BIOCOMPATIBILITY STUDIES OF PECTIN-FIBRIN NANOCOMPOSITE BEARING BALB/C MICE

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Pectin is a natural polysaccharide and the pectin scaffold system has proved to be suitable for an intended use towards biomedical applications, such as drug delivery and tissue engineering. Studies on gemcitabine loaded pectin-fibrin scaffold have shown it to be cytotoxic towards ovarian cancer cells at the *in vitro* level. Our present study aims at substantiating the biocompatibility of the pectin-fibrin composite scaffold in a mouse implantation model in order to prove the compatibility of the scaffold system *in vivo*. Composite scaffolds were implanted and the biocompatibility was assessed after the 1st, 6th and 12th week of study, respectively. Macroscopic inspection of the implantation site revealed no pathological inflammatory responses and histopathology studies depicted remarkable neutrophil accumulation within the implant in a timely manner. Furthermore, the immune response indicated significant difference with cytokines IL-1β, IL-10, and IL-17α, respectively. These results suggested that this scaffold system could be a promising targeted drug delivery system for the slow release of drugs in a mouse disease model.

Keywords: scaffold, implant, *in vivo*, macroscopic, histopathology, biocompatible

INTRODUCTION

The use of biodegradable nanoparticles as effective drug delivery devices has shown significant therapeutic potential.[1] It aims to create new tissues and organs by introducing cells, biocompatible materials, and supportive factors and is one of the current applications for the treatment of various cancer types.[2] An optimal scaffold material would provide both structural support and act as a reservoir for the release of bioactive substances.[3] This could directly influence the behavior of colonizing cells, leading to an advantage in the tissue adaptation. Recent efforts in this field have highlighted the importance of drug, protein and growth factor delivery.[4] Numerous biomaterials ranging from natural to artificial polymers have been investigated to construct scaffolds for drug delivery purposes. In order to exploit the advantages and eliminate the undesired characteristics of individual polymers, various composite scaffolds comprising two or more polymers have been considered.[2]

Pectin is a heteropolysaccharide composed of 1,4-linked-d-galactosyluronic acid residues useful for the construction of a drug delivery system. Certain pectins may possess amide groups and in others the acid groups may either be free, combined as a methyl ester, or exist as sodium, potassium, calcium or ammonium salt.[5,6] High and low methyl ester proteins with more than 50% of esterified acid units and less than 50% methyl ester groups, are the two forms of pectin extracted from citrus fruits.[6] It is an edible polysaccharide useful for the construction of drug delivery systems. Pectin functions not only as a detoxifying agent by helping in regulating and protecting the gastrointestinal (GI) tract, but also as invigorating the immune system. Pectin gets

digested by the enzyme pectinase, which is present only in the colon, and therefore remains undigested in the GI tract, which contains enzymes like protease and amylase.[7] This makes pectin an ideal drug carrier for colon-specific drug delivery, which is known to have the advantage of achieving higher biological availability because the pH in the colon is neutral and peptidase activity is relatively lower.[8,9] Biodegradable natural polymers, such as chitin and pectin, have proven to show good biocompatibility and non-toxicity.[10,11]

Fibrin is a fibrous non-globular protein involved in blood clotting, even a 60-100 mg/mL of fibrin can initiate coagulation, hampering the blood flow. Fibrin-chitosan sodium-alginate composite scaffolds have shown improved mechanical properties, providing a good composite for wound dressing applications.[12] Another study with chitosan-fibrin-collagen asymmetric scaffold has shown that fibroblasts adhered to the walls of the scaffold, suggesting good growth and excellent cell biocompatibility of the scaffold.[13] Furthermore, *in vitro* cytotoxicity studies done with gemcitabine loaded pectin-fibrin scaffold systems showed more than 70% cell death, proving the cytotoxic behavior of the drug combination towards ovarian cancer cells.[14] These results suggested the significance of the scaffold system as an efficient implantable drug delivery system for the treatment of ovarian cancer. Here in this work, the biocompatibility of pectin-fibrin scaffold system is compared with the implantable pectin scaffold system.

Inflammation is the response of tissue to injuries, which is categorized into two groups: responses to acute inflammation and those to chronic inflammation. This is mediated by a variety of soluble factors, including a group of secreted polypeptides known as cytokines. The process of inflammation is characterized in the acute phase by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes and cytokines. The development of cellular and humoral responses to pathogen describes the chronic phase of inflammation. These soluble factors are multifunctional. Cytokines are involved in the extensive networks that involve synergistic as well as antagonistic interactions and exhibit both negative and positive regulatory effects on various target cells in an autocrine as well as paracrine fashion.[15] Further, in this work, the biocompatibility of the nanocomposite scaffold is demonstrated by the studies of histopathology and immune response analysis, using ELISA assay.

EXPERIMENTAL

Materials

Pectin (from citrus) and calcium chloride (analytical grade) were purchased from Sigma-Aldrich. A Multi-Analyte ELISArray kit was purchased from QIAGEN Sciences, Maryland, USA.

Scaffold preparation

For the preparation of the pectin scaffold, the method of ionic gelation was followed.[14] Calcium chloride solution served as ionic cross-linker for the process of pectin gelation. Chitosan was added to reduce the hydrophilic nature of pectin, and increase its stability. 8% w/v of pectin solution was prepared using double distilled water by constantly stirring at 1200 rpm at 85 °C for 20-25 minutes for obtaining a monodispersive solution. The solution was allowed to cool and when the temperature approached 40 °C, 5 mL of chitosan solution (1% w/v) was added to the pectin solution by constant stirring at 1200 rpm for 15 minutes. The pH was monitored and maintained neutral. This pectin scaffold served as a control for the experiment. The composite scaffold of fibrin with pectin was prepared by redispersing the pectin scaffold in 2 mL of double distilled water. Further, the solution was added to 8 mL pectin solution (8%w/v) and kept under constant stirring speed of 1200 rpm at room temperature for 15-20 minutes. The remaining steps for the preparation of the scaffolds were the same as explained above. Thus, stable nanoscaffolds were formed. The hydrogels were transferred to 24-well tissue culture plates and freezed at -20 °C for 24 h. The frozen samples were then lyophilized (Alpha 2-4 LD plus Christ, Germany) for 24 h.

In vivo implantation studies

Animal experiments were carried out under a protocol approved by Institutional Animal Ethics Committee (Approval no: IAEC/2012/1/5), in six adult Swiss albino mice, weighing 20-30 g (Figure 1). The mice were maintained in plastic cages with paddy husk bedding in a room with ambient temperature and light. The mice were given sterilized laboratory food and filtered water.

In the present study, the *in vivo* evaluation of the prepared scaffolds was performed. Before implantation, the scaffolds were cut into sections of 0.7 cm diameter and 0.2 cm thickness. This was incubated in sterile PBS for 3 days in order to analyze the porosity of the construct.

Six female Swiss albino mice were selected and the activities were observed before implantation. The mice were anesthetized by intramuscular injections of xylazine and ketamine in the ratio 1:4 (KETMIN^{R} 50,

THEMIS Medicare limited) and a 2-cm area of the dorsal skin was cleaned and shaved. Sub-cutaneous implants (n = 2) were inserted through a 0.5 cm incision on the dorsal lower left quadrant and secured using a PROLENE 4/0 suture stitch. The National Institute of Health guidelines were followed for both the care and use of laboratory animals.

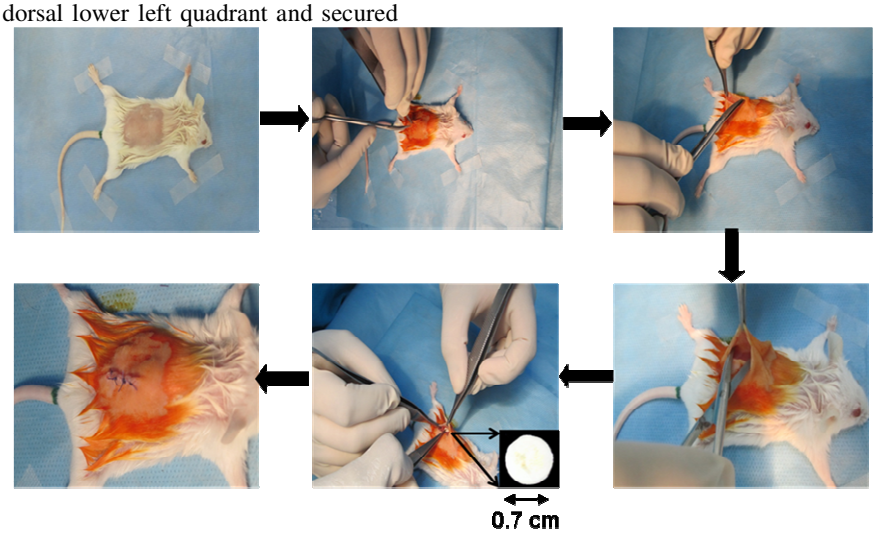

Figure 1: Implantation procedure with a 0.7 cm pectin scaffold on the dorsal lower left quadrant

Implant recovery and histology

Mice were sacrificed after periods of 1 week, 6 weeks and 12 weeks, respectively, in groups of 2 (n = 2). At the time of sacrifice, skin tissue, spleen, liver and sera were removed from each mouse to evaluate the immune response to the implants. The pectin and composite scaffold implants were recovered and the area of implantation was visually inspected for the evidence of any tissue reaction or inflammation. Recovered implants were fixed in 10% buffered formalin, dehydrated, and embedded in the paraffin blocks. The histological evaluations of the recovered implants were done using hematoxylin and eosin staining. Hematoxylin and eosin stain (H&E stain) is a popular staining method in histology. The staining method involves application of Hemalum, which is a complex formed from aluminium ions and hematein, which is an oxidation product of hematoxylin. Hemalum gives a blue color to the nuclei of cells (and a few other objects, such as keratohyalin granules and calcified material). Nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colors other eosinophillic structures with various shades of red, pink and orange.[16,17]

Immune response studies

Serum isolated from blood samples of euthanized mice was analyzed for immunological reactions. Acute and chronic inflammatory responses were studied using a Multi-Analyte ELISArray kit (QIAGEN Sciences, Maryland, USA). CBA inflammatory kits are advantageous as they allow the detection of a whole panel of cytokines in a multiplex fashion, using small volumes. The Multi-Analyte ELISArray kit is a Mouse inflammatory cytokine kit designed to simultaneously profile the level of multiple cytokines or chemokines, using the conventional and simple sandwich-based enzyme linked immunosorbant assay (ELISA) technique.

In our study, the major acute and chronic inflammatory responses were analysed with the following cytokines: IL-4, IL-1β, IL-2, IL-10, IL-12, IL-17α. Acute inflammatory immune responses were studied using two cytokines, IL-1 β and IL-17 α. IL-1 (α and β) and TNF are extremely potent inducers of acute inflammation.

RESULTS AND DISCUSSION

Implant recovery and histology analysis

Macroscopic inspection of the implantation site indicated no pathological inflammatory tissue responses to the scaffold system. Tissue overgrowth and sinusoidal congestion of implants were observed at the later stages. The total number of cells infiltrating the implant significantly decreased between week 1 and week 12 in the intra-peritonial (IP). The histological analysis of the recovered tissue indicated early neutrophil accumulation within the implant, which resolved over time. Various tissue sections were analysed and the following observable changes were noted (Figure 2).

Considering the spleen, in the first week of study, we observed that the two sections of the +control and composite showed widened red pulp and congestion within the vascular system. The 6th week of study revealed splenic tissue with

follicles and areas of haemorrhage and a few multinucleated cells. After the 12th week, histology results allowed very similar observations: widened red pulp with haemorrhage and giant cells in white pulp.

Skin tissue revealed follicular plugging and dermis showing neutrophilic infiltrate around the amorphous substance for the control samples. The test sample showed skin irregular acanthosis, follicular plugging and a focus of squamous nests with keratin pearl formation above dermis with inflammatory infiltrate. Similarly, after the 6th week of study, it was observed that the skin tissue of the control sample presented dermis with an area of dystrophic calcification and fibrosis, while the test sample showed increased follicular plugging within the dermis. After the 12th week, the scaffolds were found to be degrading; inflammatory cells with fibrosis were indicative of neutrophil accumulation. This change was due to the prolonged implantation of the scaffold system for a period of 12 weeks, which was considered to be the major criterion for confirming the biocompatibility of the scaffold.

In the case of liver tissues, after the 1st week of implantation, the control tissue samples showed congestion and ballooning degeneration of a few cells and mild lymphocytic cells infiltrated around portal tracts. The test sample showed congested vessels and sinusoids. After the 6th week, the control sample exhibited liver tissue with congested vein and sinusoids, while the test sample showed mild nucleomegaly in the hepatocytes. After the 12th week, the sections showed congested sinusoids and lymphocyte infiltrate around portal triads. As a result of the increased sinusoidal congestion and infiltration of lymphocyte, we could confirm the nature of the scaffold system over time, by considering the 1st week and 6th week results.

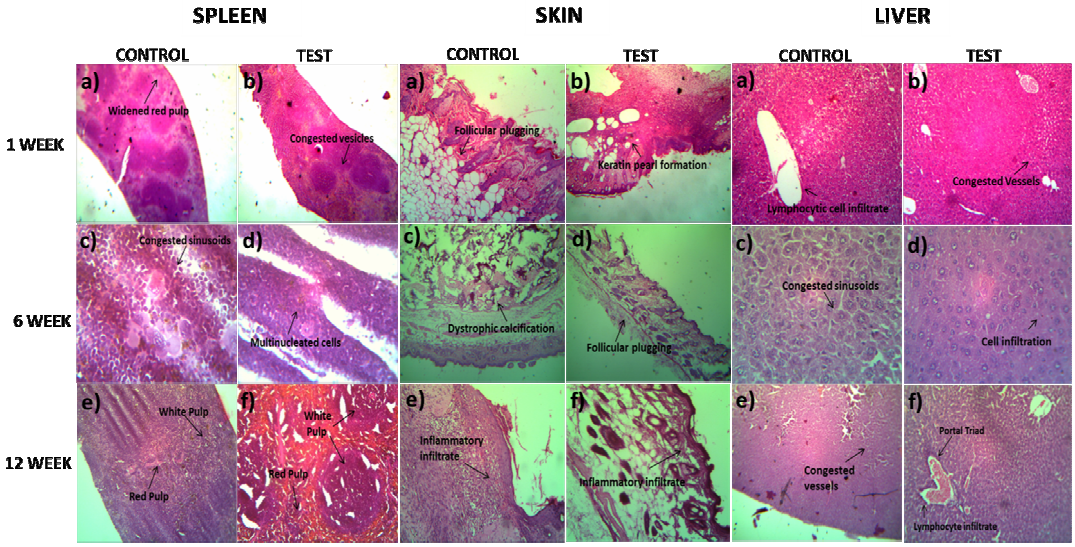

Figure 2: Histopathology images of the *in vivo* effects on the spleen, skin and liver tissue samples of control (pectin scaffold) and test (pectin-fibrin composite) groups, depicting increased rate of infiltration from the 1st week to the 12th week (20x)

Immune response analysis

The immune response was analysed for the tissue sections of the mice after the 1st week, 6th week and 12th week of implantation. The results revealed an increased reduction in the cytokine activity of the chronic inflammation linked interleukins. The following observations were made: in the case of IL-1β, there was no significant increase in the temperature of the animals during the entire course of the study; IL-1β was found to significantly decrease in the first and sixth week of study ($p < 0.05$). IL-1β is responsible for triggering fever by enhancing prostaglandin E_2 (PGE_2).[18,19] At the site inflammation, IL-1β induces release of histamine from mast cells, while our data reveal significant reduction when compared to the corresponding control groups. This was confirmed by the fact that in the sixth week of study, a significant difference was observed in the cytokine between

the control and test groups. For IL-17α, the activation results in the induction of IL-6 activity, thereby inhibiting TNF production and hence the acute inflammation response gets reduced.[20] In our study, there is a significant increase in the first week of study, thereby inhibiting acute inflammation. For the 6th week, the results revealed normal levels of IL-17α, while for the 12th week, it showed rapid reduction.

There was no evident change in the levels of IL-2 and IL-12 cytokines, which correspond to chronic inflammation. IL-2 was produced in response to antigenic stimulation, which thereby increased the suppression of IFN-γ. This indicates the absence of chronic inflammatory responses in the control and test group samples. On the other hand, IL-4 induces CD4+ T cells to differentiate into T_H2 cells, while suppressing the development of T_H1 cells.[21,22] It also acts as a B cell, T cell and mast cell growth factor, it enhances class II MHC expression on B cells. For the 1st week, there was a tremendous decrease in the levels of IL-4, while after the 6th and 12th weeks, the levels showed normal levels, indicating the inactivation of the CD4+ cells, which do not proceed to the T cell differentiation (Figure 3). IL-10 is the cytokine synthesis inhibitory factor (CSIF) that inhibits the IFN-γ production by activated T cells.[16] It acts as anticytokine by inhibiting antigen specific T cell proliferation. After the 1st week and the 6th week, no significant variations of the IL-10 cytokine from the normal levels were observed. On the other hand, increased reduction in the levels of IL-10 after the 12th week indicated the activation of T cell proliferation in the biological system.

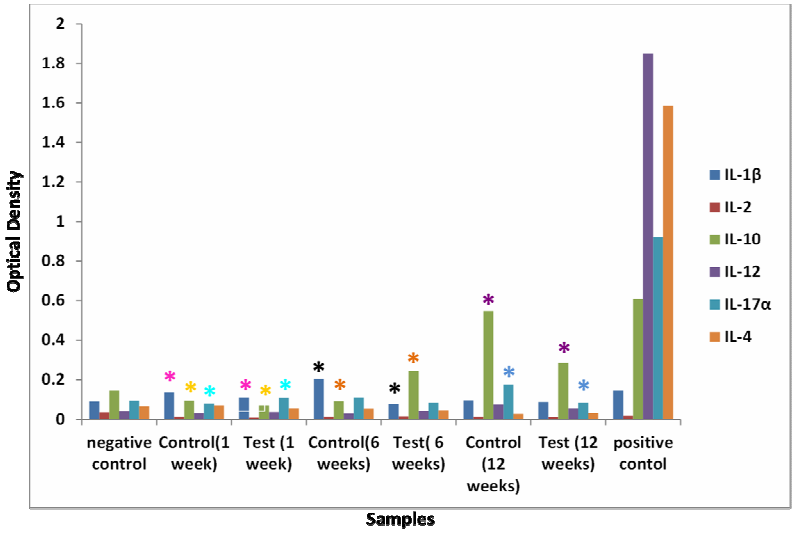

Figure 3: Immune response analysis indicating acute and chronic inflammation with greater response after the 12th week of implantation in control (pectin scaffold) and test (pectin-fibrin composite) groups

Statistical analysis

Statistical analysis of the control and test groups was carried out and the results are presented in Figure 3. The values of the immune response study were expressed as mean ± standard deviation (SD). To determine the statistical difference between groups, the two-tailed Student's t-test was performed. A probability value (p) of less than 0.05 was considered to be statistically significant. Significant differences were observed for IL-1β, IL-10, and IL-17α cytokines, respectively.

CONCLUSION

Our study elucidated the biocompatibility of a pectin-fibrin scaffold system, compared with an implantable pectin scaffold system. The macroscopic inspection of the implantation site revealed no pathological inflammatory tissue responses to the scaffold system. Tissue overgrowth and sinusoidal congestion of the implants were observed at the later stages. The total number of cells infiltrating the implant significantly decreased between week 1 and week 12 in the intra-peritonial (IP).

The histological analysis of the recovered tissue indicated early neutrophil accumulation within the implant, which resolved over time. Various tissue sections were analysed and marginal inflammatory responses were observed in the later stage of the study. The results suggest that this scaffold could be a promising targeted drug delivery system for the slow release of drugs in a mouse disease model, confirming the *in vitro* and *in vivo* strategies. The immune response results of the tissue sections revealed an increased reduction in the cytokine activity of the chronic inflammation linked interlukins. Therefore, the developed scaffold system could be an efficient implantable drug delivery system for disease management. In the future, this study can be extended to test the pectin-fibrin scaffold system in an ovarian mouse tumor model.

ACKNOWLEDGEMENTS: The authors acknowledge the Department of Science and Technology (DST), India, for the partial financial support by SERB Division (Ref. No.: SR/FT/LS-147/2009) to Vinoth-Kumar Lakshmanan. Author K. S. Snima is grateful to the Council of Scientific and Industrial Research (CSIR), India, for providing Senior Research Fellowship (09/963 (0030)/2K 13-EMK-I) for carrying out her research work. Authors Reshmi S., Reshmi T., Sarika Chandran thank the Department of Science and Technology (DST), for MTech grant support. We are also grateful to Praveen for his help in nano fabrication. We thank Amrita Centre for Nanosciences and Molecular Medicine for the infrastructure support.

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