 cells in a 24-well plate for 24 h with RPMI-1640 containing 20 ng/ml Phorbol 12-myristate 13- acetate (PMA). Thereafter fresh medium was added and cells were grown for another 24 h under similar conditions. Release of pro-inflammatory cytokines (IL-1β;, IL-6 , TNF-α and IL-10) from THP 1 cells upon contact with triplicate samples of EDCL, 0.2DCL, GlutBP and positive control (bacterial lipopolysaccharide at 20 pico gram) were carried using ELISA. The concentration of cytokines in culture supernatant

was quantified by specific ELISA kit (U-CyTech biosciences, Netherlands) as per manufacturer's instructions.

2.3.1.3 *In vivo* inflammatory response

2.3.1.3.1 Animal implantation

Animal protocol was approved by Institutional Animal Ethics Committee. Adult Wistar rats were grouped into EDCL, 0.2DCL, GlutBP groups with each group consisting of 5 animals for two durations of 21 days and 90 days. Under general anesthesia, Ventro-lateral abdomen was prepared for aseptic surgery. A linear skin incision of about 3-4cm was made, abdominal muscles were exposed. A full thickness abdominal defect of about 1.5 to 2 cm size consisting of all the layers of abdominal wall such as *external* and *internal obliquous abdominus muscle, tranverse abdominus muscle* and peritoneum was created by excising these structures. The test material in circular shape, of about 1.5 to 2 cm size was implanted in the defect created using 5/0 prolene sutures. The skin wound was closed with 3/0 silk sutures and the animal was given post operative antibiotics and analgesics for 5 days. At the end of the observation period of 21 days and 90 days, the animals were euthanized and the blood, spleen and explants samples were collected immediately and used for further studies.

2.3.1.3.2 Semi-quantitative evaluation of inflammation (Semi-quantitative assessment based on ISO 10993-6:2007(E))

For this experiment, the explants samples were processed for studying the type and intensity of the inflammation observed on Hematoxylin and Eosin stained histological sections that were scored semi-quantitatively as per the schema given in the table below . For this, occurrence and intensity of inflammatory cells such as

neutrophils, lymphocytes, plasma cells, macrophages and giant cells as observed in five random fields for each section under high power field were recorded and scored proportionately to its occurrence. A qualitative assessment of general tissue response is also made. The images were acquired using Olympus CX41 microscope and Jenoptik ProgRes C5 camera with ProgRes Capture Pro software ver 2.8.0, Jenoptik Optikal systems, GmbH, Jean, Germany. This was followed by image analysis using UTHSCSA ImageTool version3, University of Texas, Health Science Centre, Texas software, using tag and count function.

Cell type/response	**Score**				
	0	**1**	**2**	**3**	**4**
Polymorpho nuclear cells	0	Rare, 1-5 phf	5- 10 phf	Heavy infiltrate	Packed
Lymphocytes	0	Rare, 1-5 phf	5- 10 phf	Heavy infiltrate	Packed
Plasma cells	0	Rare, 1-5 phf	5- 10 phf	Heavy infiltrate	Packed
Macrophages	0	Rare, 1-5 phf	5- 10 phf	Heavy infiltrate	Packed
Giant cells	0	Rare, 1-5 phf	5- 10 phf	Heavy infiltrate	Sheets
phf = per high power (400x) field					

Table 2-1: Scoring criteria for inflammatory cells.

2.3.2 Acquired/ adaptive immune response

Acquired or adaptive immune response was studied based on the two representative tests suggested in the document ASTM F1906-98 (Reapproved 2003). To study the acquired immune response such as humoral immune response and cell mediated immune response, antibody response and lymphocyte activation in the presence of bovine pericardial proteins were studied respectively at both 21 days and

90 days in adult Wistar rats implanted with different treatment groups as described in section 2.3.1.3.1.

2.3.2.1 Materials

RPMI1640 (Himedia AL028A, India), Hi-Sep LSM1084 (Himedia LS003, India). Trypan Blue (Himedia RM1001, India), New born calf serum (PAN Biotech GmbH, Germany cat no: 0401-P100104N), DMSO (Sigma Aldrich D2650, USA). Rabbit polyclonal to rat IgG+IgM+IgA conjugated with HRP (Abcam ab8521, UK), Nunc F96 MAXISORP immunoplates (Nunc cat: 113587,Roskilde, Denmark), Sodium carbonate (Himedia RM3951, India), Pottasium chloride (Himedia RM697, India), Tween 20 (Himedia RM154, India), Bovine Serum Albumin (Himedia RM3157, India), sodium hydrogen carbonate (Nice chemicals cat no: S11929, India), Di sodium hydrogen orthophosphate (Merck India cat no: 30157), TMB 3,3',5,5' tetramethylbenzidine (Sigma Aldrich T0440, USA). Gentamycin (Himedia A005-5X,India), Amphotericin (Himedia A001A-5X, India), Penicilin and Streptomycin solution (Himedia A001A-5X India).

2.3.2.2 Humoral immune response: Antibody response (Section 5, ASTM F1906-98:Reapproved 2003)

Antibody response consisting of IgG, IgM and IgA responses against bovine pericardial proteins (prepared sterile as per section 2.1.2.5 and confirmed free of endotoxin and non-cytotoxic) induced in the animals implanted with different treatment groups such as EDCL, 0.2DCL and Glut BP were studied using indirect ELISA (section 2.2.3.1). The antibody response was studied at two time points of 21 days and 90 days as this gave an indication of the trend for this response.

2.3.2.3 Cell mediated immune response (ASTM F1906-98:Reapproved 2003)

Presence of lymphocytes sensitized against bovine pericardial proteins was studied using Lymphocyte transformation test. For this, sensitized lymphocytes were isolated from homogenized spleen of the animals implanted with different treatment groups for durations of 21 and 90 days as described in section 2.3.1.3.1. Proliferation response of these lymphocytes in the presence of bovine pericardial proteins (prepared sterile as per section 2.1.2.5 and confirmed free of endotoxin and non-cytotoxic) was studied using MTT assay at the end of 7 days incubation at 37^0C and 5% CO_2 concentration.

2.3.2.4 Lymphocyte transformation test (Section 8.1.1, ASTM F1906-98: Reapproved 2003)

In this test, sensitized lymphocyte responding specifically to an antigen will be activated and stimulate other lymphocytes to divide through released cytokines, thus amplifying the response. The proliferation of these lymphocytes is detected using MTT assay. Lymphocytes are isolated form spleen of animals which are implanted with different treatment groups as described in section 2.3.1.3.1.

2.3.2.4.1 Homogenization of spleen

Freshly removed spleen from animals implanted with different treatment groups were collected into sterile PBS containing gentamycin 50µg/ml and penicillin 10IU/ml in a sterile 50 ml centrifuge tube. The spleen was transferred to a sterile petri dish and excess tissues attached to it were removed aseptically under laminar flow bench. The spleen was then transferred to a sterile petri dish containing 3 ml complete RPMI 1640 (Himedia) with 5%FCS (Invitrogen). The spleen was chopped

into fine pieces with a sterile surgical blade. Using a circular motion, the pieces were pressed against bottom of the petri dish with the plunger of a 5ml syringe until the tissues sections were homogenized completely. Clumps in the suspension were dispersed by drawing up and expelling the suspension several times through a 5ml syringe equipped with a 20-G needle. The suspension was expelled into a 15ml centrifuge tube and centrifuged at 4^0C for 10 min at 1000 rpm (200 × *g*) and the supernatant was discarded. Pellet was re-suspended in 5 ml complete medium, centrifuged for 10 min at 1000 rpm and the pellet was again re-suspended in 5ml complete medium.

2.3.2.4.2 Isolation of lymphocytes and removal of RBC

Three ml of HiSep LSM 1084 (Himedia) was transferred to sterile 15 ml centrifuge tubes. Over this, 3 ml of the cell pellet re-suspended in RPMI was layered so as to get two distinct layers. The tubes were centrifuged at 2000rpm for 30 min at room temperature. The supernatant was aspirated carefully to remove the medium and collected the interphase to a sterile 15 ml tube. 10 ml of complete medium was added to the collected interphase and the tubes were gently inverted to ensure proper mixing. The tubes were centrifuged at 1000rpm for 10 min at room temperature. The pellet was re-suspended in 0.5 ml complete medium and 4.5ml of PBS was added to the tubes. The tubes were again centrifuged at 1000rpm for 10 min at room temperature and the pellet was re-suspended in 0.5ml complete medium. Viability of cells were determined by trypan blue exclusion staining.

2.3.2.4.3 Seeding the cells on to microtitre plates

Approximately 10^6 cells /ml concentration was used for seeding on to sterile 96 well microtitre plates. Exactly 100µl cell suspension was added to each well of

the microtitre plate. An antigen concentrations of 50µg/ml bovine pericardial protein (prepared sterile as per section 2.1.2.5 and confirmed free of endotoxin and non-cytotoxic). This antigen concentration was arrived based on preliminary experiments to obtain maximum response at minimum antigen concentration. Lymphocytes from each group of animals were taken in triplicates for this single antigen concentration. Cell control without any antigen was taken for each group for comparison. 100µl of antigen, diluted in endotoxin free sterile distilled water was added to each designated well. The plates were incubated for 7 days at 37^0c and 5% CO_2 concentration. Color changes to the medium were noted down frequently and fresh medium was introduced as required. At the end of the 7^{th} day 10 µl of cell suspension in RPMI was taken for assessing cell viability by trypan blue exclusion staining.

2.3.2.4.4 MTT assay

The 7 day incubated cultures were used for MTT assay. 200 µl of fresh medium was added to the plates and 50µl of media was replaced with 50µl of 1mg/ml MTT solution. The plates were wrapped in Aluminium foil and incubated for 4 hours at 37^0C in a humidified atmosphere. The medium and MTT were removed from the wells and centrifuge at 1000rpm for 10min at room temperature. 200µl DMSO was added to the pellet and mixed well. 25µl of glycine buffer was also added to this solution. The color developed was read at 544nm immediately using Chameleon microplate reader, Model no. 425-106, Hidex Finland.

2.4 Th1/Th2 lymphocyte pathway preference (Allman *et al* 2001, 2002)

Th1/Th2 lymphocyte pathway preference was delineated in different treatment groups by studying the type of IgG response, ie., IgG1 (Th2 response) or IgG2 (Th1 response) elicited in different treatment groups. Two different implantation conditions, viz., subcutaneous implantation for 60 days and abdominal muscle implantation for 21 and 90 days were compared to assess the relative predictive value for induced regeneration.

2.4.1 Materials

Mouse Monoclonal Secondary antibody to Rat IgG1- heavy chain (abcam ab 11668, UK), Mouse Monoclonal Secondary antibody to Rat IgG2a- heavy chain (abcam ab 11670, UK), Nunc F96 MAXISORP immunoplates (Nunc cat: 113587,Roskilde, Denmark), Sodium carbonate (Himedia RM3951, India), Pottasium chloride (Himedia RM697, India), Tween 20 (Himedia RM154, India), Bovine Serum Albumin (Himedia RM3157, India), sodium hydrogen carbonate (Nice chemicals cat no: S11929, India), Di sodium hydrogen orthophosphate (Merck India cat no: 30157), TMB 3,3',5,5' tetramethylbenzidine (Sigma Aldrich T0440, USA).

2.4.2 Estimation of IgG1 and IgG2

For assessing the IgG1/IgG2 response, Indirect ELISA (as per section 2.2.3.1) was done against fresh bovine pericardial protein antigens (prepared as per section 2.1.2.5 and confirmed free of endotoxin and non-cytotoxic) on serum samples obtained from Wistar rats which were implanted with different treatment groups and

different periods as described in section 2.3.1.3.1. HRP conjugated anti IgG1 and anti IGg2 antibodies were used as secondary antibodies. The assessment of preference for Th2 pathway which indicated a possibility for regeneration was determined by calculating the ratio of IgG1/IgG2 between different treatment groups.

2.5 M2/M1 macrophage phenotype polarization

M1/M2 polarization shown by different treatment groups was identified by looking in to the predominance of M2 or M1 phenotype of macrophages observed in the tissue sections as demonstrated by immuno-histochemistry following abdominal wall implantation at 21 days and 90 days in adult Wistar rats as described in section 2.3.1.3.1. M1 macrophage phenotype was identified by anti-rat CD80 HRP conjugated antibodies (Abcam) and M2 macrophage phenotype by anti-rat CD163 HRP conjugated antibodies (Abcam) as reported earlier (Badylak and Gilbert 2008). The ratio of CD163+ to CD80+ cells was calculated to assess the predictive value of this experiment.

2.5.1 Materials

Rabbit monoclonal to CD80 (abcam ab 53003, UK), Mouse anti Rat CD163 (Santa Cruz Biotechnology SC 58965, USA), Super Sensitive^{TM} Polymer-HRP IHC Detection System (Biogenex, San Ramon, CA.USA Cat no: QD400-60KE), Xylene (Nice Chemicals cat no: E10217, India), Protenase K (Amresco cat no: E195, India), Ethanol (Merck, India), DPX Mountant (Merck cat no: 61803502501739, Germany), Poly-L-Lysine solution (Sigma Aldrich P8920, USA). NaOH (Merck

17573, India), HCl (SD fine chem. 20125-L05, India), Haematoxilin (Merck cas no: 208-237-3, India).

2.5.2 Immuno-histochemistry

2.5.2.1 Preparation of Poly-L-Lysine coated slide

Slides were wiped clean with 70 per cent ethanol and air-dried. The dry slides were dipped in 1N hydrochloric acid followed by 1*N* sodium hydroxide for 30 minutes and rinsed in distilled water. The slides were dipped in 1:10 Poly-L-Lysine in water for 10 minutes and dried in a hot air oven at 55°C.

2.5.2.2 Immunohistochemical staining

Paraffin-embedded sections of 4-5µ were cut on to poly-L-lysine coated slides and the slides were dried at 37°C overnight in a incubator. Following deparaffinisation, the sections were incubated at 37°C for 10 minutes in proteinase K (2µg/ml) solution to unmask the antigens in the formalin fixed tissue. The solution was allowed to cool for 10 minutes and the slides were washed thrice with de-ionised water followed by buffer. The sections were incubated at 37°C for 10 minutes in 3 per cent Hydrogen Peroxide solution to block endogenous peroxidase activity. Following Hydrogen Peroxide treatment, the slides were washed three times with de-ionised water followed by buffer. The sections were covered with Power Block™ according to tissue size and incubated at room temperature for 5-10 minutes to inhibit non- specific binding of the primary antibody. Following blocking, the sections were incubated with primary antibody at 4°C in a humidified chamber for 24 hours. Following the overnight incubation, the sections were rinsed three times with PBS prior to incubation with Super Enhancer reagent at room temperature for

20 minutes and then subjected to three more washes with PBS. The sections were then incubated in Poly-HRP conjugated secondary antibody at room temperature for 30 minutes, rinsed three times in PBS and incubated in 4 per cent diaminobenzadine substrate solution (BioGenex) at room temperature for 5-10 minutes. The sections were again rinsed with buffer or de-ionised water to stop the development of the substrate and counterstained with Harris haematoxylin for 30 seconds. The sections were dried at 37 °C for 20 minutes, dehydrated and mounted in DPX mountant.

2.5.2.3 Immunohistochemical analysis (Brown *et al* 2009)

The immunostained slides were examined and imaged and the number of CD80+ and CD163+ macrophages were counted under high power using Olympus CX41 microscope and Jenoptik ProgRes C5 camera with ProgRes Capture Pro software ver 2.8.0, Jenoptik Optikal systems, GmbH, Jena, Germany. This was followed by image analysis using UTHSCSA ImageTool version 3 software, University of Texas Health Science Centre, Texas, using tag and count function. Quantitative analysis was performed by counting the number of immuno-positive cells in six matched microscopic fields at 400x magnification. The counts were expressed as mean±SD. A ratio of M2/M1 was calculated and compared to understand the regenerative preference exhibited by different treatment groups.

2.6 Regeneration studies in animal models

Regeneration was studied in adult rat abdominal wall regeneration model and adult pig vascular implantation model. The regeneration or healing response elicited by different treatment groups such as EDCL, 0.2DCL and GlutBP were studied in this experiment

2.6.1 Materials

Biebrich Scarlet-Acid Fuchsin Solution, (Sigma Aldrich Catalog No. HT15-1, USA), Biebrich scarlet, 0.9%, acid fuchsin 0.1%, in acetic acid, 1.0%. Phosphotungstic Acid Solution, (Sigma Aldrich Catalog No. HT15-2, USA), Phosphotungstic acid, 10%.(SigmaAldrich, USA), Phosphomolybdic Acid Solution, (Sigma Aldrich Catalog No. HT15-3, USA), Phosphomolybdic Acid, 10%, Aniline Blue Solution, (Sigma Aldrich Catalog No. HT15-4, USA), Xylene (Nice Chemicals cat no: E10217, India), Ethanol (Merck, India), DPX Mountant (Merck cat no: 61803502501739, Germany), Hematoxilin monohydrate (Merck cas no: 208-237-3, India), Eosin Yellow (Himedia RM115, India). Ferric chloride (Himedia RM1379, India), Sodium thiosulphate (Sigma Aldrich S 7026, USA), Acid fuchsin calcium salt (Sigma Aldrich A-3908, USA), Phosphotungstic acid (Sigma Aldrich P-4006, USA), Acetic acid (Fisher scientific, 1105, USA), Saffron (Sigma Aldrich S8381, USA).

2.6.2 Adult rat abdominal regeneration model (Shi *et al* 2011)

For adult rat abdominal regeneration model, a full thickness abdominal defect was made as described in section 2.3.1.3.1 and the regenerative response at 21 days and 90 days elicited by the different treatment groups were studied using HE staining as well as histochemical stain Accustain® Masson Trichrome (Sigma-Aldrich). This enabled identification of different structural components of abdominal wall regenerate. The qualitative observations such as presence or absence of regeneration and its micro-anatomical features were recorded and compared between treatment groups. The observations were verified by using another special stain,

Movat's Pentachrome, stained as per procedure given in section 2.1.2.3, which distinguished different structural components histochemically.

2.6.2.1 Materials

Biebrich Scarlet-Acid Fuchsin Solution, (Sigma Aldrich Catalog No. HT15-1, USA), Biebrich scarlet, 0.9%, acid fuchsin 0.1%, in acetic acid, 1.0%. Phosphotungstic Acid Solution, (Sigma Aldrich Catalog No. HT15-2, USA), Phosphotungstic acid, 10%.(SigmaAldrich, USA), Phosphomolybdic Acid Solution, (Sigma Aldrich Catalog No. HT15-3, USA), Phosphomolybdic Acid, 10%, Aniline Blue Solution, (Sigma Aldrich Catalog No. HT15-4, USA), Ferric chloride (Himedia RM1379, India), Sodium thiosulphate (Sigma Aldrich S 7026, USA), Acid fuchsin calcium salt (Sigma Aldrich A-3908, USA), Phosphotungstic acid (Sigma Aldrich P-4006, USA), Acetic acid (Fisher scientific, 1105, USA), Saffron (Sigma Aldrich S8381, USA).

2.6.2.2 Histochemical staining using Accustain® Masson Trichrome

The sections were deparaffinised and hydrated to deionized water. Mordant in preheated Bouin's Solution, Catalog No. HT10-1, at 56°C for 15 minutes. The slides were cooled in tap water (18–26°C) contained in a Coplin jar. The sections were washed in running tap water to remove yellow color from sections followed by staining in Working Weigert's Iron Hematoxylin Solution for 5 minutes. Washed in running tap water for 5 minutes and rinsed in deionized water. This was followed by staining in Biebrich Scarlet-Acid Fucshin, Catalog No. HT15-1, for 5 minutes. Rinsed in deionized water and slides were place in working Phosphotungstic/Phosphomolybdic Acid Solution for 5 minutes followed by placing

slides in Aniline Blue Solution, Catalog No. HT15-4, for 5 minutes. After this step, slides were placed in Acetic Acid, 1%, for 2 minutes and discarded solution. Finally, slides were rinsed dehydrated through alcohol, cleared in xylene and mounted. The staining characteristics observed are Nuclei — Black, Cytoplasm — Red, Muscle fibers — Red, Collagen — Blue.

2.6.3 Adult pig vascular implantation model (Cheung *et al.,* 1999, Haluck *et al.,* 1990)

The difference in healing response in another tissue site and another species was studied through aortic implantations in pig model for 6 months duration and the healing response was studied.

2.6.3.1 Materials

Super Sensitive™ Polymer-HRP IHC Detection System (Biogenex, San Ramon, CA.USA Cat no: QD400-60KE), Xylene (Nice Chemicals cat no: E10217, India), Protenase K (Amresco cat no: E195, India), Ethanol (Merck, India), DPX Mountant (Merck cat no: 61803502501739, Germany), Poly-L-Lysine solution (Sigma Aldrich P8920, USA). Rabbit polyclonal antibody to ά-actin (Santa Cruz SC 130619, USA), Rabbit polyclonal antibody to NOS3 (Santa Cruz SC 653, USA). Biebrich Scarlet-Acid Fuchsin Solution, (Sigma Aldrich Catalog No. HT15-1, USA), Biebrich scarlet, 0.9%, acid fuchsin 0.1%, in acetic acid, 1.0%. Phosphotungstic Acid Solution, (Sigma Aldrich Catalog No. HT15-2, USA), Phosphotungstic acid, 10%.(SigmaAldrich, USA), Phosphomolybdic Acid Solution, (Sigma Aldrich Catalog No. HT15-3, USA), Phosphomolybdic Acid, 10%, Aniline

Blue Solution, (Sigma Aldrich Catalog No. HT15-4, USA), NaOH (Merck 17573, India), HCl (SD fine chem. 20125-L05, India).

2.6.3.2 Animal implantation

The animal experiments were approved by Institutional animal ethics committee and CPCSEA. Under general anesthesia and using aseptic procedures, a left lateral thoracotomy was performed, the left anterior lobes of lungs were retracted and ascending aortic arch was exposed. A 3-5cm aorta was isolated with vascular side clamp and a linear incision of approximately 2 to 4cm was made on the aorta. Diamond shaped test material belonging to different treatment groups such as EDCL, 0.2DCL and GlutBP of corresponding length and about 1.5 to 2cm width was implanted into the defect by continuous 5/0 prolene sutures. Each animal had one treatment group implanted and for each treatment group, there were 3 animals The chest was closed in layers and the animal was given postoperative antibiotics and analgesics for next 7 days. The sutures were removed on 10^{th} postoperative day. At the end of 6 months, the animals were euthanized and the native aorta with the implant was resected out and fixed in 10% buffered formalin for further processing.

2.6.3.3 Histological study

The healing response was studied using routine Hematoxylin and Eosin staining, Accustain® Masson Trichrome staining (as per section 2.6.2.2) and Immunohistochemical staining (as per section 2.5.2.2). Immuno-labeling of smooth muscle alpha actin for demonstrating vascular smooth muscle cells and eNOS for demonstrating endothelial cells were done to characterize the healing response. A

qualitative assessment of healing response was made and compared between different treatment groups.

2.7 Additional experiment to compare EDCL and 0.2DCL groups by cell adhesion studies

An *in vitro* cell adhesion test was performed with test materials EDCL and 0.2DCL. L929 cells cultured on MEM medium supplemented with 10% FBS were seeded on materials EDCL, 0.2DCL, and glass cover slip (control surface) at density of 1 x 10^4 cells/cm^2 and incubated for 48 h at 37 ± 1^0C under humidified atmosphere containing 5% CO_2. After 48h, cell seeded EDCL, 0.2DCL and glass cover slips were fixed in 2.5% glutaraldehyde. Cell seeded EDCL, 0.2DCL and control cover slip were examined under ESEM. L929 cells seen on the test material EDCL were attached but not spread well and morphology not attained. 0.2DCL group showed no cell attachment or spreading on the surface. The control cover slip showed very good cell adhesion and spreading.

2.8 Statistical analysis

All quantitative estimations were expressed in mean ± SD. Significance testing for differences of means between groups were done using Single factor ANOVA. This was followed by F test for testing homogeneity of variance and Un-paired 't' Test for equal/unequal variance as required. Statistically significant difference between means was assumed whenever p value was less than 0.05. In the text exact 'p' value is reported at several instances for the sake of comparison between treatment groups.

Results and Discussion

CHAPTER 3

RESULTS AND DISCUSSION

3.1 PHASE 1: Preparation of decellularised bovine pericardial samples

The preparation of test samples consisted of decellularisation of bovine pericardium using a non-detergent based proprietary process. This was done to avoid deleterious effect of residual detergents which may elicit inflammatory response as reported earlier (Cebotari *et al.,* 2010). Care was taken to procure bovine pericardium with the least bioburden to minimize microbial contamination thereby minimizing the chances of avoiding endotoxin and other 'pathogen associated molecular pattern' substances which might affect the study. The different stages involved in this phase were decellularisation and its confirmation, glutaraldehyde crosslinking and its confirmation and preliminary biocompatibility testing. This phase also included characterization of the test samples. Glutaraldehyde crosslinked non-decellularised bovine pericardium was used for comparison since this xenograft has been used for more than four decades for clinical use in human patients and extensive literature is available on its biological properties.

3.1.1 Characterization of decellularised bovine pericardium

Decellularization could be satisfactorily achieved by the method used. Nuclear remnants could not be demonstrated by Hematoxylin and Eosin (Figure 3-1) as well as by Hoechst 33258 staining indicating satisfactory decellularisation (Figure 3-2).

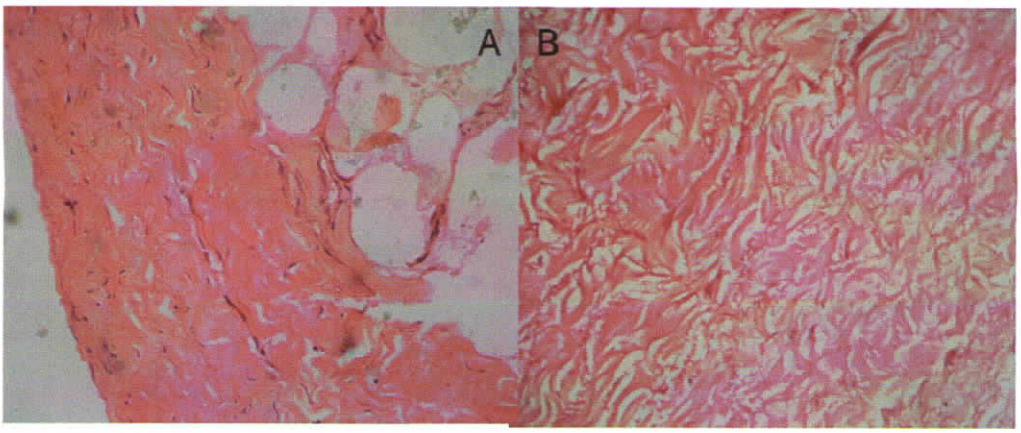


Figure 3-1: Confirmation of decellularization with Hematoxylin-Eosin staining. A: Non decellularised bovine pericardium showing intact nucleus. B: Decellularized bovine pericardium showing absence of nucleus. HE 400X.

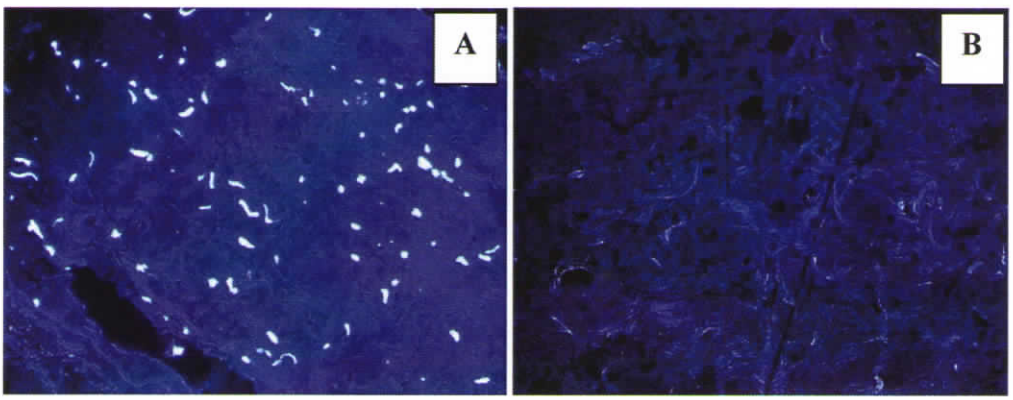


Figure 3-2: Confirmation of decellularisation with Hoechst 3328 staining. A: Non decellularised bovine pericardium showing intact nucleus. B: Decellularised bovine pericardium with no nuclear remnants. Hoechst 33258 400X.

Previous studies have utilized standard histological staining with Hematoxylin and Eosin as the first line of inspection to determine if nuclear structures can be observed (Gilbert *et al.,* 2006). Presence of DNA was also verified by staining the specimen with Hoechst 33258 (Kakkar & Grover, 2005) to confirm decellularization.

DNA extraction yielded a residual DNA content of 7±3.5 ng per mg in decellularised bovine pericardium compared to 97±2 ng /mg in fresh bovine pericardium (triplicate samples). Extractable DNA from decellularised tissue could

not be demonstrated in 1% agarose gel electrophoresis probably because of extensive DNA fragmentation (Figure 3-3).

Figure 3-3: 1% Agarose gel showing extractable DNA form decellularised bovine pericardium; 1st lane: molecular weight marker, 2nd and 3rd lane: DNA from decellularised bovine pericardium, 4th lane: DNA from fresh bovine pericardium (arrow head- 500bp).

Minimal criteria reported for removal of DNA following decellularisation are <50ng dsDNA per mg ECM dry weight, <200bp DNA fragment length and lack of visible nuclear material in tissue sections stained with 4',6-diamino-2-phenylindole or H&E (Crapo *et al.*, 2011). By the decellularization technique employed in this study, these minimum criteria could be achieved.

3.1.2 Structural analysis

Decellularised pericardium mainly consisted of well separated collagen bundles with a few elastin fibers as demonstrated by Movats Pentachrome staining (Figure 3-4). Image analysis revealed a collagen content of approximately 92% and

elastin content of 8%. Surface study using ESEM revealed morphological differences between the normal bovine pericardium and the decellularized pericardium.

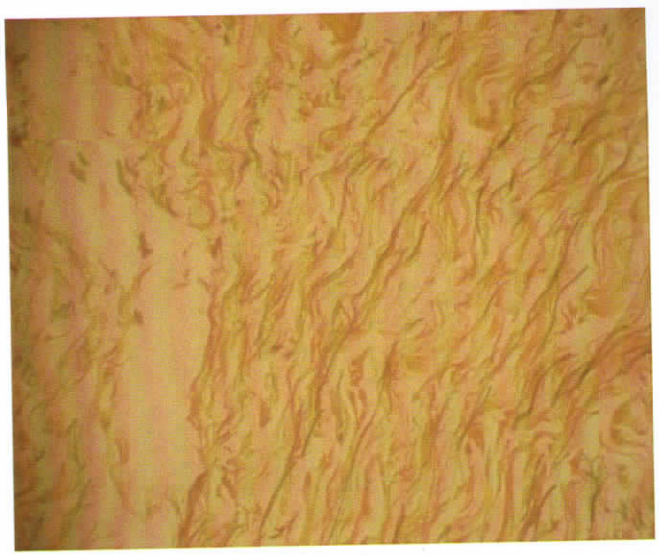


Figure 3-4: Decellularised bovine pericardium showing separated collagen bundles (stained yellow)with a few elastin fibers (stained black).Movats Pentachrome200X.

The normal bovine pericardium had a smooth surface which is having corrugations probably on account of glutaraldehyde fixation. The decellularized pericardium showed eroded surface with many crevices and fiber architecture was visible as linear structures more or less arranged in a uniform pattern resembling collagen bundles (Figure 3-5). Collagen is identified as the most abundant protein within mammalian ECM and is mainly constituted by collagen type I which is also widely used for therapeutic applications (Vanderrest and Garrone, 1991). Bovine pericardium is reported to contain 90% collagen and consisting predominantly of type 1 collagen (Naimark *et al.,* 1992; Schoen *et al.,* 1986,) by hydroxyproline estimation method.

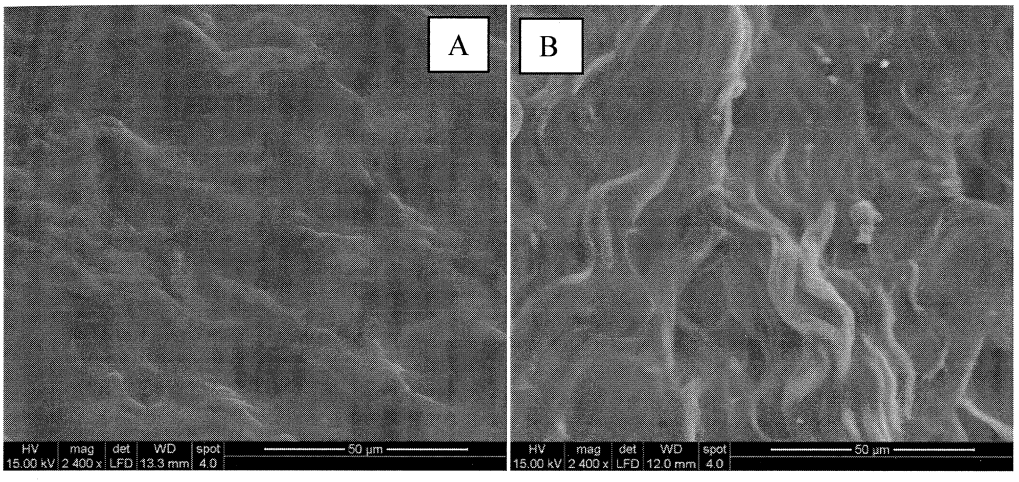
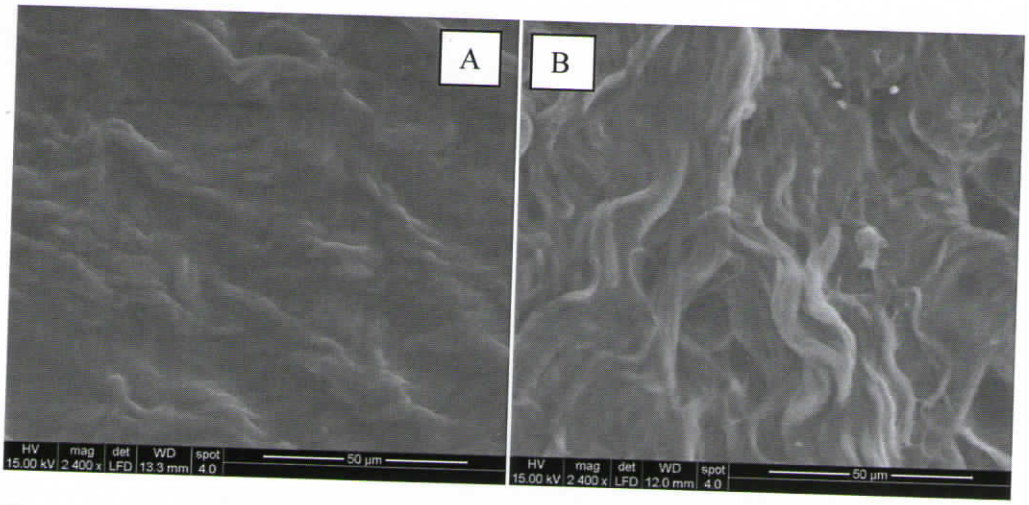


Figure 3-5: ESEM analysis of pericardial surfaces, A: Non decellularised bovine pericardial surface, B: Surface of decellularised bovine pericardium.

3.1.2.1 Extractable protein from decellularised tissue

The extractable protein content of decellularised pericardium in comparison to fresh bovine pericardium (n=9) has significantly reduced ($p<0.001$) from 0.241±0.02 mg/100mg wet tissue 0.102±0.026mg/100mg following decellularisation. It was further reduced to 0.09± 0.015 mg /100mg wet tissue following three days normal saline wash; however this reduction in protein content was not statistically significant. SDS-PAGE revealed several faint protein bands immediately after decellularisation and after the intense wash procedure only a faint band at around 66KD was only noticed (Figure 3-6). Fresh bovine pericardium showed several protein bands of which the band at 66KD region was the most prominent one.



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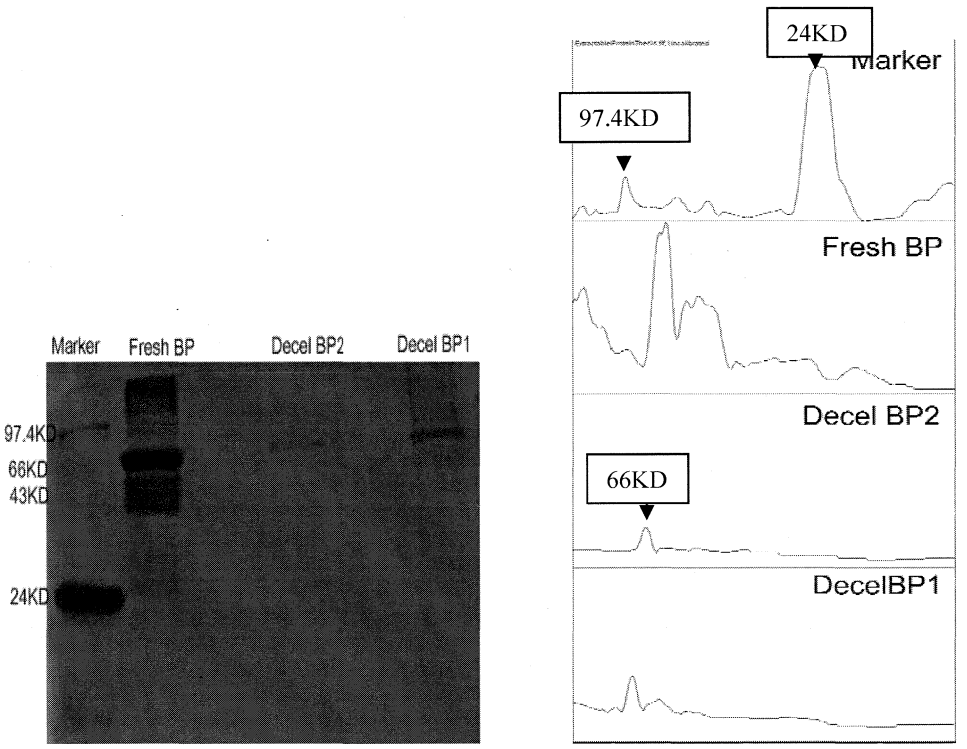


Figure 3-6. SDS-PAGE of extractable protein. Marker: molecular weight marker, Fresh BP: fresh bovine pericardial protein, Decel BP2: proteins from decellularised bovine pericardium after final wash, Decel BP1: proteins from decellularised bovine pericardium after immediate decellularisation

Bovine pericardium has been identified to contain 31 putative antigenic proteins capable of T cell-dependant antibody response as revealed by 2D electrophoresis and IgG production. (Griffith *et al.,* 2008, Mitchison, 2004, McHeyzer-Williams & McHeyzer-William, 2005). Out of these, two putative antigens such as albumin (Mol wt 69278.5D) and allergen bos d6 (69248.4D) has molecular weights in range observed in the case of decellularised bovine pericardium used in this study (Griffith *et al.,* 2008) . In this study endotoxin free, sterile, fresh bovine pericardial proteins were used to evaluate antibody response and cell mediated immune response to get a more representative picture rather than using a specific antigen such as bovine serum albumin.

3.1.3 Confirmation of glutaraldehyde crosslinking of decellularized and normal bovine pericardium

The glutaraldehyde crosslinking has yielded the following samples

- EDCL (non crosslinked decellularised bovine pericardium)
- 0.2DCL (minimally glutaraldehyde crosslinked decellularised bovine pericardium)
- 0.6DCL (moderately glutaraldehyde crosslinked decellularised bovine pericardium)
- GlutBP (fully glutaraldehyde crosslinked non-decellularised bovine pericardium).

In this study, different concentrations (0.2% and 0.6%) of glutaraldehyde were used for short duration (10 minutes) fixation of decellularised pericardium and the difference in biological response elicited by them was studied in comparison to un-cross linked decellularised bovine pericardium and completely crosslinked normal bovine pericardium. It was reported that the degree of crosslinking determines the degradation rate of chemically crosslinked acellular tissue and as a consequence it can significantly affect its regeneration pattern (Liang *et al.,* 2004). It was also reported that modification of the crosslinking process to produce a lower crosslinking rate can produce "growable" materials that are easily infiltrated with cells, degraded and remodeled as part of the wound healing response. (Noishiki *et al.,* 1989). Hence in this study glutaraldehyde crosslinking at three levels, mild (0.2DCL), moderate (0.6DCL) and complete (GlutBP) is done. To avoid

inflammatory response due to residual glutaraldehyde left after partial cross-lining, it was treated with 8% Glutamic acid as reported earlier (Grabenwoger *et al.,* 1992). The treated tissue was further incubated in 70% ethanol to extract residual tissue cholesterol and phospholipids which might induce calcification (Vyavahare *et al.,* 1997). This step also sterilized the tissue which was confirmed by routine sterility testing.

The change in their chemical and physical properties on account of glutaraldehyde treatment was assessed through studies on Collagenase susceptibility, estimation of Shrinkage temperature, Contact angle measurement and Thermogravimetric analysis and is presented below.

3.1.3.1 Collagenase susceptibility

Resistance to enzymatic degradation of collagenous tissue indicated the level of glutaraldehyde crosslinking and was routinely used reproducible method to assess the extent of glutaraldehyde crosslinking (Nimni *et al.,* 1987).

In this study its converse, *ie*., susceptibility to Collagenase enzyme was studied as it was hypothesized the susceptibility to tissue Collagenase such as matrix metalloproteinase will help in the positive tissue remodeling and may finally aid the induced regeneration elicited by decellularised scaffold. It was assumed that by altering the degree of crosslinking, changes in Collagenase susceptibility can also be achieved. Since glutaraldehyde crosslinking is also aimed at reducing the immune response on account of the residual protein, an optimum level of crosslinking is preferred which will provide adequate remodeling with simultaneous suppression of immune response.

By varying the concentration and duration of glutaraldehyde treatment, different extent of collagen-crosslinking could be obtained as presented here. Data on residual weight of different groups following Collagenase II digestion is given in table 3-1.

Residual weight (mg)	24 hours	72 hours	7Days
EDCL	0.3±0.2	-	-
0.2DCL	4.9±0.4*	1.8±0.3*	1.3±0.2
0.6DCL	46.6±1.5*	35.1±4.3*	17.9±1.9*
GlutBP	49±1*	49±1	48±1

Table 3-1: Residual weights in different groups with time following Collagenase type II digestion.*Statistically significant at $p<0.05$.

Following Collagenase type II digestion of 50mg samples, EDCL group (un-cross linked sample) had maximum degradation (>99% weight loss) in 24 hours. In comparison minimally cross-linked group, 0.2DCL had 90.2% weight loss, indicating mild chemical crosslinking. Moderately cross linked sample, 0.6DCL group was seen more resistant to degradation with only 6.8% weight loss at this period. GlutBP group had maximum Collagenase resistance with minimum weight loss (2%). At 72h residual weight in EDCL group was not measurable. 0.2DCL, 0.6DCL and Glut control groups showed a cumulative weight loss of 96.5%, 29.8% and 2% indicating progressive degradation in 0.2DCL and 0.6DCL groups. At 7 days the rate of weight loss has slowed down in 0.2DCL group with only an additional 1% weight loss was noticed at this time. 0.6DCL group showed further weight loss during this period reaching a cumulative weight loss of 64.2%. GlutBP

continued to lose weight at very low rate reaching about 4% at 7 days. The data on degradation profile is given in table 3-2.

Treatment groups	EDCL (un-cross linked sample)	0.2DCL (minimally crosslinked sample)	0.6DCL (moderately crosslinked sample)	GlutBP (fully crosslinked sample)
Susceptibility to Collagenase type 2 (*in vitro*)%	>99%* degradation in 24 hours; no measurable residue left at 72 hours.	90.2%* degradation in 24 hours; 96.5% degradation in 72 hours; 97.4% in 7 days.	6.8%* degradation in 24 hours; 29.8% in 72 hours; 64.2% in 7 days.	2%* degradation in 24 hours, 2% in 72 hours; 4% in 7 days.
Shrinkage temperature, ^{0}C	64.58±0.47*	66.31±0.48*	70.3±0.55*	77.5±0.91*
Contact angle, degrees	29±0.57*	33.5±0.86*	45.3±0.88*	-

Table: 3-2, showing the changes in the physical and chemical properties brought about by glutaraldehyde crosslinking. *Statistically significant difference at $p<0.05$.

3.1.3.2 Shrinkage temperature

Heat of denaturation with associated shrinkage of collagenous tissue indicated the level of glutaraldehyde crosslinking (Nimni *et al.,* 1971; Nimni, 1975; Strawich *et al.,* 1975). This is one of oldest methods used to assess level of glutaraldehyde crosslinking. In this experiment, a setup was created to produce controlled increase in the temperature of the bath water and the temperature at which a change in dimension of the test sample placed between glass slide was observed and was digitally recorded. This method gave considerable repeatability. Data thus

obtained pertaining to shrinkage temperature is given in table 3-2. Un-cross linked sample (EDCL) showed minimum shrinkage temperature of 64.58±0.47°C. This has significantly increased by 1.73°C in minimally cross linked group (0.2DCL). Moderate cross linking (0.6DCL group) has further elevated the shrinkage temperature significantly by 3.99°C. Maximum shrinkage temperature of 77.55±0.91°C was noted in completely cross linked sample (GlutBP). The above data further confirms that chemical crosslinks were produced by glutaraldehyde treatment and there is variation in the degree of crosslinking between 0.2DCL, 0.6DCL and GlutBP group.

3.1.3.3 Contact angle measurement

Contact angle measurement is used to study surface modification of polymer materials to indicate hydrophilic/hydrophobic nature. This also indicated chemical crosslinking in the case of tissue engineering matrices (Ghanbaria *et al.,* 2010). In this experiment, contact angle formed by the sessile drop of distilled water on different treatment groups are reported. Contact angle reading in EDCL group was 29 ± 0.57^0 which was the lowest ($p<0.001$). The contact angle reading in 0.2DCL and 0.6DCL were 33.5 ± 0.86^0 and 45.3 ± 0.88^0 respectively, which were significantly higher compared to in EDCL group (Table 3-2). This indicated definite structural modification on the measured surface because of short duration glutaraldehyde cross-linking. Furthermore contact angle on 0.6DCL group was significantly higher ($p<0.001$) compared to 0.2DCL group indicating a higher degree of surface change because of the treatment. EDCL group with no crosslinking was seen as the most hydrophilic one. Contact angle of GlutBP group could not be measured as sessile drop could not be formed on its surface.

3.1.3.4 Thermogravimetry

Thermogravimetric analysis determines the temperature of structural degradation and gives an indication degree of chemical crosslinking in bovine pericardium (Shamis *et al.,* 2009). Thermogravimetric analysis indicated an initiation of structural degradation around 152^{0}C in uncross linked sample (EDCL) compared to 206.4^{0}C in minimally cross-linked sample (0.2DCL). The initial weight loss noticed in both the samples can be due to water loss. Likewise the temperature of final structural degradation has increased from 244.76^{0}C in un-cross linked sample (EDCL) to 267.2^{o}C in 0.2DCL (minimally cross linked sample) . This shift in the degradation temperature observed in minimally cross linked sample can be due to

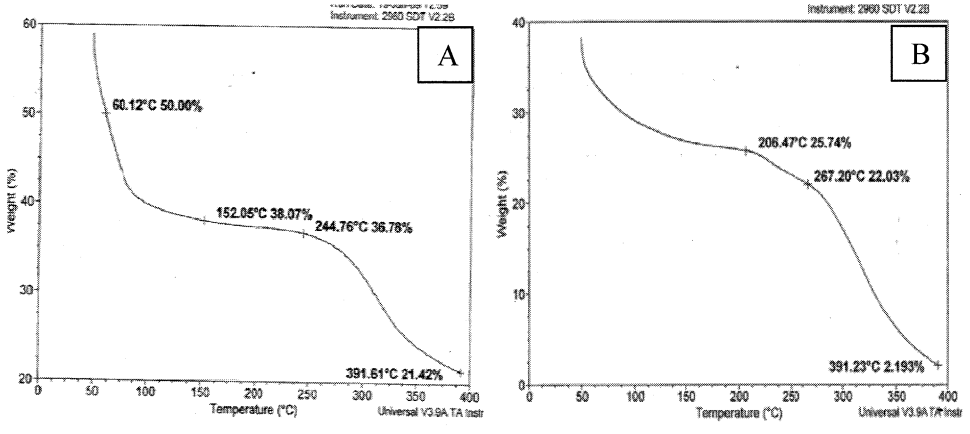


Figure 3-7: Thermogram of un-crosslinked decellularised bovine pericardium, EDCL (A) and minimally cross linked decellularised bovine pericardium,0.2DCL (B).

formation of chemical cross links.

Thus the above experiments confirms that by decellularisation and glutaraldehyde treatment, test samples with different level of chemical crosslinking such as EDCL (un-crosslinked decellularised bovine pericardium), 0.2DCL (minimally crosslinked decellularised bovine pericardium), 0.6DCL (moderately

crosslinked decellularised bovine pericardium) and GlutBP (fully crosslinked normal bovine pericardium) could be produced.

3.1.4 Preliminary screening of samples for biocompatibility

The test samples were tested for its sterility, presence of endotoxin, cytotoxicity by direct contact as well as through indirect method on extract to assure that the responses observed during further experiments are solely on account of the material property and not because any contaminants. The test samples were seen sterile and no viable organism could be detected even after 14 days culture. Endotoxin level detected was 0.1 EU/ml in the test samples. As per USP25 NF 21<85>, a level of 0.5 EU/ml is considered non-pyrogenic.

3.1.4.1 Cytotoxicity testing: Direct contact method (ISO10993-5).

Test samples EDCL 0.2DCL and 0.6DCL were seen non cytotoxic to L929 fibroblasts by direct contact method. This indicated that the residual glutaraldehyde following short duration cross linking using both 0.2% and 0.6% Glutaraldehyde could be effectively neutralized by PBS wash and Glutamic acid treatment. However, the cell density was less in the case of 0.6DCL compared to EDCL or 0.2DCL groups. Hence cytotoxicity testing on extract was performed on 0.6DCL and GlutBP samples. GlutBP sample was seen moderately cytotoxic probably because of residual glutaraldehyde. Positive control was severely cytotoxic and negative control was seen non-cytotoxic (Figure 3-8). Cytotoxic nature of glutaraldehyde treated tissue was reported earlier (Gendler *et al.,* 1984).

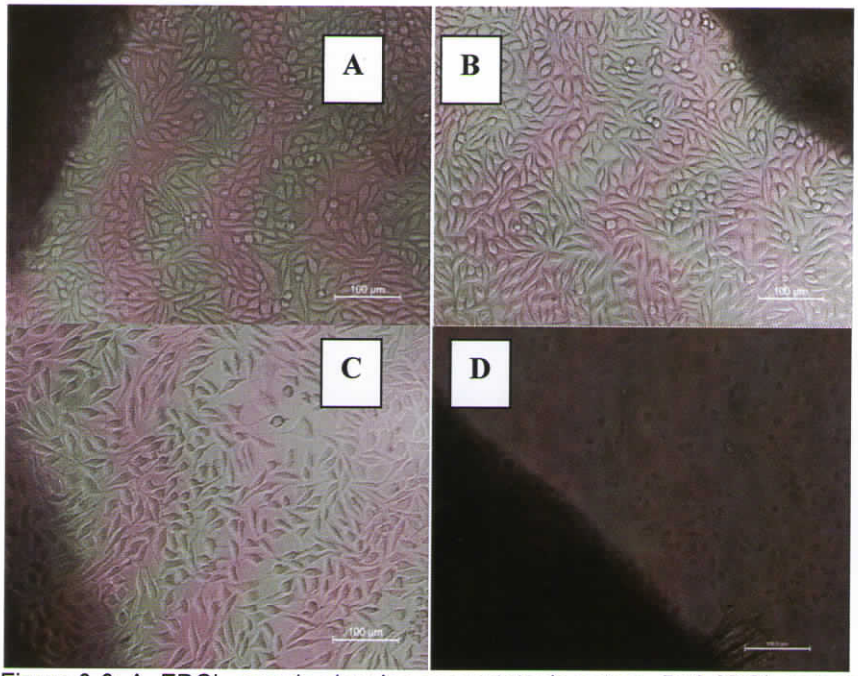
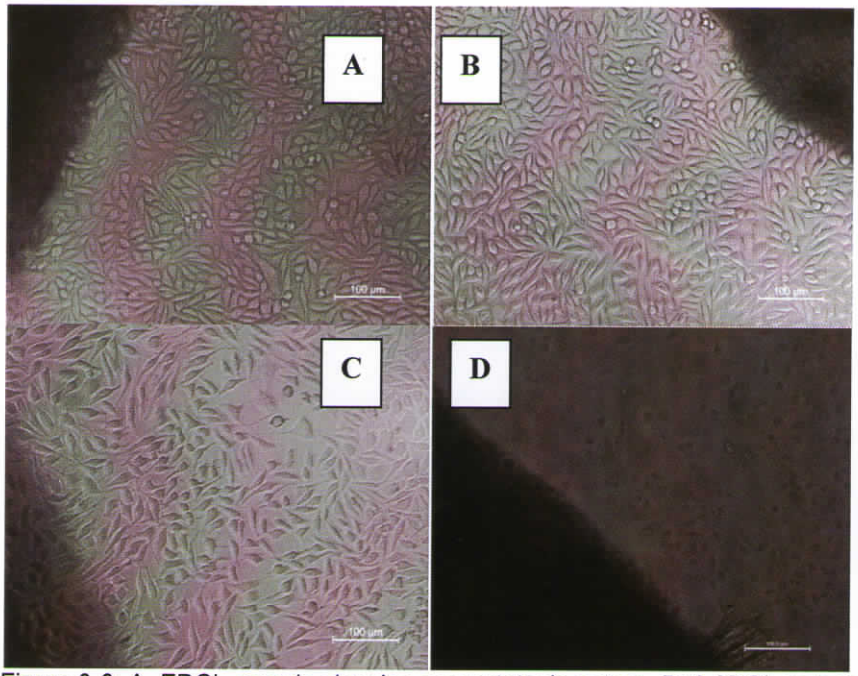


Figure 3-8: A. EDCL sample showing non cytotoxic nature B: 0.2DCL sample showing non-cytotoxic nature. C: 0.6DCL sample showing non-cytotoxic nature.. D:Glutaraldehyde control sample showing moderate cytotoxicity.

3.1.4.2 Cytotoxicity testing on extract:

The MTT assay of L929 cells after contact with 50% and 25% extract of decellularised bovine pericardium, 0.6DCL showed 96% and 97% metabolic activity respectively compared to GlutBP which had 14% and 15.1% metabolic activity respectively. This clearly showed that the extract of GlutBP samples has significantly affected the metabolic activity of fibroblast cells and hence it is toxic in nature compared to 0.6DCL sample.



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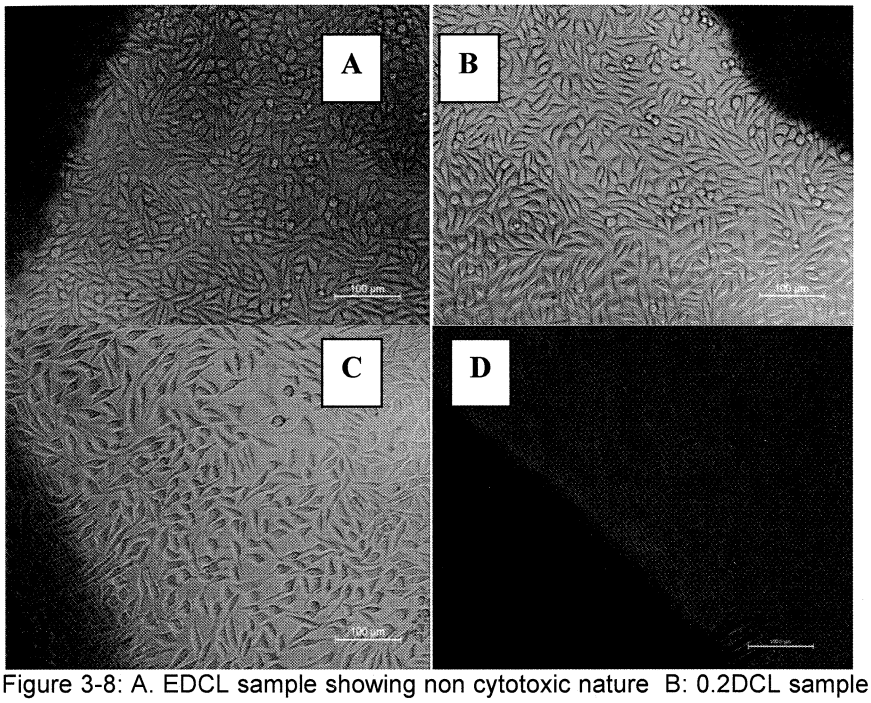

Figure 3-8: A. EDCL sample showing non cytotoxic nature B: 0.2DCL sample showing non-cytotoxic nature. C: 0.6DCL sample showing non-cytotoxic nature.. D:Glutaraldehyde control sample showing moderate cytotoxicity.

3.1.4.2 Cytotoxicity testing on extract:

The MTT assay of L929 cells after contact with 50% and 25% extract of decellularised bovine pericardium, 0.6DCL showed 96% and 97% metabolic activity respectively compared to GlutBP which had 14% and 15.1% metabolic activity respectively. This clearly showed that the extract of GlutBP samples has significantly affected the metabolic activity of fibroblast cells and hence it is toxic in nature compared to 0.6DCL sample.

3.2 PHASE 2: Confirmation of modulation of immune response and tissue response of test samples consequent to chemical modification

The objective of glutaraldehyde crosslinking of decellularised bovine pericardium was to produce samples with different immune response. The experiments described below were performed to confirm that following this treatment significant variation in the biological response including immune response could be produced. The results showed that there was significant difference between treatment groups with respect to its *in vivo* responses such as antibody response, delayed hypersensitivity response, calcification response and tissue response when studied by 60 days subcutaneous implantation in juvenile rat model. Nearly 1.5cm^2 material of the same group was implanted in each animal weighing around 60-80g body weight to study this response.

3.2.1 Antibody response by Indirect ELISA

Immunogenicity of glutaraldehyde tanned bovine pericardium has been previously tested by estimating the antibody response against the extractable protein from the material (Dahm *et al.,* 1990). In this experiment, antibody response against sterile endotoxin free fresh bovine pericardial protein was estimated using In-direct ELISA. Considerable variation in response was noted among different treatment groups as given in Figure 3-9. Antibody response in uncross-linked sample, EDCL was significantly higher than 0.2DCL group ($p<0.01$), 0.6DCL group ($p<0.0001$) and GlutBP group ($p<0.02$). Minimally crosslinked sample 0.2DCL, had significantly lower antibody response compared to all the other groups. Interestingly

0.6DCL group showed a higher antibody response compared to 0.2DCL group ($p<0.01$), whereas it was noticed comparable to GlutBP group. Less antibody response of 0.2DCL group compared to EDCL ($p< 0.004$) indicate reduction in antibody production in response to partial cross linking. Antibody response elicited in BP protein immunized animal was also studied to validate the In-Direct ELISA procedure.

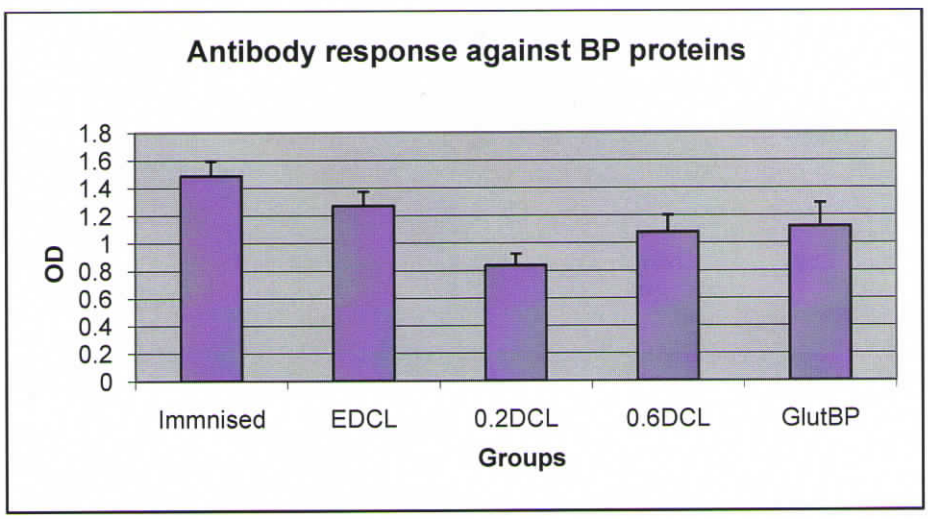

Figure 3-9: Chart showing difference in the antibody response against bovine pericardial protein observed in different treatment groups.

3.2.2 Delayed hypersensitivity response

The test methodology adapted in this experiment was based on the protocol suggested by Luo & Dorf, (1993). In this study, Type IV hypersensitivity reaction is studied following introduction of an antigen in a pre-sensitized animal. In this experiment, sensitization of the animal takes place during the 60 days implantation period. Sterile, endotoxin free, fresh bovine pericardial protein is injected into the footpad of the animal to detect hypersensitivity response. In this experiment (Figure 3-10) at 0 hour, immediately after injection there was no significant difference

between treatment groups with respect to foot pad thickness. At 1h, statistically significant increase in the foot pad thickness was noted in EDCL group followed by 0.6DCL (p=0.003) compared to both 0.2DCL (p= $0.5x10^{-5}$) and GlutBP $2.43x10^{-9}$) groups. Footpad thickness in 0.6DCL group was significantly higher compared to both 0.2DCL and GlutBP groups. There was no significant difference between 0.2DCL and GlutBP groups at this period. At 24 hours, EDCL group continued to be significantly higher compared to 0.6DCL (p=0.001), 0.2DCL (p=0.0003) and GlutBP group (p=0.0033). There was no significant difference between0.6DCL, 0.2DCL and GlutBP groups at this period. At 48h, EDCL remained as the highest reactive group compared to 0.6DCL group p=(0.0001), 0.2DCL (p= 0.0009), GlutBP ($p=3.1x10^{-6}$). There was no statistically significant difference in foot pad thickness between 0.2DCL, 0.6DCL and GlutBP groups at this period. (Figure 3-10)

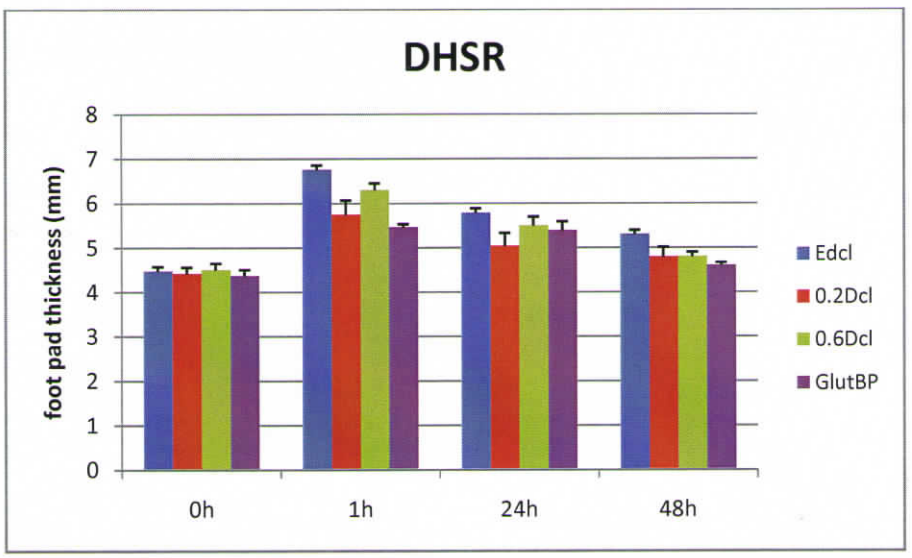

Figure 3-10: Chart showing the difference in delayed hypersensitivity response observed between treatment groups.

The above two experiments conclusively prove that by varied glutaraldehyde crosslinking, samples with different immune response could be produced which can

be utilized for further experiments. It was also noted that EDCL group was seen consistently more immunogenic compared to all the other groups.

3.2.3 Calcification response

A previous study has reported direct relation between calcification and antibody response (Human & Zilla, 2001). Hence calcification potential of different treatment groups was studied in this experiment. The result of calcification experiment is given in Table 3-3. EDCL group explant had minimum calcium content of 42.7±16.9μg/100mg of wet tissue; followed by 0.2DCL group explant which had a calcium content of 102±59μg/100mg wet tissue. 0.6DCL group showed an intermediate response with a calcium content of 433±243μg/100mg of wet tissue. Maximum calcium content of 1579±727μg/100mg of wet tissue was noticed in glutaraldehyde treated tissue. All the differences in means noted between the groups were statistically significant at $p<0.05$. Schoen *et al.*, (1986) have reported a calcium level of 199μg/mg tissue dry weight at 112 day explant in rat subcutaneous model.

Treatment groups	EDCL (un-cross linked sample)	0.2DCL (minimally cross-linked sample)	0.6DCL (moderately cross linked sample)	GlutBP (fully cross linked sample)
In vivo calcification response (μg/100mg wet tissue)	42.7±16.9*	102±59*	433±243*	1579±727

Table 3-3: Depicting differences in calcification response consequent to glutaraldehyde treatment.

The relationship between calcification and antibody response was noticed only in glutaraldehyde crosslinked samples immaterial of whether partially or completely cross linked. This relation was not observed in un-cross linked sample (EDCL) which showed a significantly higher antibody response with minimum calcification.

3.2.4 Tissue response

Subcutaneous implantation of test samples provided valuable information on the type of healing response produced consequent to different glutaraldehyde crosslinking of decellularised BP in comparison to un-crosslinked decellularised BP and fully crosslinked un-decellularised BP. This experiment also provided inputs for selecting groups which would be tested for regenerative response. The tissue response studied on Hematoxylin and Eosin stained paraffin sections is presented below (Figure-3-11). The sections were qualitatively assessed for peri-implant necrosis, inflammatory response, calcification, peri-implant fibrosis, tissue incorporation and angiogenesis in the implanted material as well as in the periphery.

EDCL implants did not show peri-implant necrosis. A very thin capsule could be appreciated around the implant. There was moderate to severe diffuse infiltration of mononuclear cells into the implant. No focal inflammatory cell accumulation in the periphery could be appreciated. Implant calcification could not be observed. Angiogenesis was noticed in the periphery as well as the interior of the implant. Cells with fibroblast morphology could be appreciated in the implant.

0.2DCL explant did not show any peri-implant necrosis. A thin fibrous capsule was noted around the implant. There was mild to moderate diffuse infiltration of mononuclear cells in to the implant is noticed. Macrophages could be identified at the site of implant degradation. Implant Calcification could not be

observed. Implant showed excellent in-growth of cells with fibroblast morphology. Angiogenesis is noticed in the implant as well as the periphery. Implant calcification could not be observed.

0.6DCL explants showed thicker fibrous capsule compared to 0.2DCL group. No peri-implant necrosis could be observed. Mononuclear cell infiltration of moderate intensity composed of lymphocytes, macrophages and occasional multinucleated giant cells was noticed at several places around the implant.

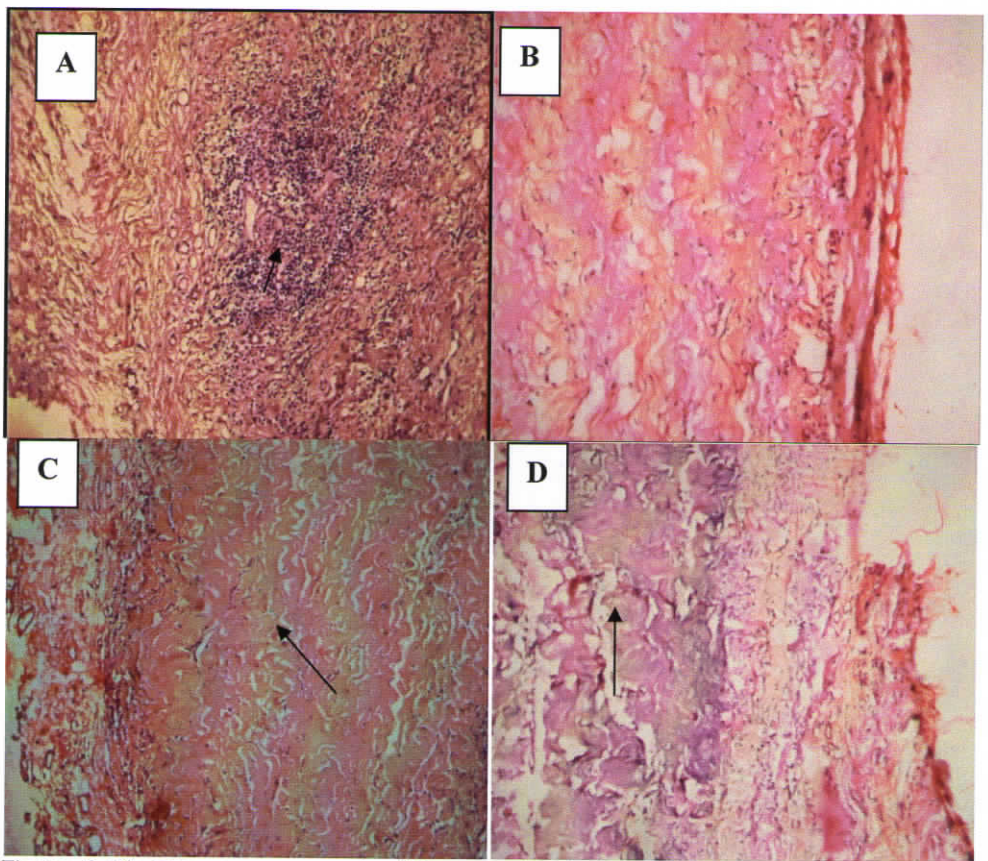

Figure 3-11: Tissue response to different implants at 60 days in rat subcutaneous implantation; **A**: EDCL group: showing moderate to severe inflammatory response in the interior of implant (arrow). **B**: 0.2DCL group showing thin capsule formation, minimum inflammatory response and uniform host cell incorporation **C:** 0.6DCL group showing thicker capsule formation, focal inflammatory response and acellular interior (arrow). D: GlutBP showing thicker capsule, inflammatory response in the interphase an acellular interior (arrow). HE 200X.

Implant calcification could be observed as small discrete deposits in the collagen fiber. Angiogenesis could be appreciated only in the implant periphery. Some sections showed implant areas devoid of host cell incorporation or angiogenesis.

GlutBP: Thick capsule was noticed around the implant. Certain areas showed peri-implant necrosis. Moderate to severe inflammation evidenced as mononuclear cell infiltration could be seen at the periphery of implant. The implant interior was remarkably acellular. Angiogenesis was seen only in the periphery of the implant. Calcium deposit could be observed in many sections.

Effect on chemical crosslinking on tissue response to acellular matrices are reported earlier. It was shown that degree of crosslinking determines the degradation rate of acellular tissue and consequently its tissue regeneration pattern (Liang *et al.*, 2004). In the above experiment un-crosslinked EDCL group and minimally crosslinked 0.2DCL group exhibited connective tissue incorporation into the scaffold interior. Whereas moderately crosslinked 0.6DCL group showed regions devoid of cell incorporation and fully crosslinked GlutBP group showed total acellularity. Similar observation on the angiogenic response was also evident with angiogenesis being present only in EDCL and 0.2DCL group. Yao *et al.*, (2008) have observed that chemical crosslinking contributes substantially to angiogenic properties of collagen matrices. Above observations confirmed that different degrees of glutaraldehyde crosslinking resulted in varying immune response and tissue responses.

3.3 PHASE 3: Study of immune response and its effect on regeneration

Based on the observations in Phase 2 of the study, treatment groups EDCL (un-crosslinked decellularised bovine pericardium), 0.2DCL (partially crosslinked decellularised bovine pericardium) and GlutBP (fully crosslinked normal bovine pericardium) were selected for studies on immunogenicity and regeneration. 0.2DCL group had minimum inflammatory and immune response besides excellent tissue incorporation and neo-angiogenesis within the implant which appears congenial for regeneration. While EDCL had excellent healing characteristics such as excellent tissue incorporation into the scaffold matrix and neo-angiogenesis within the implant, at same time presented a picture of moderate to severe chronic inflammatory response together with significantly high antibody production against bovine pericardial proteins (Section 3.2.1). 0.6DCL group was rejected for Phase 3 since it presented a picture of poor cell incorporation into the scaffold matrix and no neo-angiogenesis into the scaffold. Inflammatory, calcification and antibody responses were also significantly higher compared to 0.2DCL group. GlutBP group was included in the study since it presented the other extreme of the desirable healing response needed for induced regeneration such as lack of cell incorporation and neo-angiogenesis into the scaffold and moderate to severe chronic inflammation.

Both innate and acquired/ adaptive immune responses were studied in this phase. For studying the *in vitro* innate immune response, cytokine release from activated macrophage on exposure to different treatment groups were estimated. This was followed by semi-quantitative assessment of inflammatory response against

different treatment groups following intramuscular implantation into abdominal muscle of adult Wistar rats at 21 and 90 days.

3.3.1 Innate immune response

Innate immune response was studied using in vitro macrophage activation study and *in vivo* study to assess inflammatory response elicited by different treatment groups.

3.3.1.1: *In vitro* Macrophage activation study

Macrophages besides being an important component of innate immune response as antigen presenting cells have an important role in eliciting specific immune response. Hence macrophage activation study was conducted. Cytokine release profile of activated macrophage in response to exposure to different treatment groups showed varied response (Figure 3-12). Positive control group (LPS) showed the highest cytokine release thus validating the test system. With respect to the TNFα and IL1β, highest macrophage activation was seen in GlutBP group. This was seen statistically significant compared to EDCL and 0.2DCL groups. There was no significant difference in response between EDCL and 0.2DCL with respect to the above cytokines. In the case of IL6, 0.2DCL group showed a higher response when compared to GlutBP group ($p=0.004$) and EDCL group (p0.005). Anti-inflammatory cytokine IL10 was seen highest in GlutBP group, when compared to EDCL group ($p=0.03$) and 0.2DCL group ($p=0.02$). Minimum IL10 release was seen in EDCL group. There was no significant difference between EDCL and 0.2DCL group.

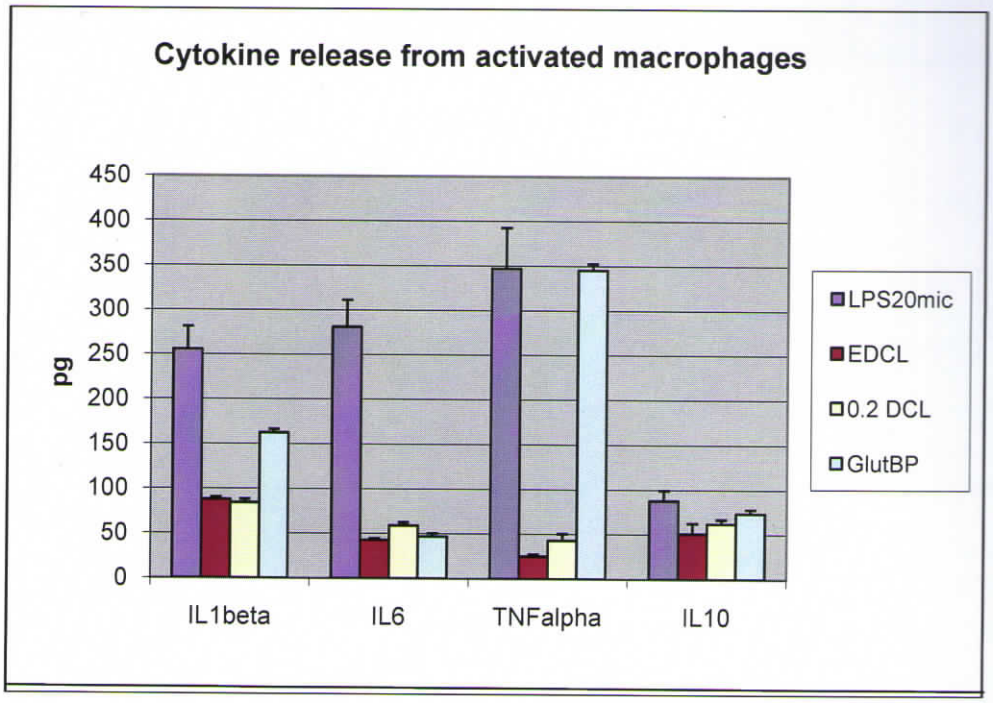

Figure 3-12: Chart showing cytokine release from activated macrophages on exposure to different treatment groups.

The data showed a definite increase in the release of inflammatory cytokines such as TNFα and IL1β in completely cross-linked samples (GlutBP) compared to partially crosslinked samples (0.2DCL) and un-crosslinked sample (EDCL). Partially crosslinked or un-crosslinked didn't differ in their macrophage activation potential with respect to the above cytokines. Interestingly, IL6 release was seen highest in the partially crosslinked sample compared to un-crosslinked and completely crosslinked samples. Data on IL-10 release showed no difference between un-crosslinked (EDCL) and partially crosslinked (0.2DCL) group. It has been previously observed that chemically modified protein antigens induce inflammatory cytokines (Yang *et al.,* 1993). In contrast to the above observation, a study showed no significant variation between in *vivo* cytokine release of inflammatory and anti-inflammatory cytokines in response to different polymeric materials (Schutte *et al.,* 2009).

3.3.1.2 *In vivo* inflammatory response (Semi-quantitative assessment based on ISO 10993-6:2007(E))

As an extension to *in vitro* macrophage activation study to assess the innate immune response, *in vivo* inflammatory responses elicited by different treatment groups was studied. Inflammatory response to biomaterial is considered as innate immunity expressed in a *in vivo* situation (Anderson *et al.,* 2008). In this experiment, an intramuscular site was selected as it provides better opportunity to evaluate inflammatory response in a highly vascularised tissue. Cholvin *et al.,(*1986), reported that muscle being a highly vascular tissue produced tissue responses which is more cellular compared to subcutaneous implantation. A period from 7 -30 days is suggested for making observations. However others have suggested a period beyond 10-14 days to avoid the effect of surgical trauma (Anderson *et al.,* 2008). The observations were made on 21 days and 90 days as it provided information on the trend in this response. 21 days provided a chronic picture of inflammation without the effect of surgical trauma or surgical trauma interfering with the inflammatory response. All the animals survived the implantation period un-eventfully. None of the animals exhibited hernia at the implantation site indicating intactness of the implants belonging to different treatment groups.

3.3.1.2.1 Tissue response to different treatment groups

The inflammatory response observed following abdominal muscle implantation of different treatment groups at 21 and 90 days is given in Figure 3-13. At 21 days, EDCL showed moderate to severe inflammatory response at inter-phase as well as mononuclear infiltration into the graft. There was excellent host cell incorporation and neo-angiogenesis within the graft. At 90 days the inflammation

has subsided to mild to moderate intensity with evidence of structural organization and neo-angiogenesis. New muscle fibers could be noticed laid along the graft collagen bundles.

0.2DCL group showed at 21 days moderate to severe inflammation evidenced by mononuclear cell infiltration. Muscle degeneration at host tissue-implant interphase was also noticed. Graft was uniformly infiltrated with mononuclear cells as well as fibroblasts. Angiogenesis could be noticed in the scaffold. At 90 days, inflammation has subsided to mild to moderate intensity and consisted of mononuclear cells. Graft was cellular and organized collagen bundles and neo-angiogenesis could be noticed within the graft.

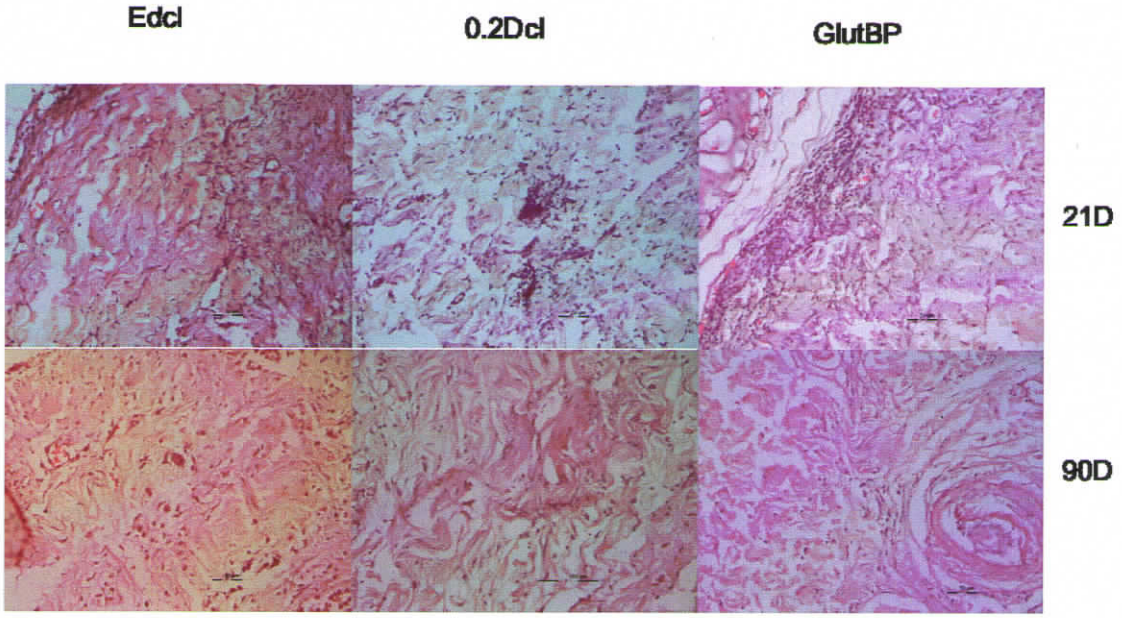

Figure 3-13: Micrograph showing distinct tissue response between groups at 21 and 90 days.

At 21 days, GlutBP showed necrosis, hyalinization of adjoining muscle bundles and severe inflammation at host tissue-implant interphase. The graft interior was acellular and angiogenesis was limited to graft periphery. At 90 days, the

inflammation has subsided substantially from mild to moderate intensity. Necrosis of adjoining muscles was still visible. Graft interior was still acellular.

3.3.1.2.2 Semi-quantitative evaluation of inflammation

In all the groups, the severity of inflammatory response subsided significantly by 90days (Figure 41-14). However there was no significant change at 90 days with respect to neutrophils in case of 0.2DCL and EDCL and macrophages in case of EDCL and GlutBP when compared to 21 days. In case of plasma cells, there was significant increase at 90 days in EDCL group ($p=0.02$) in comparison at 21 days. The predominant inflammatory cell noticed was macrophage (score 2.2 to 3.4) followed by lymphocyte (score 2 to 3.3), plasma cells (score 1 to 1.2) and neutrophils (score 0.44 to 1.7). Giant cells could not be observed in any of the groups at both periods. At 21 days GlutBP group had significantly higher neutrophilic infiltration compared to both EDCL and 0.2DCL group ($p=0.002$ and 0.0003 respectively). There was no significant difference between EDCL and 0.2DCL groups at this period. At 90 days the same trend of high neutrophilic count continued in the case of GlutBP compared to other groups at significance level of $p=0.006$ for EDCL and $p=0.15$ for 0.2DCL. Continuous presence of neutrophils has been noted previously on glutaraldehyde treated dermal collagen in rats (Ye *et al.*, 2010). There was no significant difference between EDCL and 0.2DCL at 90 days. In the case of lymphocytes, no observed significant difference between groups is evident at both periods. However only EDCL showed significant increase of lymphocytes compared to 0.2DCL ($p=0.049$) at 90 days. In case of plasma cells, there was no significant difference between groups at 21 days. At 90 days, EDCL showed a significant increase in plasma cell count compared to both GlutBP and

0.2DCL groups (p= 0.001 and 0.01 respectively). There was no significant difference between GlutBP and 0.2DCL at this period. At 21 days, macrophages showed a significant increase in case of both EDCL and 0.2DCL compared to GlutBP. (p=0.002 and 0.0002 respectively). There was no significant difference between EDCL and 0.2DCL at this period. At 90 days, EDCL showed an increased macrophage response compared to both GlutBP and 0.2DCL group (p=0.002 and 0.02 respectively).

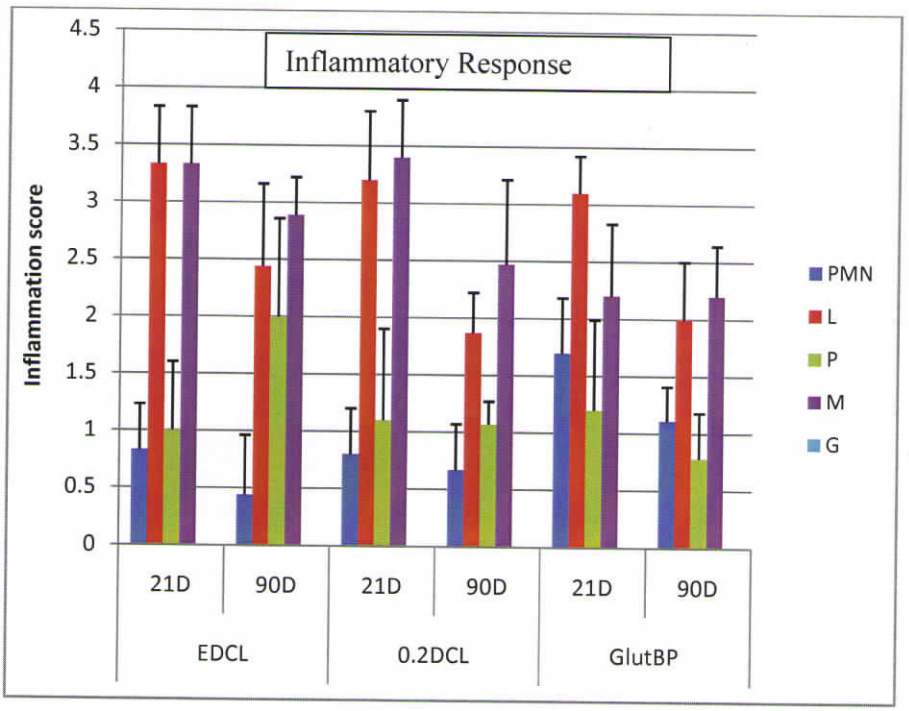

Figure 3-14: Chart showing comparison of inflammatory response between different treatment groups at 21 and 90 days. (PMN: polymorpho nuclear cells, L: lymphocytes, P: plasma cells, M: macrophages, G: Giant cells)

A wide range of host response has been observed in decellularised matrix on account of the variation in source of biological scaffold, decellularisation method and methods of terminal sterilization. (Gilbert *et al.*, 2006; Reing *et al.*, 2010). Though intense acute inflammatory response, predominance of mononuclear response and absence of foreign body giant cells have been observed in the tissue

response to various decellularised tissue matrices, the end results have been either regeneration or scar formation (Valentin *et al.,* 2006). In the present implantation study, the different treatment groups have produced varied tissue response and inflammatory response. With respect to tissue response, both EDCL (un-crosslinked decellularised bovine pericardium) and 0.2DCL (minimally cross-linked decellularised bovine pericardium) showed a similar picture of healing at 21 days. At 90 days, regeneration such as new skeletal muscle formation in the scaffold could be observed only in EDCL group. Whereas GlutBP has elicited an adverse tissue response which is not congenial for induced regeneration (Badylak *et al.,* 2007). With respect to inflammatory response, it was noted that both EDCL and 0.2DCL group showed a predominance of macrophage response compared to GlutBP at 21 days. But this picture has changed at 90 days and macrophages were seen highest in EDCL group alone. Interestingly, the initial macrophage response noted in 0.2DCL group has reversed and at 90 days both GlutBP and 0.2DCL showed a similar response with respect to macrophage infiltration. Macrophage has been associated with neo-angiogenesis and *in vivo* regeneration. Roh *et al.,* (2010) has reported an inflammation mediated process of vascular remodeling in the case of tissue engineered vascular graft. In this study they have observed that monocytes/macrophages were the predominant cellular infiltrate in tissue engineered vascular grafts and by secreting MCP-1, human BMC seeded scaffold attracted monocytes into the site. This rapid monocyte recruitment has been has been shown to be intricately involved in postnatal vessel formation (Bergmann *et al.,* 2006). In conjunction, VEGF expression within the scaffold was noticed throughout the duration of monocyte/macrophage infiltration suggesting a continued secretion of

VEGF by infiltrating cells.(Roh *et al.,* 2010). It is opined that mononuclear infiltration associated with remodeling of decellularised implants has been confused with inflammatory response (Badylak *et al.,* 2007).

Apart from the above observations it is important to note that EDCL group showed progressive immune stimulation compared to both 0.2DCL and GlutBP group as evidenced by significant increase in plasma cell (score increased from 1 to 2) and higher lymphocyte count at 90 days compared to 21 days (Figure 3-14). This clearly indicated that inspite of immune-stimulation by un-crosslinked decellularised bovine pericardium, induced regeneration identified as new skeletal muscle formation ensued.

3.3.2 Acquired/ adaptive immune response

Acquired or adaptive immune response was studied based on two representative tests suggested by the document ASTM F1906-98 (Reapproved 2003). To study the acquired immune response such as humoral immune response and cell mediated immune response, antibody response and lymphocyte activation in the presence of bovine pericardial proteins were studied respectively, both at 21 days and 90 days. Bovine pericardial protein used was freshly prepared, sterile filtered and confirmed free of endotoxin. Endotoxin is considered deleterious for both *in vitro* and *in vivo* testing of biomaterials (Gorbet & Sefton, 2004).

3.3.2.1 Humoral immune response: Antibody response (Section 5, ASTM F1906-98:Reapproved 2003)

Antibody response was studied in adult rats implanted with different treatment groups for periods of 21 days and 90 days. 21 days was considered

sufficient to elicit an antibody response following implantation. Nearly $3cm^2$ material was implanted in each animal to study this response. The secondary antibody used was raised against anti-rat heavy chain of immunoglobulin which can detect all the three types of antibodies such as IgG, IgM and IgA. This enabled detection of the total antibody induced against bovine pericardial proteins in the animal. The difference between treatment groups and its trend is illustrated in Figure 3-15. In comparison to 21 days, at 90 days EDCL group showed no significant elevation of antibody response. Whereas 0.2DCL group showed significantly reduced antibody response ($p=0.014$) at 90 days in comparison to 21 days. Similar observation was noted in GlutBP and the reduction in antibody response noticed at 90 days was highly significant ($p= 1.79x10^{-5}$). On comparing different treatment groups, at 21 days GlutBP showed highest antibody response in comparison to EDCL ($p=0.004$), and 0.2DCL groups ($p= 3.48x10^{-5}$). EDCL showed second highest antibody response which was significantly higher than 0.2DCL group ($P= 0.02$). 0.2DCL had the lowest antibody response at 21 days. At 90 days, GlutBP showed highest antibody response followed by EDCL group. However there was no statistically significant difference between GlutBP and EDCL at this period. 0.2DCL group showed significantly reduced antibody response compared to both GlutBP ($p= 0.01$) and EDCL ($p=0.006$).

The observations clearly indicate a sustained antibody response for EDCL, whereas for both 0.2DCL and GlutBP there was a significant reduction at 90 days. GlutBP group was seen the most antigenic with respect to humoral response both at 21 and 90 days in contrast to subcutaneous implantation where EDCL group was seen more antigenic. Immunogenicity of glutaraldehyde treated bovine pericardium

(Dahm *et al.*, 1990) or collagenous implants (Maede & Silver, 1990) was reported earlier.

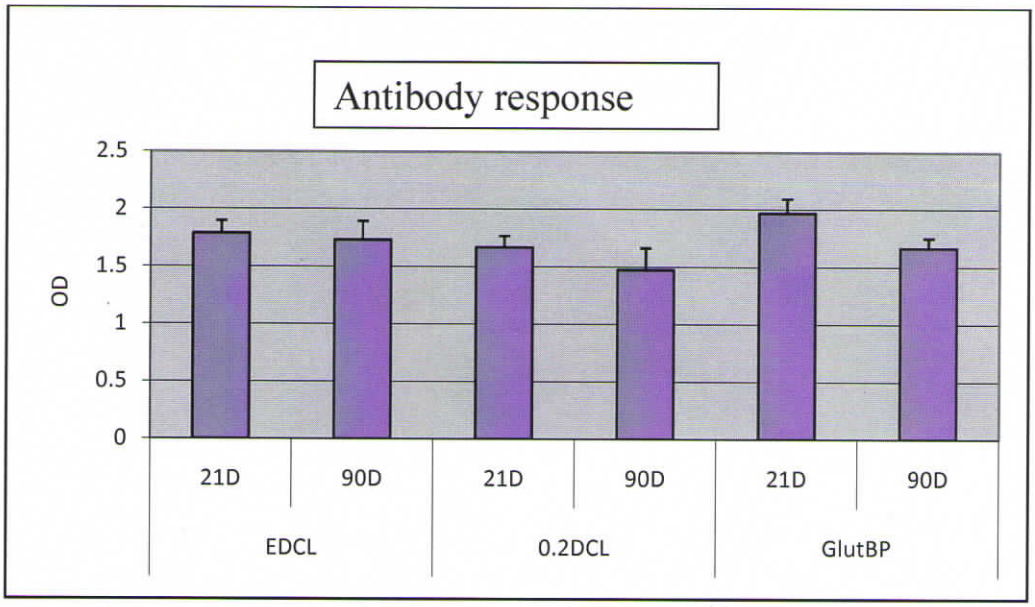

Figure 3-15: Chart depicting IgG, IgM and IgA response against fresh bovine pericardial proteins in different treatment groups observed in rat abdominal implantation model at 21 and 90 days.

3.3.2.2 Assessment of Cell mediated immune response using Lymphocyte transformation test (Section 8.1.1, ASTM F1906-98: Reapproved 2003)

Presence of lymphocytes sensitized against bovine pericardial proteins was studied using Lymphocyte transformation test. Sterile and endotoxin free fresh bovine pericardial protein was used in this test to avoid artifacts (Gorbet & Sefton, 2004). In this test, sensitized lymphocyte responding specifically to an antigen, in this case bovine pericardial proteins, was activated releasing cytokines. This stimulated other lymphocytes to divide in response to released cytokines, thus amplifying the response. The proliferation of these lymphocytes was detected using

MTT assay. Lymphocytes were isolated from spleen of animals implanted with different treatment groups.

The result of this test is illustrated in Figure 3-16. At 21 days, GlutBP group showed highest proliferative response followed by EDCL in the presence of fresh bovine pericardial protein. 0.2DCL group showed no significant proliferative response either at one month or at 3 months. At 90 days, the proliferative response of EDCL group still persisted indicating continued activation, whereas in the GlutBP group there was no significant proliferative response in the presence of fresh bovine pericardial protein at this period.

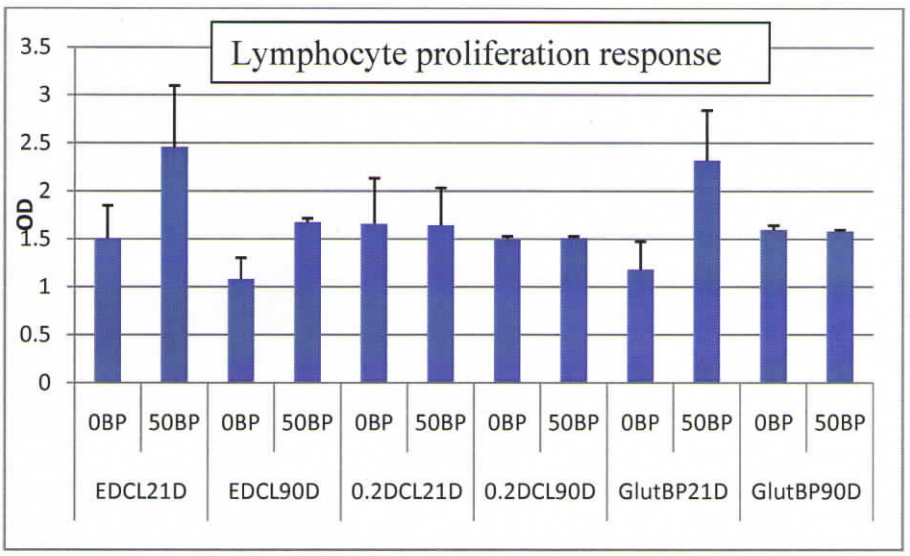

Figure 3-16: Chart showing difference in cell mediated immune response between different treatment groups at 21 and 90 days.

The above data also showed that immune-stimulation was sustained in the case of EDCL group compared to GlutBP or 0.2DCL group. 0.2DCL group was observed as the least responsive.

3.4 Th1/Th2 lymphocyte pathway preference

The Th1/Th2 preference shown by different treatment groups were assessed by studying the IgG2 and IgG1 antibody response as suggested by Allman *et al.*, (2001, 2002). The IgG1-IgG2 response elicited against fresh bovine pericardial protein was measured using Indirect ELISA. The preference for Th2 pathway was assessed by estimating levels of IgG1 and IgG2 in the serum of different treatment groups and the ratio of IgG1 to IgG2 was calculated. A higher ratio indicated better preference for Th2 response. Difference in Th2 preference was assessed in both subcutaneously implanted rats as well as abdominal muscle implanted rats. In subcutaneously implanted rats (Figure 3-17), EDCL group showed maximum IgG1to IgG2 ratio followed by 0.2DCL group. GlutBP group showed minimum ratio. 0.2DCL group showed an intermediate response. The differences observed were statistically significant at $p<0.05$. IgG1/IgG2 responses in abdominal muscle implanted animals were less conclusive. There was no significant difference within treatment groups, between 21 and 90 days. At 21 days, GlutBP group showed a significant increase in the IgG1/IgG2 ratio when compared to both EDCL ($p=0.002$) and 0.2DCL groups ($p=0.002$). There was no significant difference between EDCL and 0.2DCL groups. The same trend continued at 90 days also with GlutBP group showing a higher ratio at $p=0.003$ and 0.004 when compared to EDCL and 0.2DCL respectively.

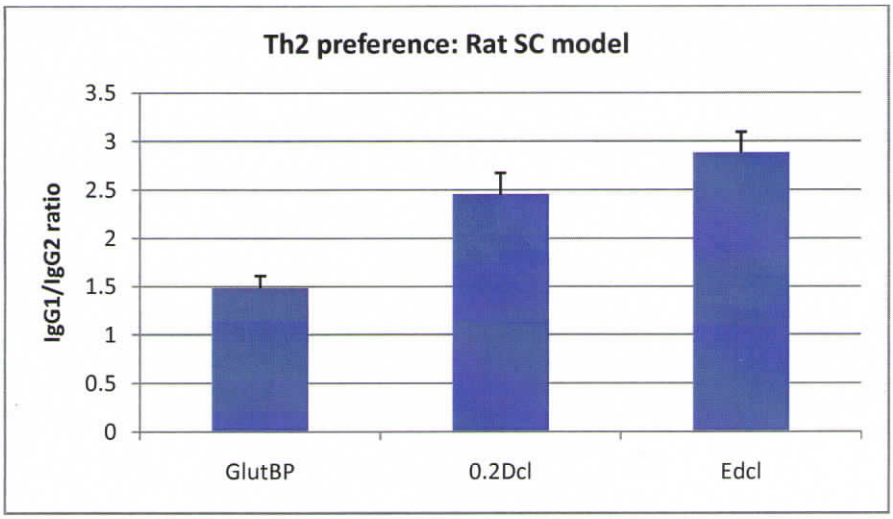

Figure 3-17: Th2 preference observed in different treatment groups in rat subcutaneous implantation model.

As observed in this experiment, the data obtained from subcutaneous implanted animals did not exactly correlate with that of abdominal muscle implanted ones. In subcutaneous experiment, the highest Th2 preference was noted in EDCL group and the least in GlutBP group. Whereas in abdominal muscle implantation model, highest response is seen in GlutBP group and there was no significant difference between EDCL and 0.2DCL group, which were equally low. If viewed trend wise, although not significant, only EDCL and 0.2DCL group showed an increasing Th2 trend, while a significantly decreasing trend for Th2 preference was noticed in GlutBP group. As a ECM scaffold material, only on SIS-ECM Th1/Th2 have been characterized. Th2 response has been associated with induced regeneration in this case. (Badylak & Gilbert, 2008).

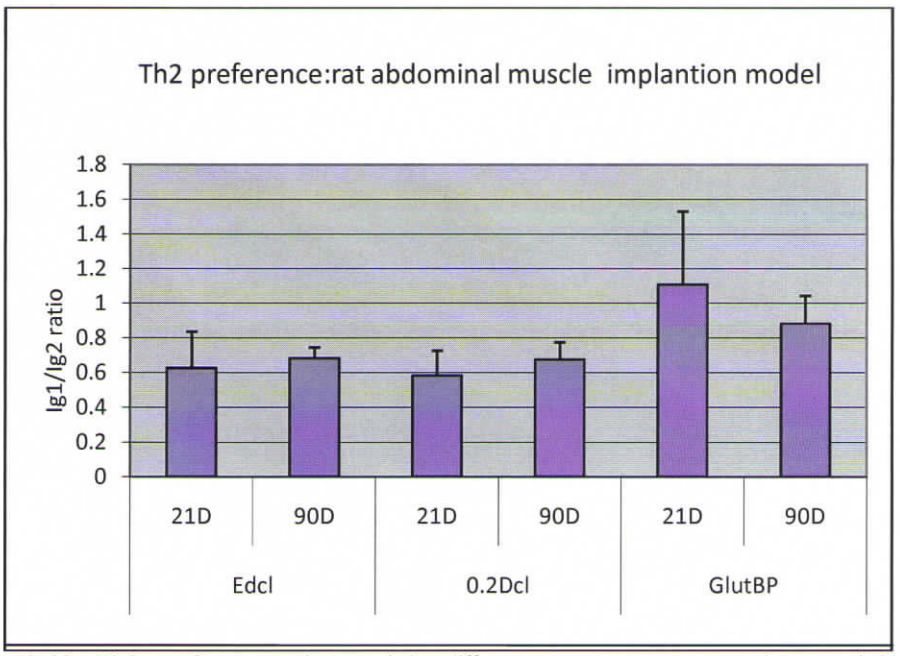

Figure 3-18: Th2 preference observed in different treatment groups in rat abdominal implantation model at 21 and 90 days.

It has been observed that signals between macrophages and fibroblasts can exacerbate, suppress, or reverse fibrosis. Fibroblasts and macrophages are reported to be activated by T cells or inhibited by engaging a negative feedback loop to reduce fibrosis through Th2 cytokine secretion (Barron & Wynn, 2011).

3.5 M2/M1 macrophage phenotype polarization

M1/M2 polarization in different treatment groups was identified by observing the predominance of M1 or M2 phenotype of macrophages in the tissue sections. Both rat subcutaneous implantation model (60 days) and rat abdominal muscle implantation model (21 and 90 days) were utilized for this study. M1 macrophage phenotype was identified by anti-rat CD80 HRP conjugated antibodies (Abcam) and M2 macrophage phenotype by anti-rat CD163 HRP conjugated antibodies (Abcam) as reported earlier (Badylak and Gilbert, 2008). The number of CD80+ and CD163+

macrophages (Figure 3-19) were counted using tag and count function of UTHSCSA ImageTool version 3.0 software on 400X digital images is presented below. The ratio of CD163+ to CD80+ cells was calculated to provide to indicate the predictive value of this experiment with respect to induced regeneration.

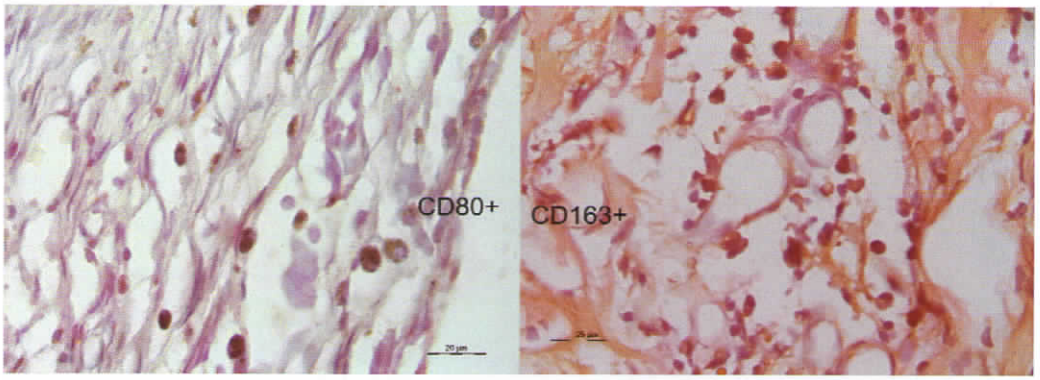

Figure 3-19: Micrograph showing immune-labeled CD80+ and CD163+ cells (1000X).

It is reported that the pro-inflammatory cytotoxic macrophage phenotype, M1 is characterized by cells that are associated with classic signs of inflammation whereas, the anti-inflammatory macrophage M2, promotes immunoregulation, tissue repair and constructive remodeling (Badylak and Gilbert, 2008).

In the rat subcutaneous implantation model (Figure 3-20), CD80+ cells (M1) were seen prominent in GlutBP group compared to both EDCL ($p= 0.001$) and 0.2DCL groups ($p= 0.003$). There was no statistically significant difference between EDCL and 0.2DCL groups with respect to CD80+ cells (M1). In the case of CD163+ cells (M2), most prominent response was observed in EDCL group compared to both GlutBP ($p=6.05 \times 10^{-9}$) and 0.2DCL group ($p=0.01$). 0.2DCL group also showed a higher response compared to GlutBP group ($p=0.0009$) with respect CD162+ cells (M2).

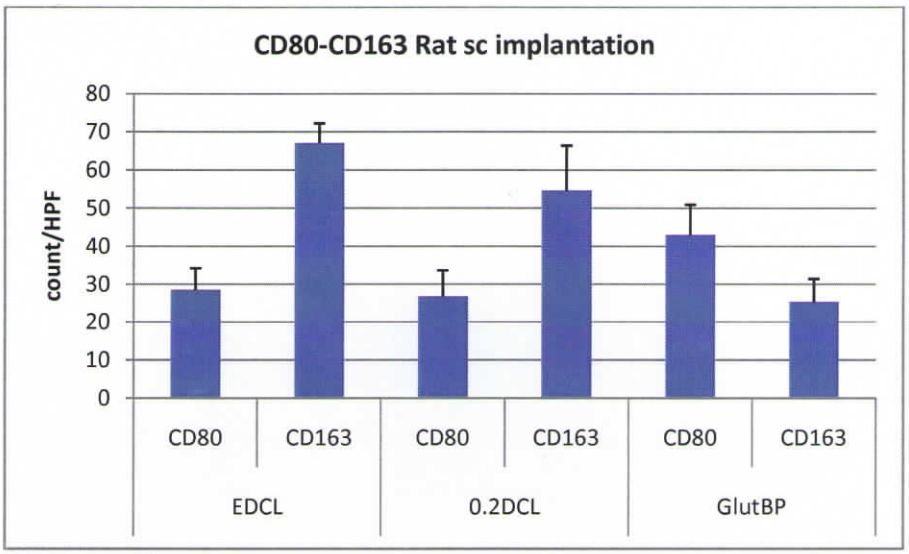

Figure 3-20: Chart showing prominence of CD80+-CD163+ cells in different treatment groups following subcutaneous implantation rat for 60 days.

Predictive value of the above experiment with respect to induced regeneration was arrived by comparing the ratio of CD163+ cells (M2) to CD80+ cells (M1) as given in Figure 3-21. GlutBP group showed the lowest M2/M1 ratio in comparison to GlutBP group (p=0.0006) and 0.2DCL group (0.0003) indicating negative possibility for induced regeneration. Whereas EDCL group showed the highest ratio of M2/M1 indicating a strong possibility for induced regeneration followed by 0.2DCL group. However there was no significant difference between EDCL and 0.2DCL with respect M2/M1 ratio. In the rat abdominal implantation model, predominance of CD80+ (M1) or CD163+ cells (M2) were compared within the same group between 21 and 90 days as well as between groups within the same time duration. In general, the trend in CD80+cell counts varied between groups whereas there was consistent reduction in count in the case of CD163+ cells.

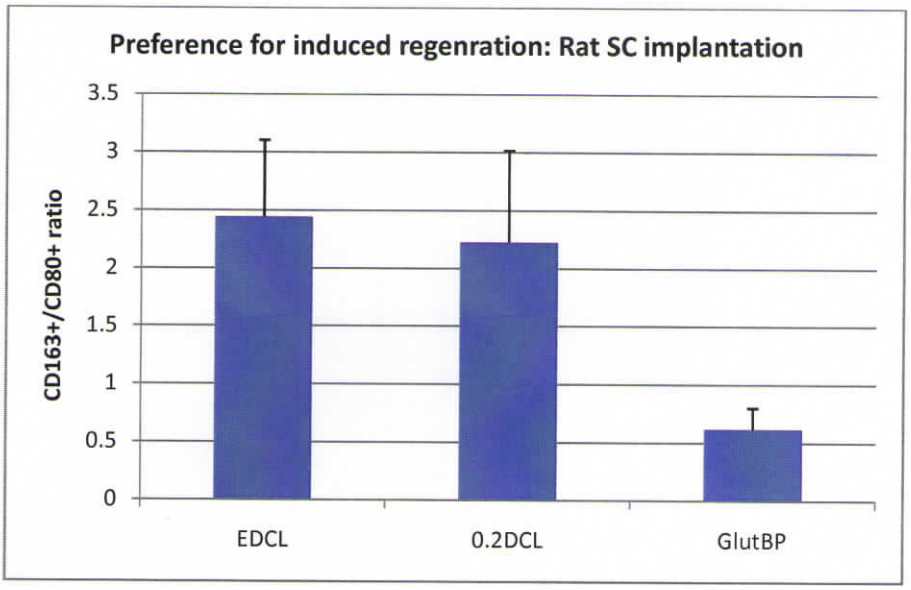

Figure 3-21: Chart depicting M2 preference exhibited by different treatment groups in rat subcutaneous implantation model

With respect to preference for induced regeneration, EDCL group showed a predominance of CD163+ cells (M2) at both 21 and 90 days indicating a trend for regeneration at both these periods. Whereas GlutBP group showed a predominance of CD80+ cells (M1) at both 21 and 90 days indicating a preference for inflammatory pathway. Interestingly, 0.2DCL group showed predominance of CD163+ cells (M2) at 21 days and this trend got reversed at 90 days with the predominance of CD80+ cells (M1) at this period, indicating a drift from regenerative pathway to inflammatory pathway. (Figure 3-22). Similar observations were previously reported in the case of SIS-ECM where chemical crosslinking with carbodiimide resulted in a switch from an M2 dominant profile to an M1 dominant profile (Badylak *et al.*, 2008).

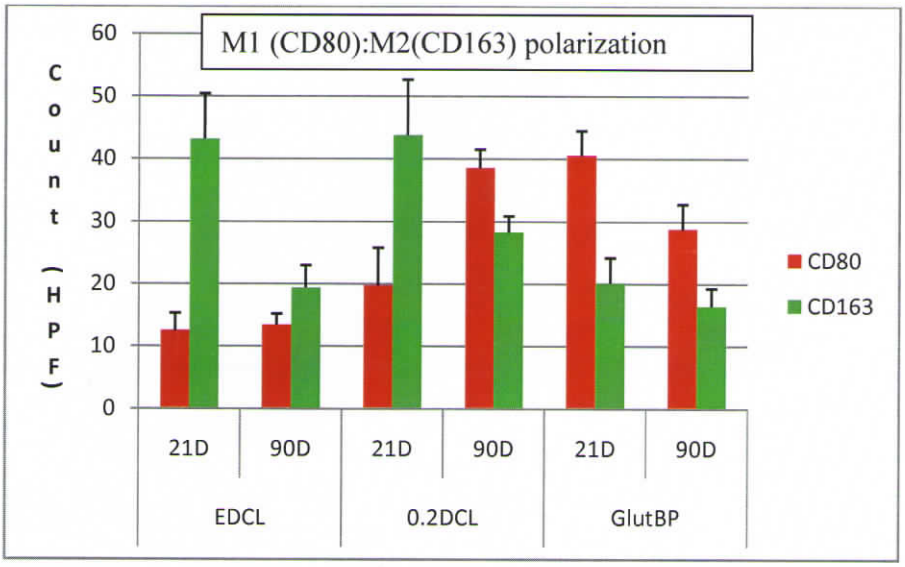

Figure 3-22: Chart depicting M1 (CD80+) – M2 (CD163+) polarization in different treatment groups at 21 and 90 days in rat abdominal implantation model.

Analyzing in detail the difference within group between 21 and 90 days , CD80+ cells in EDCL, group showed no significant difference in counts between 21 and 90 days. While in 0.2DCL group, Cd80+ cell count was significantly higher at 90 days ($p= 5x10^{-6}$), when compared to 21 days indicating a trend towards inflammatory pathway (M1) in this group. Cd80+ cell count reduced significantly ($p=1.8x10^{-5}$) at 90 days compared to 21 days in GlutBP group. With respect to CD163+ cells, there was significant reduction in count at 90 days when compared to 21 days in the case of EDCL group ($p=1.6x10^{-9}$) and 0.2DCL group ($p=0.006$). There was no statistically significant reduction in the count of CD163+ cells at 90 days when compared 21 days in the case of GlutBP group.

When compared between groups with respect to CD80+ cells at 21 days, GlutBP showed highest count compared to both EDCL ($p=4.2x10^{-11}$) and 0.2DCL group ($p= 1.x10^{-7}$). Interestingly 0.2DCL group showed significantly higher count of CD+ cells when compared to EDCL group ($p=0.002$) at 21 days. At 90 days CD80+

Cd163+ (M2)/ CD80+ (M1) ratio between groups at 21 and 90 days gave a clear picture of regenerative preference exhibited by different groups discounting the influence of degree of macrophage density at these periods. At 21 days EDCL group showed significantly higher M2/M1 ratio compared to both 0.2DCL ($p=0.014$) and GlutBP ($p=3.6x10^{-6}$) groups. At this period 0.2DCL group also showed significantly higher ratio when compared to GlutBP group ($p=1.5x10^{-5}$). At 90days, the same trend continued with EDCL showing a significantly higher ratio compared to 0.2DCL ($p=0.008$) and GlutBP ($p=0.002$). However, the level of significance has reduced in case of 0.2DCL group when compared to GlutBP group. Within different treatment groups, compared to 21 days, both EDCL and 0.2DCL has shown statistically significant reduction in the M2/M1 ratio ($p=1.5x10^{-5}$ and $p=0.005$ respectively) when compared to their response at 90 days. GlutBP group showed no significant change in response between 21 and 90 days. Studies on host response to implanted biological scaffold suggest that macrophages differentiate towards a phenotype that is associated with either cytotoxic inflammation or constructive remodeling (Mantovani *et al.,* 2004; Mantovani *et al.,* 2005). The pro-inflammatory cytotoxic macrophage phenotype, M1 is characterized by cells that are associated with classic signs of inflammation whereas, the anti-inflammatory macrophage M2, promotes immunoregulation, tissue repair and constructive remodeling (Badylak and Gilbert, 2008). In the above experiment there was clear preference for the un-crosslinked decellularised bovine pericardium for M2 macrophage phenotype both in the subcutaneous implantation model as well as abdominal muscle implantation model clearly indicating a possibility for induced regeneration. In the later model, this preference was exhibited both at 21 and 90 days consistently. Whereas the fully

crosslinked bovine pericardium exhibited a predominance of M1 macrophage phenotype consistently in both models and at both durations indicating no possibility for induced regeneration. Most interesting observation was made in the case of mildly crosslinked decellularised bovine pericardium. It exhibited an initial preference for M2 response at 21 days in rat abdominal model, and this trend was reversed at 90 days to M1 macrophage phenotype indicating a no possibility for induced regeneration. Similar observations were made by Badylak *et al.,* 2008 with carbodiimide crosslinked SIS. The reason for this observation is not explained so far. It is suggested that this change in M2 to M1 response can be due to breakdown of glutaraldehyde crosslinks around later periods leading to leaching of glutaraldehyde in the vicinity which might have induced an M1 response.

Summarizing the findings in the phase 2 experiments it may noted that, although the un-crosslinked decellularised bovine pericardium (EDCL) produced maximum antibody response, it was predominantly IgG1; a non-complement fixing antibody which is associated with Th2 pathway favoring induced regeneration. With respect to inflammatory response also, it may be noted that EDCL group although produced maximum macrophage infiltration, it was predominantly M2 response again indicating a preference for induced regeneration. Glutaraldehyde treated bovine pericardium exhibited the other extreme of Th1 preference and M1 preference indicating no possibility for induced regeneration. Interestingly mildly crosslinked decellularised bovine pericardium induced minimum antibody response that too similar to that of un-crosslinked decellularised bovine pericardium with respect to the type and pathway preference (Th2). Only exception noticed was in the

macrophage phenotype preference noticed in this group, where M1 preference was noticed only in abdominal implantation model, that too at 90 days only.

3.6 Induced Regeneration in animal models

The ability of different treatment groups such as EDCL, 0.2DCL and GlutBP groups to induce regeneration in an adult animal was studied in rat abdominal regeneration model as described by Shi *et al.,* 2011, at 21 and 90 days. Besides this the healing response in a vascular defect was studied by aortic implantation in pig aortic patch model at 6 months duration as described by Haluck *et al.,* 1990.

3.6.1 Adult rat abdominal regeneration model

The difference in induced regeneration between different treatment groups at 21 and 90 days is illustrated in Figure 3-24. There was considerable variation in induced regeneration observed between 21 and 90 days within the same group as well as between treatment groups at both durations. The skeletal muscle regenerate was identified by its characteristic staining features observed in Hematoxylin-Eosin sections such as striated appearance on account of its anisotropic and isotropic bands. Masson Trichrome staining also produced red stained structure with characteristic striations of skeletal muscle tissue.

In the EDCL group, at 21 days muscle bundles were seen arising at the peritoneal surface of the graft along the mesenteric attachments confined at the graft-tissue interphase. Graft interior was devoid any new muscle formation at this period. At 90 days, skeletal muscle tissue noticed on the peritoneal surface along with the mesenteric attachment has further expanded and was seen growing toward the centre

of the graft as well as the defect (arrow). Moreover new muscle bundles were noticed arising in the graft interior (open arrow) which is contiguous with the muscular edges of the defect. This new extension of muscle bundle was also seen

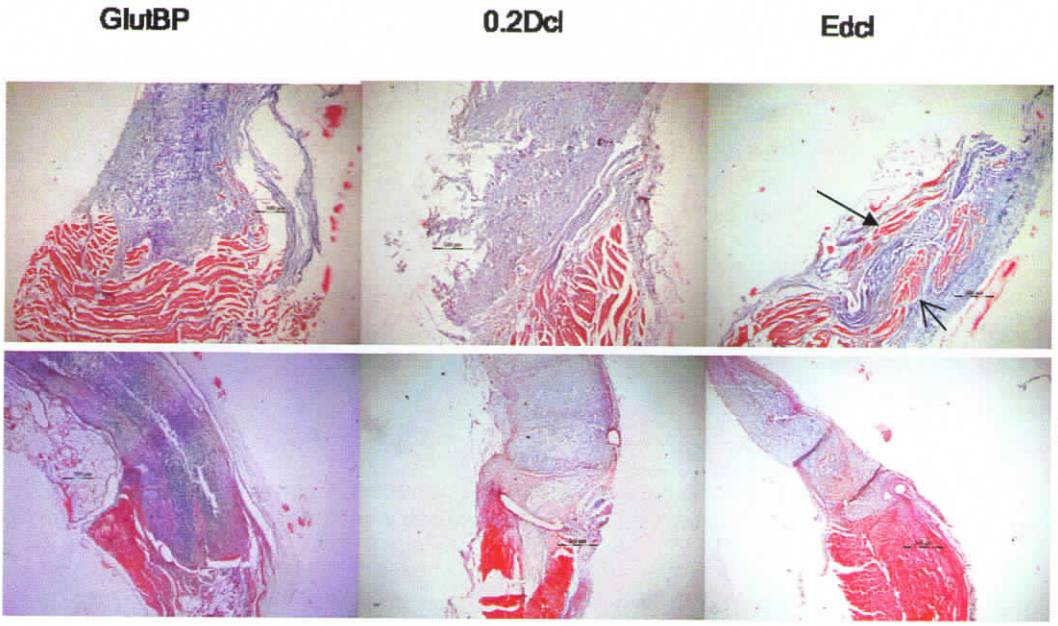

Figure 3-24: Representative micrographs showing differences in regeneration induced by different treatment groups at 21 and 90 days. Masson's Trichrome 40X.

growing towards the centre of the muscular defect. The extent of regeneration varied from collection of cells with heterochromatic round nucleus and eosinophilic cytoplasm (Figure 3-25), linear orientation of these cells to form fibers (Figure 3-26), formation of islands of skeletal muscles along and within collagen bundles of the scaffold (Figure 3-27), formation of skeletal muscle bundles which are seen fusing to form much bigger bundle up to formation of continuous skeletal muscle bands in the interior of the scaffold bridging the defect (Figure 3-28). Neo-angiogenesis can also be appreciated along with the events of skeletal muscle formation (Figure 3-29).

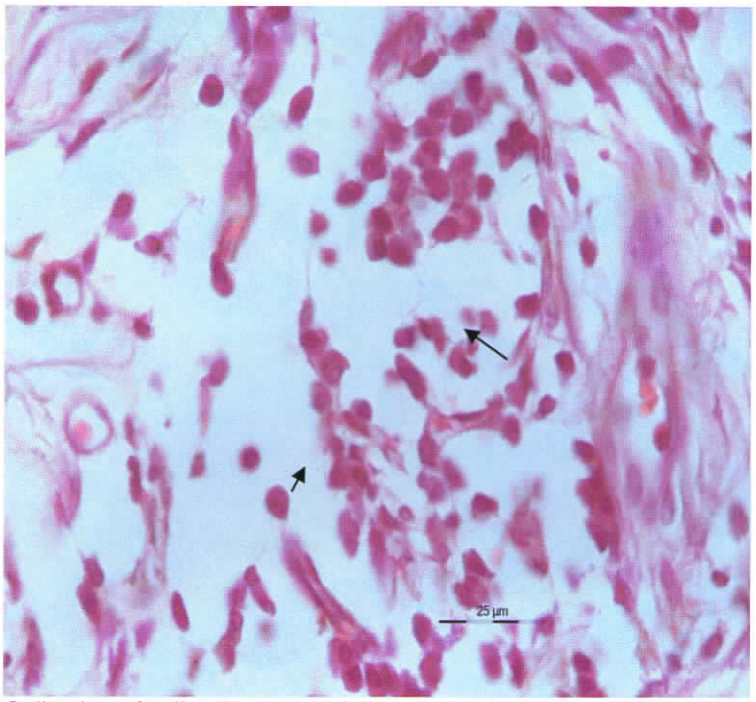

Figure 3-25: Collection of cells with eosinophilic cytoplasm and heterochromatic nucleus in EDCL group at 90 days. Formation of new capillaries can also be appreciated (arrow) in this section. HE 1000X.

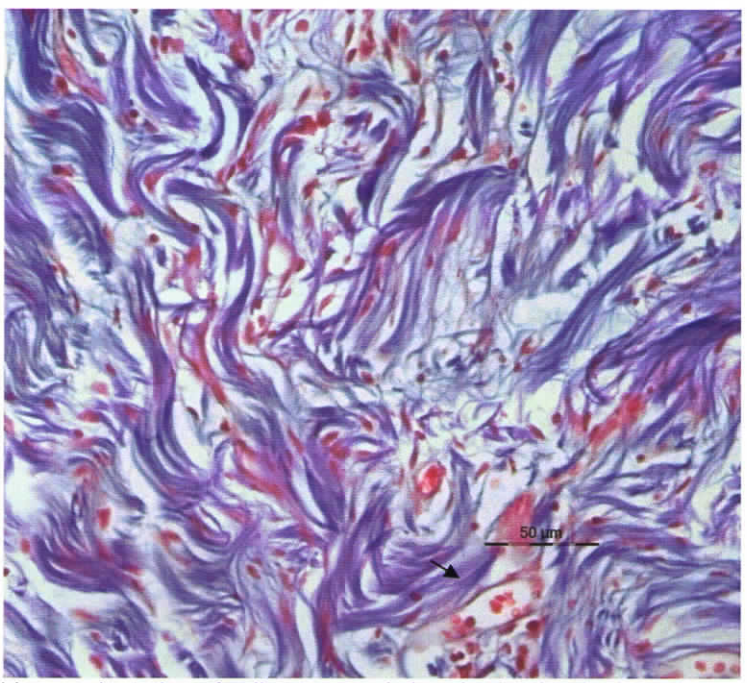

Figure 3-26: Linear orientation of cells at several sites (stained red) along the scaffold (stained blue) in EDCL group at 90days. Neo-angiogenesis is also visible (arrow). Masson Trichrome 400X.

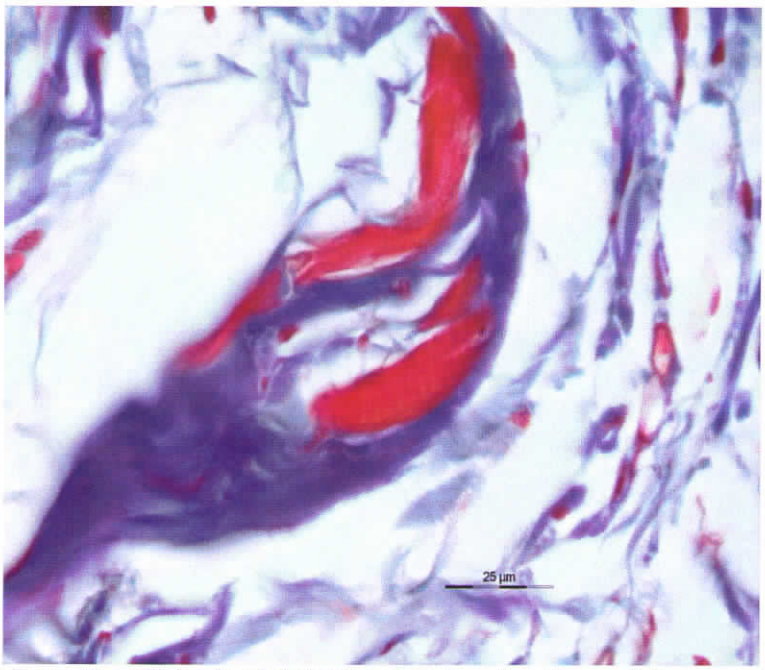

Figure 3-27: Formation of islands of skeletal muscle bundle with parallel arranged myofibrils (stained red with striations) within the collagenous scaffold (stained blue) in EDCL group at 90 days, Masson Trichrome 1000X.

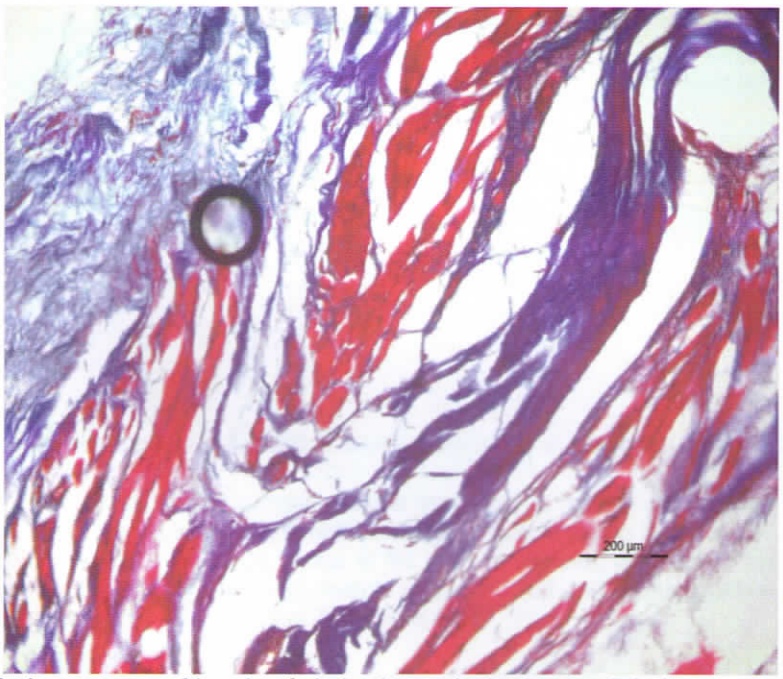

Figure 3-28: Appearance of bands of skeletal muscle (stained red) in between collagenous scaffold (stained blue) in EDCL group at 90 days. Masson Trichrome 100X.

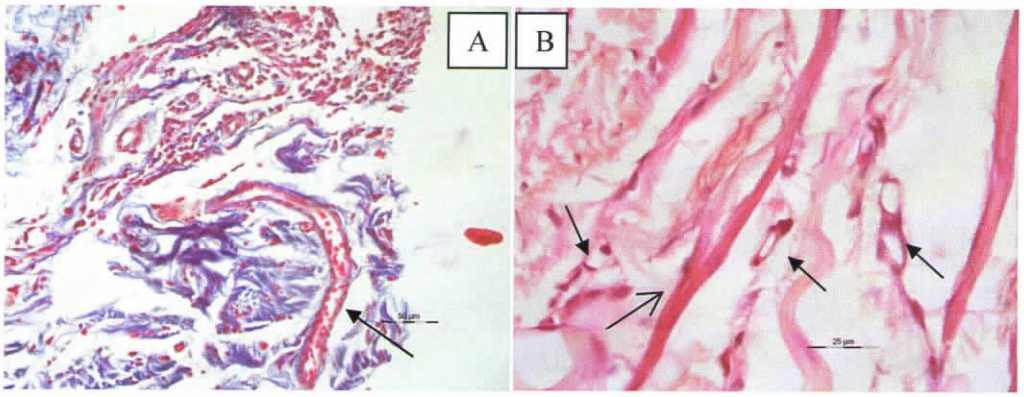

Figure 3-29: EDCL group, 90 days- A, Formation of new blood vessels (arrow) around collagenous scaffold (stained blue), Masson Trichrome 400X. B, Formation of skeletal muscle fiber (open arrow) besides numerous capillaries (arrow). HE 1000X

In the case of 0.2DCL group, at 21 days, at the host tissue-implant interphase on the peritoneal surface, new muscle fibers could be observed along the mesenteric attachments (Figure 3-30:A). At 90 days, these new muscle fibers have receded and the graft is collagenous and is devoid of any skeletal muscle tissue (Figure 3-30:B).

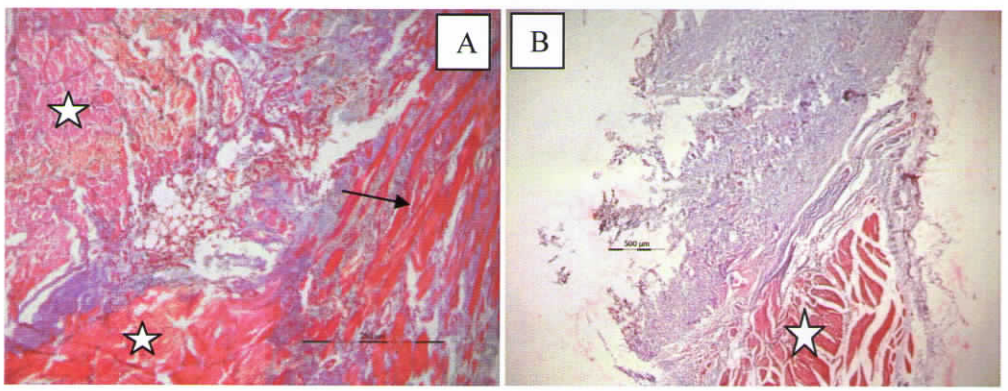

Figure 3-30: A, 0.2DCL group showing initial muscle formation at mesenteric surface (red stained and marked with arrow) at 21 days. Native skeletal muscle is red and marked with star. Masson Trichrome 200X B, 0.2DCL group at 90 days is showing absence of muscle tissue at mesenteric surface. Native skeletal muscle is stained red and marked with arrow. Collagenous scaffold is stained blue. Masson Trichrome 40X

GlutBP group also showed a similar picture of the presence of muscle fibers in the peritoneal surface at the interphase of the graft growing along the graft. Graft interior is devoid any skeletal muscle tissue at 21 days. At 90 days, the muscle fibers noticed on the peritoneal surface at interphase have receded and no skeletal muscle tissue could be noticed within or on the graft (Figure 3-31).

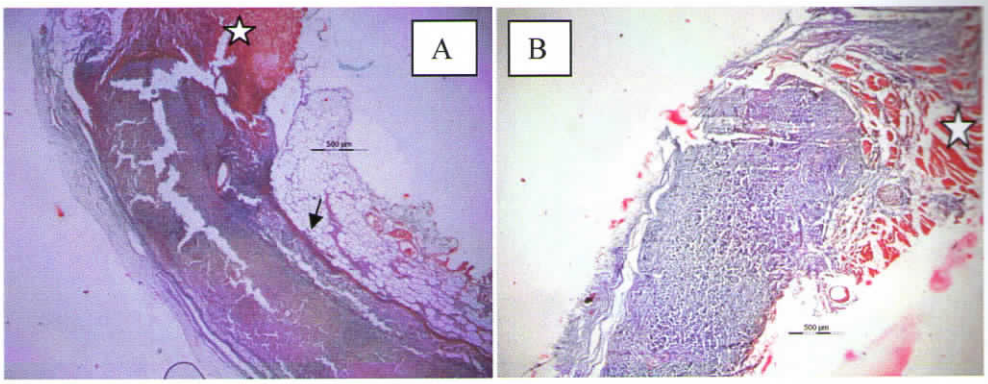

Figure 3-31: A, GlutBP group at 21 days showing a thin band of muscle tissue at mesenteric surface (arrow). Native skeletal muscle is marked with star Movats Pentachrome 40X. B, GlutBP showing absence of thin muscle in the mesenteric surface. No new muscle tissue is seen within the scaffold (stained blue). Masson Trichrome 40X.

3.6.2 Adult pig vascular implantation model

Cheung *et al.,* 1999 has described this model using autologous pericardium after different processes.

In this study, bovine pericardium of different treatment groups were implanted as a patch in the descending aorta and the healing response was studied. It is important to note that none of the treatment groups regained the native aortic structure at the end of 6 months. Similar pattern of healing was observed in all the groups consisting of an endothelialised neointima with subintimal layer of smooth muscles and a collagenous adventitia. Elastin was conspicuously absent in the regenerate. (Figure 3-32). Healing noticed was more over similar in the case of

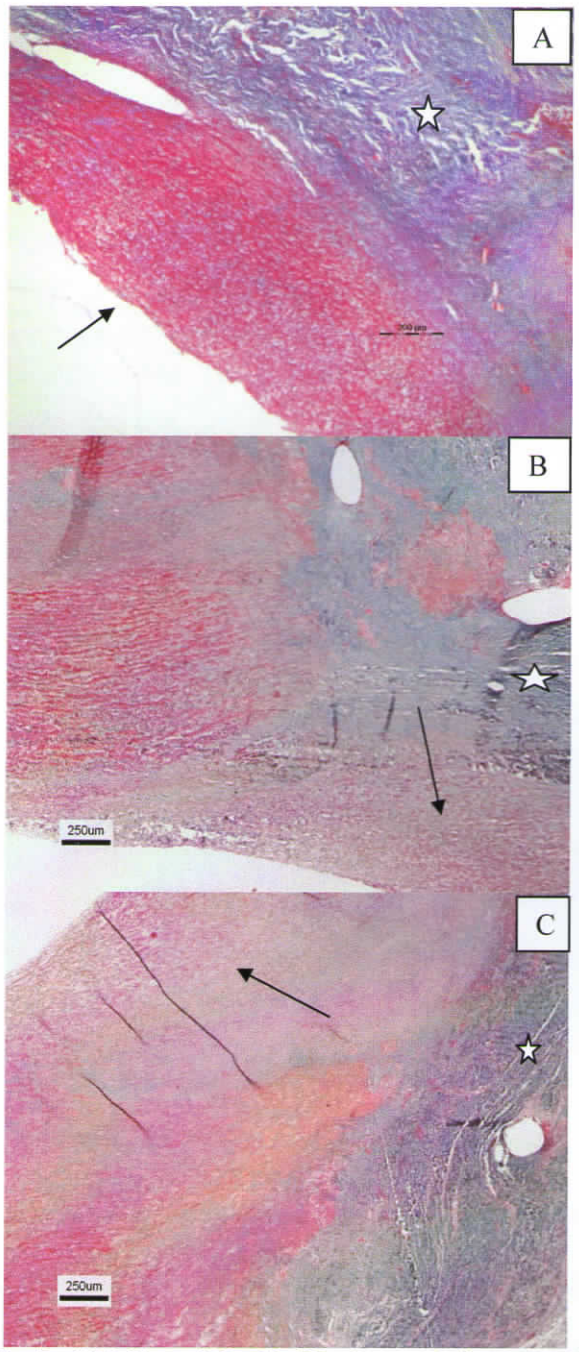

Figure 3-32: A- EDCL, B- 0.2DCL and C- GlutBP. Arrows indicate neointima. Star indicate scaffold. Massons Trichrome 200X.

EDCL and 0.2DCL with thinner neointima and well populated scaffold with a collagenous adventitia. In the case of GlutBP, neointima was thicker with profoundly acellular scaffold and collagenous adventitia. The reported regeneration in tissue

engineered vascular graft is remarkably similar consisting of endothelial cells lining the inner lumen which is invested by a smooth muscle layer. There was no elastin in the regenerated vessel (Roh *et al.,* 2010). In EDCL group, the graft area consisted of smooth muscle cells growing along and in between the collagenous matrix (Figure 3-33). In the case of 0.2DCL, the graft area appeared as organized collagenous structure in (Figure 3-34). There were fewer smooth muscle cells compared to EDCL. GlutBP group was largely acellular (Figure 3-35) with minimum smooth muscle cells in the scaffold.

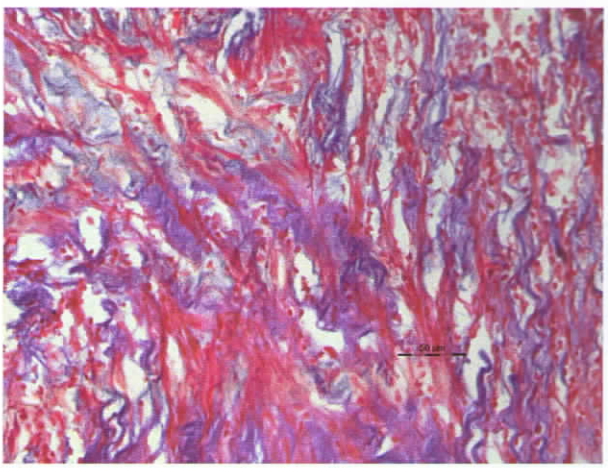

Figure 3-33: EDCL showing organized collagenous structure (blue stained) with more smooth muscle incorporation (red stained).Masson Trichrome 400X.

Haluck *et al.,*1990 has described vascular healing in pig model. They have observed that the graft was covered with alpha actin positive smooth muscle cells with a coat of von Willebrand positive endothelial cells. Newly deposited collagen was observed in between the fibers of scaffold collagen. Nevertheless in all these studies structural regeneration of aorta was not reported (Cheung *et al.,* 1999).

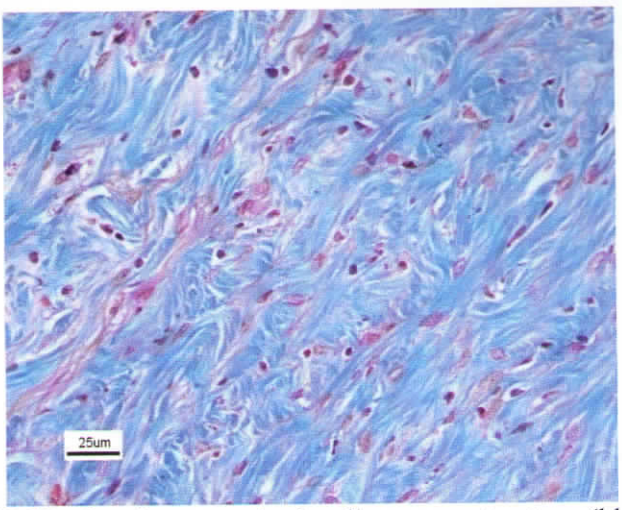

Figure 3-34: 0.2DCL showing organized collagenous structure (blue stained) with less amount of smooth muscle (red stained) in the scaffold. Masson Trichrome 400X.

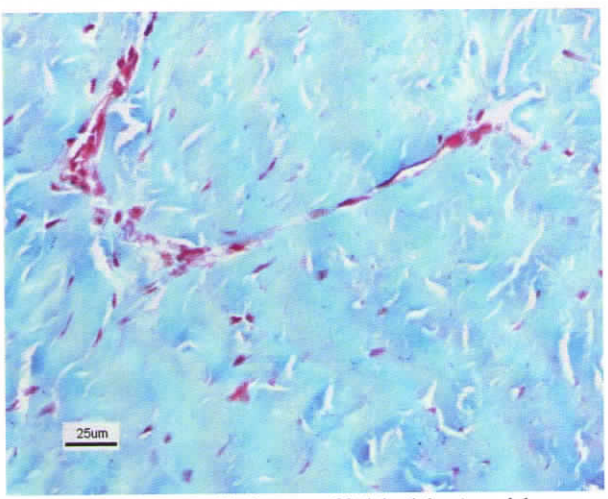

Figure 3-35: GlutBP showing acellular scaffold (blue) with very few cells in the scaffold (red stained). Masson Trichrome 400X.

Immuno-labeling studies revealed more detailed picture of the difference in healing response observed between groups. Results of the immune-labeling studies are shown in Figure 3-36.

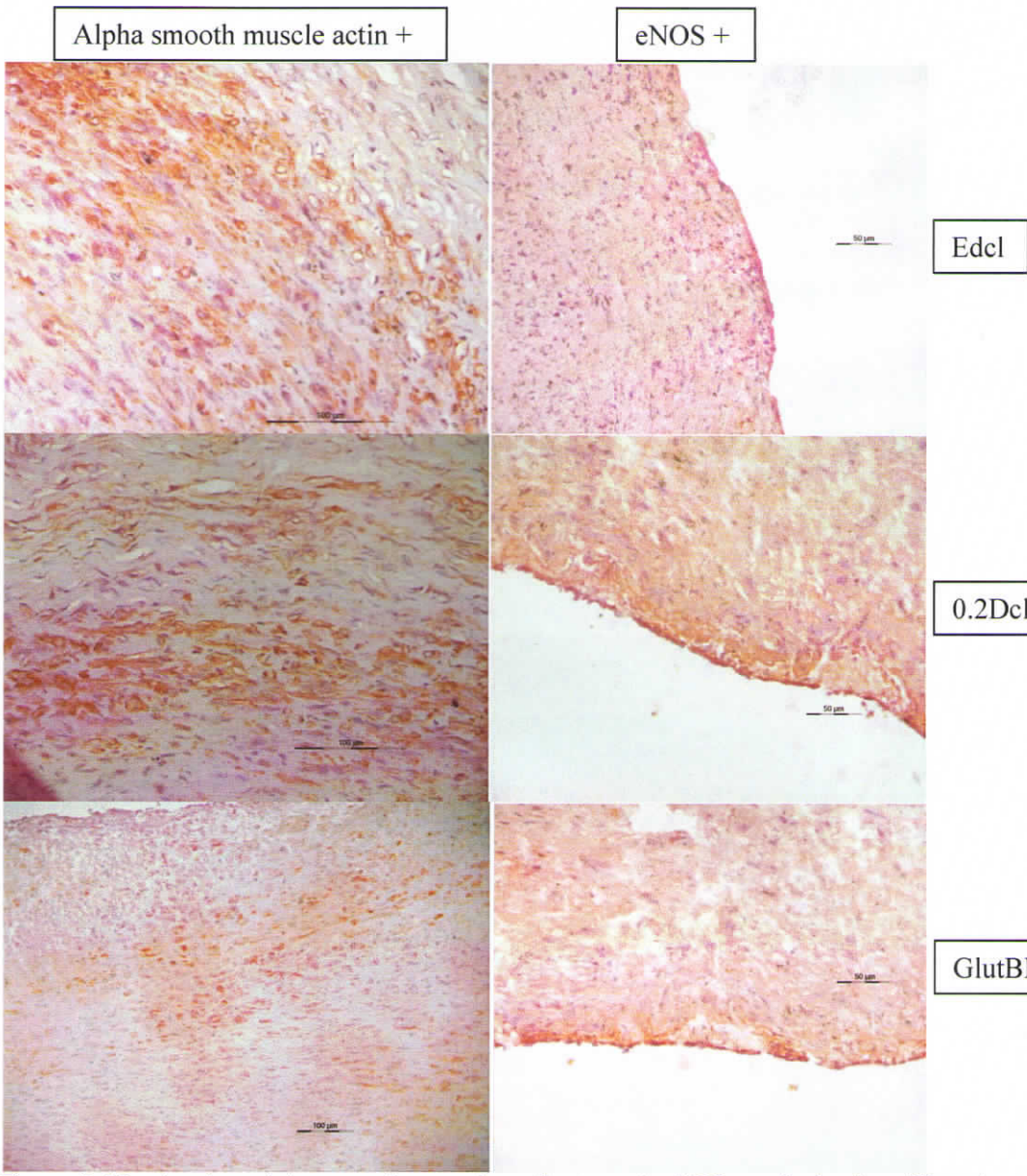

Figure 3-36: showing alpha smooth muscle actin + and eNOS+ cells in the different treatment groups.

All the groups showed smooth muscle in the neointima as demonstrated by immune labeling of smooth muscle alpha actin. The amount of smooth muscle cell varied between groups. EDCL showed more abundant smooth muscle population compared to 0.2DCL and GlutBP. GlutBP group showed minimum amount of alpha smooth

muscle actin + cells. Endothelialization was present in all the groups demonstrated by ENOS+ cells lining the lumen of the vessel. Similar observations on vascular healing were made by Cheung *et al.*,1999 and Haluck *et al.*, 1990.

Nevertheless, the structure of native aorta consists of different layers such as tunica interna, tunica media and tunica externa composed of elastin and collagen fibers. Longitudinally oriented smooth muscle cells are numerous (Figure 3-37, 3-38) in the tunica interna and media. The tunica externa is thin and also contains elastic and collagen fibers. By comparing the structure of native aorta with the healing response noticed in different treatment groups it is evident that there is no structural regeneration in this model.

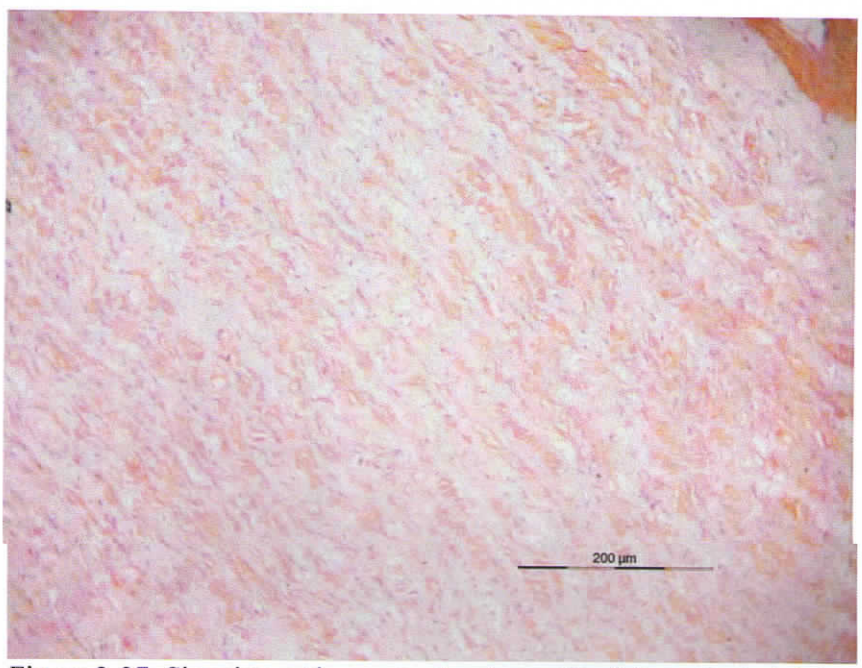

Figure 3-37: Showing native aorta structure with alpha actin + staining for smooth muscle cells.

Figure 3-38: Showing native aorta structure. Movats Pentachrome staining showing red stained smooth muscle cells attached to yellow stained collagen. Presence of GAG can also be noticed as blue stained material at 400X magnification.

3.7 Additional experiment to compare EDCL and 0.2DCL groups by cell adhesion studies

An *in vitro* cell adhesion test with L929 fibroblast cells was performed with test materials EDCL (un-crosslinked decellularised bovine pericardium) and 0.2DCL (partially crosslinked decellularised bovine pericardium) to study the basic cell interaction since these two groups were comparable in their innate immune response and general tissue response. Both the groups showed excellent tissue in-growth into the scaffold, minimum calcification and neo-angiogenesis into the scaffold material which are desirable properties for induced regeneration. These groups differed mildly in their Collagenase type II susceptibility and adaptive immune response, with EDCL being more immunogenic compared to 0.2DCL. Yet, there was no

observed regeneration in the case of 0.2DCL group in rat abdominal implantation model compared to EDCL. These observations have prompted this experiment to find out whether the partial glutaraldehyde crosslinking has inactivated the cell adhesion property of the scaffold.

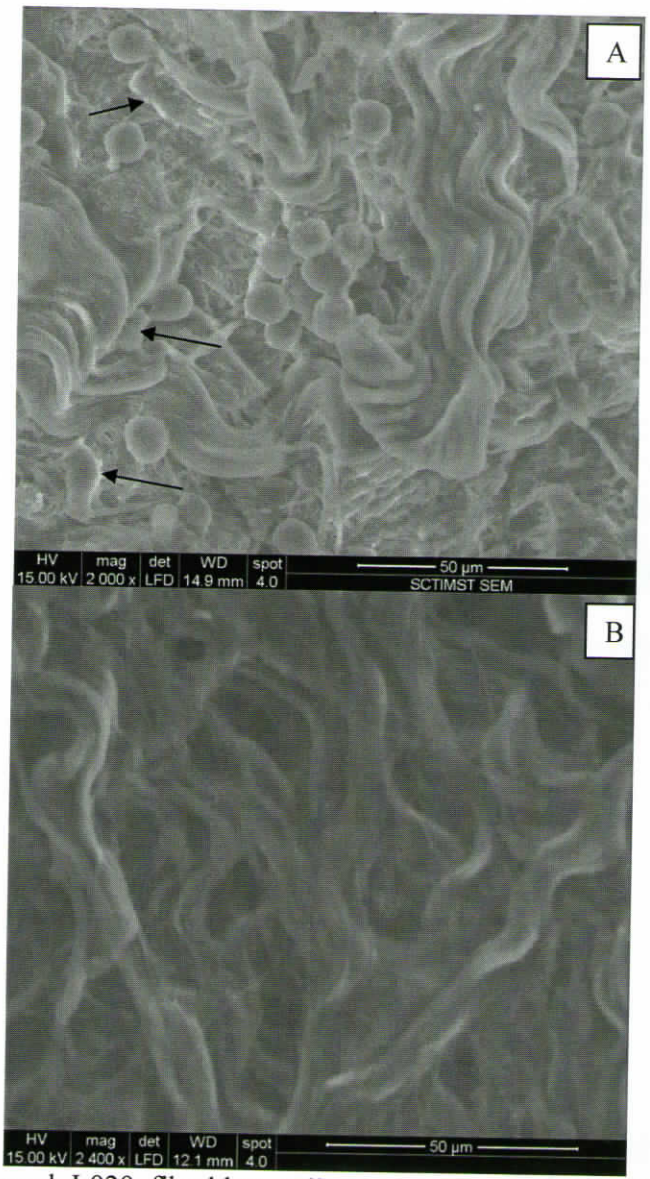

Figure 3-39: Round L929 fibroblast cells are seen attached to EDCL scaffold. Spreading of cells is also noticed occasionally (arrow).B- No cells are seen attached to 0.2DCL scaffold.

It was noted that after 48h of incubation, L929 cells was seen attached well on the test material EDCL and a few cells have spread and attained the morphology even at this period (Figure 3-37). 0.2DCL group on the other hand showed no cell attachment at all or spreading on the surface, indicating that the partial glutaraldehyde crosslinking might have modified the Arg-Gly-Asp (RGD) sequences required for the integrin mediated cell adhesion (Ruoslahti & Pierschbacher, 1987). However this difference has not affected their *in vivo* cell incorporation and neo-angiogenesis into the scaffold material. It was previously observed that crosslinked decellularised bovine pericardia have low cell adhesion. It was wrongly attributed to small pore size, low porosity and lack of extra cellular matrix (ECM) after native cell extraction of bovine pericardium. Hence modifications such as increasing the scaffold pore size and porosity and conjugation of RGD polypeptides have been attempted to promote attachment of hMSCs (Dong *et al.,* 2009). It is important to note that in this study, un-crosslinked decellularised bovine pericardium has shown the cell adhesion property indicating that RGD sequences on the ECM has been preserved which might have contributed to the cell adhesion properties.

Another reason for this absence of cell induced regeneration in mildly crosslinked decellularised bovine pericardium can be due to inactivation of matrix-bound growth factors following glutaraldehyde treatment. It is known that component growth factors such as such as VEGF, bFGF and TGF-β are released during scaffold degradation and exert their biological effects as they are dissociated from their binding proteins and activated (Hodde & Hiles, 2001; Hodd *et al.,* 2001; Hodde *et al.,* 2002a; Hodde *et al.,* 2002b; McDevitt *et al.,* 2003; Voytik-Harbin *et al.,* 1997). Moreover cryptic peptides such as Endostatin, Angiostatin, Anastellin

fragment III1C, Canstatin, Restin, Tumstatin, ABT 510 released by the degradation process also initiate and sustain the recruitment of circulating bone marrow derived cells that actively participate in long-term tissue remodeling (Badylak *et al.,* 2001; Zantop *et al.,* 2006).

Summary and Conclusions

CHAPTER 4

SUMMARY AND CONCLUSIONS

4.1 Summary & conclusion

Spontaneous regeneration in adult mammal is not reported so far. Both adaptive and innate immune response has been attributed a role in this lack of spontaneous regeneration. Of the different approaches, 'Induced regeneration' is one approach where regeneration instead of scarring can be brought in adult mammal following injury at organ level through the use of scaffolds. Regeneration can be induced in adult mammals with the help of decellularised xenografts used as a scaffold. Decellularised tissue represents a relatively new biomaterial whose biological performance is still uncertain and not well understood. Decellularised tissue produced from different sources and by different methods was known to produce a host of healing response ranging from scarring to more desirable regenerative response. It is hypothesised that this range of response can be on account of the difference in their immune response. Immunogenicity of decellularised xenograft was known and it was reported that the immune response against incompletely decellularised xenograft was seen much more prominent compared to isografts or even allografts. Despite many studies, it was still not possible to truly predict biocompatibility of one decellularised material over another. A more detailed investigation of host immune response, the ECM constituents that affect the response and the effect of these factors upon scaffold remodeling and outcomes was warranted.

Hence it was felt that xenograft immunogenicity is one of the major concerns and its effect on the regenerative potential of decellularised xenograft is not explored adequately. This study attempted to find out the effect of both innate and acquired immune response of decellularised xenograft on its *in vivo* regeneration potential in adult animal models.

To approach this problem, this study was carried out in 3 phases.

In the **first phase** decellularised bovine pericardial samples were prepared with different biological properties. The different stages involved in this phase were decellularisation and its confirmation, glutaraldehyde crosslinking and its confirmation, characterization of samples and its preliminary biocompatibility testing. By the method adopted in this study, decellularisation could be done effectively. And through differential glutaraldehyde crosslinking, test samples with different level of chemical crosslinking such as Edcl (un-crosslinked decellularised bovine pericardium), 0.2Dcl (minimally crosslinked decellularised bovine pericardium), 0.6Dcl (moderately crosslinked decellularised bovine pericardium) and GlutBP (fully crosslinked normal bovine pericardium) could be produced. The test samples differed in characteristics such as Collagenase resistance, Shrinkage temperature, Contact angle and Thermal degradation. The test samples were seen sterile, endotoxin free and non-cytotoxic and hence were considered for the second phase of the study.

Second phase of the study involved assessment of change in immune response and tissue response consequent to chemical modification of test samples. The results showed that there was significant difference between treatment groups

with respect to its *in vivo* responses such as antibody response, delayed hypersensitivity response, calcification response and tissue response when studied by 60 days subcutaneous implantation in juvenile rat model. In this experiment un-crosslinked decellularised bovine pericardium was seen most immunogenic and mildly crosslinked decellularised bovine pericardium as least immunogenic. Both these groups also exhibited minimum calcification and excellent healing response. Moderately crosslinked decellularised bovine pericardium and completely crosslinked bovine pericardium showed adverse tissue response and more calcification and intermediate immune response. Based on the above observations it was confirmed that consequent to different glutaraldehyde crosslinking, immune response and tissue response could be varied between groups which could be used for the Phase 3 of the study.

In the **third phase** of the study, based on the observations in Phase 2 , treatment groups such as un-crosslinked decellularised bovine pericardium (Edcl), partially crosslinked decellularised bovine pericardium (0.2Dcl) and fully crosslinked normal bovine pericardium (GlutBP) were selected for studies on immunogenicity and regeneration. Both innate and acquired/ adaptive immune responses were studied in this phase and it was correlated with regenerative response in adult animal models. Innate immune response was assessed by studying cytokine release from activated macrophage on exposure to different treatment groups. *In vivo* innate response was studied by assessing *in vivo* inflammatory response. Macrophage activation and inflammatory cytokine release was seen maximum in glutaraldehyde treated bovine pericardium. Decellularised pericardial groups, both

un- and mildly crosslinked did not release significant amount of cytokines and were similar in response. With respect to tissue response, both un-crosslinked decellularised bovine pericardium and minimally cross-linked decellularised bovine pericardium showed a similar picture of healing. However, induced regeneration could be observed only in un-crosslinked group. Whereas glutaraldehyde treated bovine pericardium has elicited an adverse tissue response which is not congenial for induced regeneration. With respect to inflammatory response, it was noted that both un-crosslinked and mildly crosslinked group showed a predominance of macrophage response compared to glutaraldehyde treated pericardium initially. Interestingly, at later period only un-crosslinked decellularised bovine pericardium showed a predominant macrophage response. The macrophage phenotype observed in this response was predominantly of M2 phenotype. Apart from the above observations it was also noted that un-crosslinked group showed progressive immune stimulation compared to both mildly crosslinked and glutaraldehyde treated group as evidenced by significant increase in plasma cell and lymphocyte count at 90 days compared to 21 days. Acquired or adaptive immune response was studied by assessing the antibody response to valuate humoral immune response and by conducting Lymphocyte transformation test for valuating cell mediated immune response. Indirect ELISA was conducted using bovine pericardial proteins for evaluating humoral immune response. The observations clearly indicated a sustained antibody response for un-crosslinked decellularised pericardium compared to both mildly crosslinked and glutaraldehyde treated pericardium. The antibody response noted in un-crosslinked decellularised bovine pericardium was predominantly non-complement fixing IgG1 isotype. Cell mediated immune response was studied using

Lymphocyte transformation test which evaluated the proliferative response of sensitized lymphocytes. The above data also showed that immune-stimulation was sustained in the case of un-crosslinked group compared to mildly crosslinked or glutaraldehyde treated group. Mildly crosslinked group was seen as the least responsive in this regard. The pathway preference for this immune response was studied by assessing Th1/Th2 and M1.M2 polarization. In the case of Th1/Th2 preference, the data obtained from subcutaneous implanted animals did not exactly correlate with that of abdominal muscle implanted ones. In subcutaneous experiment, the highest Th2 preference was noted in un-crosslinked group and the least in glutaraldehyde group. Whereas in abdominal muscle implantation model, highest response is seen in glutaraldehyde group and there was no significant difference between un-crosslinked and mildly crosslinked group, and both were equally low. If viewed trend wise, although not significant, only un-crosslinked group and mildly crosslinked group showed an increasing Th2 trend, while a significantly decreasing trend for Th2 preference was noticed in glutaraldehyde treated group. In the case of M1/M2 polarization studied using both subcutaneous and abdominal muscle implantation models; un-crosslinked group exhibited a tendency for M2 polarization and Glut BP group for M1 polarization. Mildly crosslinked group showed an intermediate response.

Both '**Induced regeneration**' and '**healing response**' was studied using rat abdominal regeneration model and pig vascular implantation model respectively. At 21 days, in rat abdominal regeneration model, indications of regeneration could not be observed in all the groups, although extension of muscle bands could be seen at

the peritoneal side through the mesenteric attachments in all the groups. At 90days, regeneration could be appreciated in uncross-linked decellularised bovine pericardium group alone, which was not observed in either in mildly cross-linked decellularised bovine pericardium or fully cross-linked bovine pericardium. In the pig aortic patch implantation model, none of the treatment groups regained the native aortic structure at the end of 6 months. Similar pattern of healing was observed in all the groups consisting of an endothelialised neointima with subintimal layer of smooth muscles and a collagenous adventitia. Elastin was conspicuously absent in the regenerate. It was also noticed that by mildly crosslinking decellularised bovine pericardium, it loses its cell adhesion property although the significance of this on induced regeneration is not known.

4.2 Conclusion

From the above findings, it is concluded that un-crosslinked decellularised bovine pericardium induces regeneration in rat abdominal implantation model in 3 months. Un-crosslinked decellularised bovine pericardium did not stimulate macrophages *in vitro*, but produced an inflammatory response *in vivo*. Yet this group produced induced regeneration. Mildly crosslinked decellularised bovine pericardium which showed a similar *in vitro* and *in vivo* response, did not produce any induced regeneration. Hence it is concluded that Innate immune response have an ambiguous effect on induced regeneration. However, the type of macrophage phenotype induced during this inflammation is also important. Acquired immune response seems to have no effect on induced regeneration, as un-crosslinked decellularised bovine pericardium stimulated both humoral and cell mediated

immune response un-ambiguously and yet produced induced regeneration. On the other hand, mildly crosslinked decellularised bovine pericardium was the least immunogenic and yet induced regeneration could not be observed in this group. It is also important to note the type of antibody produced (IgG1 or IgG2) is also relevant while correlating with induced regeneration since the former produced induced regeneration while the later failed to do so. With respect to predictive value of Th1/Th2 or M1/M2 response, it was observed that Th2 preference is indicative only in subcutaneously implanted animals and not in abdominal muscle implanted ones. M1/M2 response is indicative of regeneration in both subcutaneous and abdominal muscle implanted animals. As animal model of induced regeneration, adult rat abdominal wall regeneration model appears useful compared to pig aortic implantation model as structural regeneration failed to happen in this model even at the end of 6 months. Nevertheless a difference in healing response between groups was appreciated in this model. It is also important to note that even mildest chemical crosslinking is reversing the trend for regeneration in the case of decellularised bovine pericardium. Mildest crosslinking is also affecting the cell adhesion properties of decellularised bovine pericardium.

Significance of the Study and Future Direction

CHAPTER 5

SIGNIFICANCE OF THE STUDY AND FUTURE DIRECTION

5.1 Significance of the study

This study had attempted to delineate the effect of immune response on induced regeneration. It was clearly demonstrated that, not the intensity of immune response; innate or acquired, but the type which is more important as far as induced regeneration is concerned. It was unambiguously proved that decellularised biological scaffold, although it produced chronic inflammation and activated antibody mediated as well as cell mediated immune response, induced *in vivo* regeneration in adult mammal. It is important to note that this biological scaffold exhibited a selective preference for non-complement binding IgG1 phenotype and an immuno-regulatory phenotype of M2 macrophages during *in vivo* studies.

It may be noted that at present, despite many studies, it was still not possible to truly predict biocompatibility of one decellularised material over another (Badylak *et al.,* 2009). Through this study it was shown that for evaluation of a biological scaffold, it is important to consider the type of innate or acquired immune response rather than the intensity. This study had also demonstrated that, it is equally important to demonstrate *in vivo* regeneration capacity of a biological scaffold to accept it as a tissue engineering material. This should be done to prove that by

decellularisation process the growth factors embedded in the ECM is not destroyed and the regenerative capacity is preserved.

5.2 Future direction

The influence of M2 macrophage phenotype or the Th2 preference on the mechanism of induced regeneration is not explained in the literature. The effect of cell adhesion property on induced regeneration is also vaguely explained. Understanding the basic mechanism of the above processes will enable design and development of biological scaffold, or even synthetic scaffolds which will promote 'induced regeneration'. Thus scaffolds with induced regenerative property can be ultimately used for guided tissue regeneration in clinical practice.

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APPENDIX I- List of reagents

1. DNA Isolation

TE buffer (100 ml)

10 mM Tris.Cl P^H 7.4

1 mM EDTA.

DNA loading buffer: 10x

20% Ficoll 400

0.1M EDTA (P^H8.0)

1% SDS

0.25% bromophenol blue

0.25% xylene cyanol

Ethidium bromide stock solution

50 mg of ethidium bromide was dissolved in 100 ml of H_2O. Used diluted 1:1000.

Stored at 4°C Protected from light

Lysis buffer (5ml)

1M Tris HCl PH 8.0	50µl
0.2M EDTA PH8.0	2µl
10% SDS	100 µl
Protenase K 20mg/ml	100 µl

Make up to 5 ml with de-ionised water.

2. MTT Assay

MTT stock

The MTT powder was dissolved (1mg/ml) PBS (Sterile filtered).

When not in use protect the reconstituted MTT reagent from light and store at 4°C. (Can be stored for several months)

3. SDS-PAGE

Acryl amide Solution (30%) (W/V)

Acryl amide 29.2 g

N,N1-Methylene bis acryl amide 0.8 g

Volume made up to 100 mL with distilled water. The solution was filtered and stored in dark bottle at 10°C.

Lower Tris Buffer (1.5 M, pH 8.8) (W/V)

Trisbase (1.5M) 36.34 g

Distilled Water 200 mL

Upper Tris Buffer (1.0M pH 6.8) (W/V)

Tris base (0.5 M) 12.1 g

Distilled water 200 mL:

Sodium dodecyl sulphate (SDS) (10%) (W/V)

SDS 10.0 g

Distilled water 100 mL

Ammonium Sulphate (10%)(W/V)

Ammonium per sulphate 0.1 g

Distilled Water 1.0 mL

Should be prepared freshly prior to use

Sample loading dye (W/V)

Stacking Buffer 1.25 mL

Glycerol 1.0 mL

2-Mercaptoethanol 0.5 mL

SDS 150 mg

Bromophenol blue 50 mg

Volume make up to 100 ml with distilled water

Tris Glycene Electrophoresis tank buffer,(pH 8.4)

Tris base 25 mM 3.0 g

Glycene 250 mM 18.6 g

SDS 10gm

Composition for Separating and stacking gel SDS PAGE

Composition	Separating gel 15% (10 mL)	Stacking gel (5%) (5mL)
Water	2.3	3.4
Acryl amide	5	0.83
1.0M Upper Tris buffer		0.63
1.5M Lower Tris Buffer	2.5	
10% SDS	0.1	0.05
10% APS	0.1	0.05
TEMED	0.004	0.005

4. Protein Visualization. Silver staining

Fixative (V/V)

Formaldehyde (35%) 35 mL

Methanol 40 mL

Sodium thio sulphate 0.02% (W/V)

Sodium thiosulphate 0.02 g

Distilled Water 100 Ml

Silver nitrate (0.1%)(W/V)

Silver Nitrate 0.1 g

Distilled Water 100 mL

Developing Solution

Sodium thiosulphate (0.02%) 3.0 mL

Formaldehyde (35%) 75 µl

Sodium Carbonate 4.5 g

Distilled Water 147 ml

Stopping solution

Citric Acid 22 g

Developing Solution50 ml

5. ELISA

Antigen diluting buffer P^H 9.6 or carbonate-bicarbonate buffer

Na_2CO_3-1.59G

$NaHCO_3$-2.93G

Dissolve in 1l deionised water

Washing buffer pH 7.4

Dissolve 160g NaCl, 4g KCl,22.4g Na_2HPO_4in deionised water to 1L (20X) used working soln- 5 ml+95 ml water

Secondary antibody dilution buffer

0.01M PBS P^H 7.0

0.05%tween 20

1L BUFFER +0.5ml tween 20

Blocking buffer (pH 7.2-7.4)

PBS containing;

1mM EDTA

0.25% BSA

Store at 4^0c.

6. Movart's Pentachrome

Biebrich Scarlet-Acid Fuchsin Solution,

Biebrich scarlet, 0.9%, acid fuchsin 0.1%, in acetic acid, 1.0%.

Phosphotungstic acid solution,

Phosphotungstic acid, 10%.

Phosphomolybdic acid solution,

Phosphomolybdic Acid, 10%

Aniline blue solution,

Aniline blue, 2.4% and acetic acid, 2%.

7. Antibiotics used in cell culture medium

Solution 1: 100X

10000u penicillin+10mg streptomycin (Himedia-A001A-5X100ML)

Solution 2 5X :

250µg/ml amphotericin B solution (Himedia-A011-5X20ML)

Solution 3: 5X

Gentamycin 50 µg/ml (Himedia-A005-5X20ML)

Working solution:

1ml A+200 µl B+200 µl C

Total of 1400 µl of this working solution is added to 100ml RPMI 1640.

LIST OF PAPERS PUBLISHED

1. Umashankar PR, Arun T , Kumary TV (2011). Short duration glutaraldehyde crosslinking of decellularised bovine pericardium improved biological response. *J Biomed Mater Res*; 97A(3):311-320.
2. P.R. Umashankar, Mohanan.P.V and Kumari.T.V. Glutaraldehyde treatment elicits toxic response compared to decellularisation in bovine pericardium. Accepted for publication in Toxicology International.
3. Umashankar PR, Arun T, Kumary TV. Inflammatory or immune response do not deter *in vivo* regeneration induced by decellularised bovine pericardium. Communicated to J Biomed Mater Res- Part A.