

Original Article

Regeneration of Full Thickness Skin Defects Using Decellularized Fish Swim Bladder Scaffolds Developed by *Sapindus Mukorossi* Fruit Pericarp Extract

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Decellularized tissue scaffolds are better option for the regeneration of large wounds. Fish-derived biological materials are safer than mammal-derived biological materials. Various chemicals including detergents are used in the process of decellularization which causes alteration of tissue architecture and composition. We investigated the *Sapindus mukorossi* fruit pericarp extract (SPE) (5%) for decellularization of the fish swim bladder (FSB). The FSB samples were incubated in 5% SPE under continuous agitation for 96h at room temperature. The processed samples were observed by histological examination, DAPI staining, scanning electron microscopy (SEM), quantification of DNA, hydroxyproline and hemocompatibility determination. Further, these decellularized FSB scaffolds were transplanted on full thickness skin wounds of group III New Zealand white rabbits. The wounds were left open in group I (Sham) and reconstructed by autograft in group II (n=6 in each group). The fresh FSB samples were completely decellularized at 72 hours interval as the decellularized tissue samples did not show any nuclei. DNA quantity was reduced ($P<0.05$) in decellularized samples as compared to fresh samples and FSB scaffolds were found to be hemocompatible. Complete healing was observed on day 21 in decellularized implanted wounds (group III). However, complete healing was recorded on day 28 in group II and the wounds of group I were still in the process of healing. Our results suggested that the 5% SPE solution effectively decellularized the fresh fish swim bladder at 72 h interval and the scaffolds were well tolerated by the animals.

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Introduction

The integument may be damaged by acute or chronic wounds, burn, diabetic, pressure and venous ulcers. In most cases, small skin wounds are healed either by primary closure or by second intention. But large skin defects require adequate wound dressing/skin grafts and skin substitutes to accelerate the healing process with minimum scar formation. Autologous skin flap transplantation is the gold standard procedure for reconstruction of skin defects but it causes donor site morbidity [1]. Allografts

and xenografts are unsuitable for reconstruction of large skin defects because they elicit severe immune reaction as compared to autografts [2].

Extracellular matrix (ECM) scaffolds prepared by decellularization process are widely employed in tissue engineering research as well as in regenerative medicine [3,4]. The goal of the decellularization technique is to reduce genetic material that could trigger immunological rejection while leaving the scaffold undamaged for tissue and organ restoration. Various organs and tissues have been decellularized to get natural ECM using chemical, enzymatic, or mechanical disintegration. Several methods have been used for developing natural tissue and organ scaffolds using variety of chemicals that are harmful to the environment [5]. sodium dodecyl sulfate (SDS), Triton X-100, and sodium deoxycholate (SDC) effectively decellularize the tissues but SDS residues inhibits

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metabolic activity, proliferation and viability of the cells within the scaffold [6].

Sapindus mukorossi is a critically significant ethno - medicinal tree and has various biological and pharmacological features like anti-inflammatory, antiparasitic, antiviral, antifungal, antibacterial, anticancer, antihyperglycemic, antihyperlipidemic, and hepatoprotective effects [7,8]. Because of its biosurfactant potential, the dried fruit of *S. mukorossi* has a soapy texture due to the presence of saponins. It has traditionally been used to make Ayurvedic shampoos, detergents, and hand wash. The saponins are secondary metabolites that are employed as herbal detergents due to their amphiphilic properties [9]. The saponins from *Sapindus mukorossi* have an LD50 of more than 5000 mg/kg in wistar rats, and the results of a dermal irritation test in rabbits showed that after 14 days of continuous dermal irritation by saponins from *Sapindus mukorossi*, the average score of dermal irritation per day of each rabbit is zero [10]. The sample is classified as “practical nontoxic” and “non dermal irritant” according to the toxicity classification criterion in “Hygienic Standard for Cosmetics” (Ministry of Public Health of China, 2002). *Sapindus* saponin has a CMC of 0.04 to 0.05 wt%, which is significantly lower than synthetic surfactants like Triton X100 (0.13) and SDS (0.224) [11].

In recent years, collagen of fish origin has been attracting attention as a medical biomaterial due to the low risk of transfer of pathogen infection compared to animal collagen. The swim bladder constitutes about 3-5% of the total body weight of the fish, and it is a rich source of Type I collagen [12]. The swim bladder is mainly composed of collagen I, glycosaminoglycan (GAG), and elastin, especially rich in elastin, in accordance with higher elastic modulus in comparison to bovine pericardium. These superior properties of swim bladder-derived material with excellent hemocompatibility and cytocompatibility endow it a great candidate as cardiovascular biomaterials [13].

The present study was undertaken to develop a protocol for efficient decellularization of fish swim bladder using aqueous extract of soap nut fruit pericarp (SPE) and to authenticate the scaffolds for regeneration of full thickness skin defects in New Zealand white rabbits.

Materials and Methods

Preparation of SPE

Sapindus mukorossi fruit pericarp extract (5 wt %) was prepared as per method suggested by Goyal and coworkers [14]. Briefly, *Sapindus mukorossi* fruits were purchased and authentication was performed by the College of Horticulture and Forestry of our University. Fruit pericarp powder (5g) was soaked in 100 ml phosphate buffer saline (PBS) for 24h at room temperature, agitated on magnetic stirrer for 6 h and then filtered using stainless steel sieve. This filtered extract was further centrifuged at 8,500 rpm for 10 min to get suspended free SPE which was used for decellularization of fish swim bladder.

Collection and decellularization of FSB

Fish swim bladder (n=3) of Rohu fish (*Labeo rohita*) was procured and rinsed thrice using antibiotic mixed sterile PBS (pH 7.4) to remove blood clots. The above process was completed within 3 h. The FSB was cut into 2 x 2 cm² pieces, transferred in a sterile plastic container having 100 mL of 5% SPE extract and loaded over magnetic stirrer for processing up to 96h at room temperature. The tissue samples were collected at 12, 24, 48, 72 and 96h time intervals for assessment of decellularization efficiency of SPE solution. The

processed samples were characterized by histology (H&E, Masson's trichrome and PAS), fluorescent DAPI staining, scanning electron microscopy (SEM), DNA quantification, hydroxyproline and hemocompatibility determination, and compared with native tissues. The decellularized samples were stored at -20°C in 0.1% amikacin mixed PBS solution for further use.

Characterization of decellularized FSB scaffolds

Microscopic examination and DAPI staining

The processed tissues were fixed in 10% formalin prepared in PBS, dehydrated in ethyl alcohol, treated with xylene, embedded in paraffin and finally cut in to 5-micron thick tissue sections. The tissue sections were stained with hematoxylin and eosin (H&E), Masson's trichrome and Periodic Acid Schiff (PAS) and graded using histological scoring system [15]. Briefly, visible nuclei per square micrometer: high (+++), moderate (++), low (+), none (-); collagen fibers arrangement: compact (+++), mildly loose (++), moderately loose (+), heavily loose (-); debris: more (+++), moderate (++), mild (+), none (-) and porosity: highly porous (+++), moderate (++), mild (+). For DAPI staining, the tissue sections were washed with 0.2% Tris buffered saline Tween 20 (TBS-T) and dried. Thereafter, the slides were stained with 4, 6-diamidino-2-phenylindole 2HCl (DAPI) solution and incubated in dark for 10 min at room temperature. Nuclear material emits cold blue fluorescence under fluorescent microscope.

Extraction of DNA and quantification

The extraction of DNA from native and decellularized FSB tissues was performed using DNA extraction kit following the manufacturer's instructions (Dneasy blood and tissue kit, Quagen India Pvt. Ltd., New Delhi). After extraction, DNA concentration and purity were quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA).

Quantitative evaluation of hydroxyproline

The hydroxyproline content in native and decellularized FSB was quantified as per the protocol developed by Reddy and Enwemeka [16]. In brief, a known quantity of dried samples (n=6) were homogenized, centrifuged at 8000rpm and supernatant was collected. At the same time 100 µl of known hydroxyproline concentrations standard samples and test samples were taken in different test tubes. Subsequently, 100 µl of 0.05M copper sulphate and 2.5N sodium hydroxide was added to each test tube, and heated for 5 minutes in water bath at 40°C. Thereafter, 100 µl of hydrogen peroxide (6%) was added and left in the water bath for 10 min. After thawing under tap water, 400 µl of 3N H₂SO₄ and 200 µl of 5% p-dimethylaminobenzaldehyde were added and OD was taken at 550nm.

Hemocompatibility determination

Fresh blood was collected from healthy New Zealand white rabbits in disodium EDTA vials and centrifuged at 800 rpm for 10 minutes to get the red blood cell (RBC) pellet. The pellet was washed with 1X PBS until getting a clear supernatant. The decellularized FSB scaffolds (n=3) were then incubated in the diluted RBCs (1:4) at 37°C for 2 hours and centrifuged at 1200 rpm for 10 minutes to get the supernatant. The whole blood was diluted with distilled water and 1X PBS which acts as a positive and negative control, respectively. The absorbance (A) of the supernatants was measured at 540 nm and percentage of haemolysis was calculated as:

$$\text{Hemolysis (\%)} = (\text{Abs}_{\text{sam}} - \text{Abs}_{\text{NC}}) / (\text{Abs}_{\text{PC}} - \text{Abs}_{\text{NC}}) * 100$$

where Abs_{sam}, Abs_{NC} and Abs_{PC} are sample absorbance, absorbance

of negative control and positive control respectively.

Animals, wound creation and transplantation of FSB scaffolds

Eighteen New Zealand white rabbits of either sex, weighing about 2 Kg were allocated into three groups having six rabbits in each group. After aseptic preparation of the dorsal thoracic area, a full thickness skin defects (20x20mm²) was created under surgical anesthesia using intramuscular administration of xylazine (6 mg/Kg) and ketamine (60 mg/Kg). The wounds of group I (Sham) animals were covered with a sterile non-adhesive dressing (Cloxigras™ - Gujrat Healthcare, Changodar, Ahemdabad-13). The wounds of groups II and III were reconstructed immediately with autograft and double layer of decellularized FSB scaffolds, respectively and covered with sterile non-adhesive dressing as in group I. The wound healing was assessed on the basis of following parameters.

Warmth at wound/ transplanted site

The warmth of the open wound and graft transplanted site was noted at day 0, 3, 7, 14, 21 and 28 by a non-contact infrared thermometer (Dikang HG01).

Wound area and percent contraction

On days 0, 3, 7, 14, 21, and 28, the wound area was measured using transparent sheet having graph, and percent contraction (PC) was estimated using formula given by Schallberger and coworkers [17].

$$PC (\%) = 100 - (\text{total wound area on day "n"} / \text{wound area on day 0}) * 100$$

Hematological observations

On post-operative days 0, 3, 7, 14, 21 and 28, blood smears were prepared and stained with leishmann stain for differential leukocyte count (DLC). The counts were expressed in per cent.

Planimetry and macroscopic observations

Color images of the site were captured from a fixed distance on days 0, 3, 7, 14, 21, 28 to examine the wound shape, color, scar formation and other changes and a score was given [15]. In a nutshell, Graft/wound color: 1-pink, 2-pinkish pale 3-pale, 4-light brown, 5-dark brown, 6-black; Graft/ wound desiccation: 1-similar to normal skin, 2-slight dry, 3-moderate dry, 4-leathry; Wound edges: 1-pink, 2-brown, 3-black; Graft sloughing: 1-no slough, 2-partial sloughing, 3-complete sloughing; Surface/marginal granulation: 1 -mild, 2-moderate, 3-severe; Scar formation: 1-no scar, 2-mild, 3-moderate, 4-severe. The group with the lowest score was deemed to be the most effective in wound healing.

Histopathological observations

On days 3, 7, 14, 21, and 28, biopsy samples were taken from the implantation site for examination of histological alterations that occurred during the healing process. Tissue samples were taken in phosphate buffered 10% formalin saline and processed as previously stated. These tissue sections were stained with H&E, Masson's trichrome and Alcian blue for evaluation of collagen fiber density, thickness and their arrangement at the healing site, concentration of sulphated glycosaminoglycans and scored as per the scoring system given by Gangwar and coworkers [18]. The group with the lowest score was deemed to be the most effective in wound healing.

Data analysis

The data were analyzed using Statistical Program for Social Sciences (SPSS) software version 17.0 and expressed as Mean $\pm$ Standard



Figure 1: Macroscopic structure of decellularized fish swim bladder (DFSB)

Error. Statistical significance was determined by one-way analysis of variance. The difference ($P < 0.05$) was considered statistically significant.

Results and Discussion

Macroscopic observations

Macroscopic structure of decellularized fish swim bladder is presented in figure 1. At 72h the decellularized FSB (DFSB) tissue was soft and swollen. The grafts become milky white after decellularization which may be due to removal of cells from the tissues.

Microscopic observations

Microscopic observations of the H&E and Masson's trichrome stained native and decellularized FSB tissues are presented in figure

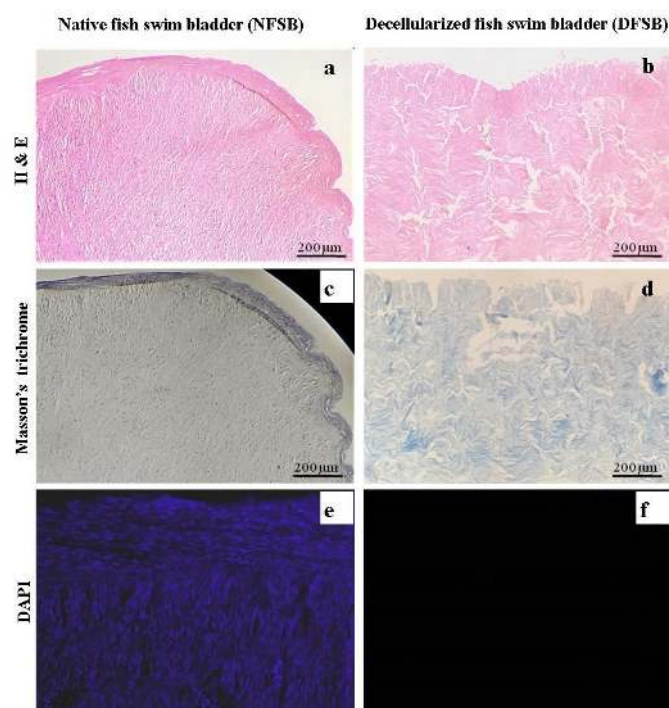


Figure 2: Representative micrograph of NFSB and DFSB stained with Hematoxylin and Eosin (a,b), Masson's trichrome (c,d) and 4',6-Diamidino-2-phenylindole (DAPI) (e,f); 10x and scale bar = 200µm

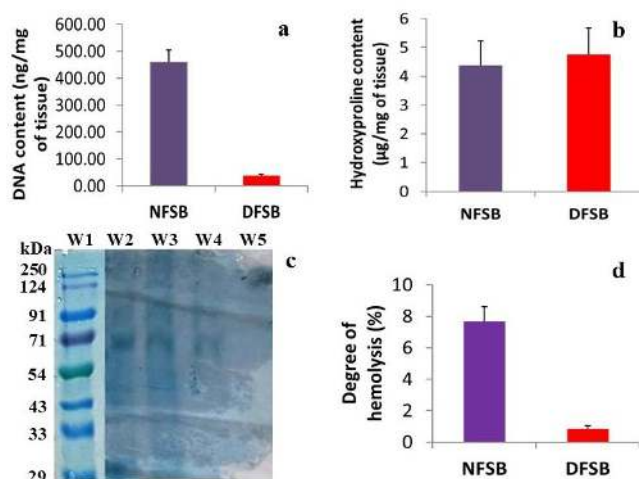


Figure 3: Characterization of DFSB: Quantification of residual DNA (a), Quantification of hydroxyproline (b), SDS-PAGE of fish swim bladder: Lane W1, Marker; Lane W2, NFSB; Lane W3, NFSB processed in 5% SPE for 24hours; Lane W4, 48 hours; Lane W5, 72 hours (DFSB) (c) and degree of hemolysis (d) p < .05 is considered significant

2 a-d. At 72 hours, the DFSB tissue sections were devoid of nuclei and cellular debris while retaining ECM architecture. The collagen fiber distribution was looser with good porosity. Exhaustive use of chemicals/reagents/enzymes may have a negative impact on the scaffold's quality. The presence of saponins (detergents) in *Sapindus mukorossi* might be responsible for FSB decellularization [19,20]. Surfactants disrupt lipid-protein and lipid-lipid interactions but usually leave protein-protein interactions intact, resulting in the proteins' functional conformations remaining unchanged [21].

DAPI staining

The DAPI stained native FSB tissues showed large number of spilled cold blue fluorescent nuclei (figure 2e). However, DFSB did not show any fluorescent nuclei (figure 2f). If the nuclear materials are not noticed in 4', 6-di-amidino-2-phenylindole, the tissue is supposed to be decellularized [22]. Combine treatment of swim bladders using DNase-I and electrophoresis; freezing-thawing and detergent, and freezing thawing and DNase-I treatments could effectively remove cells from the tissue [23].

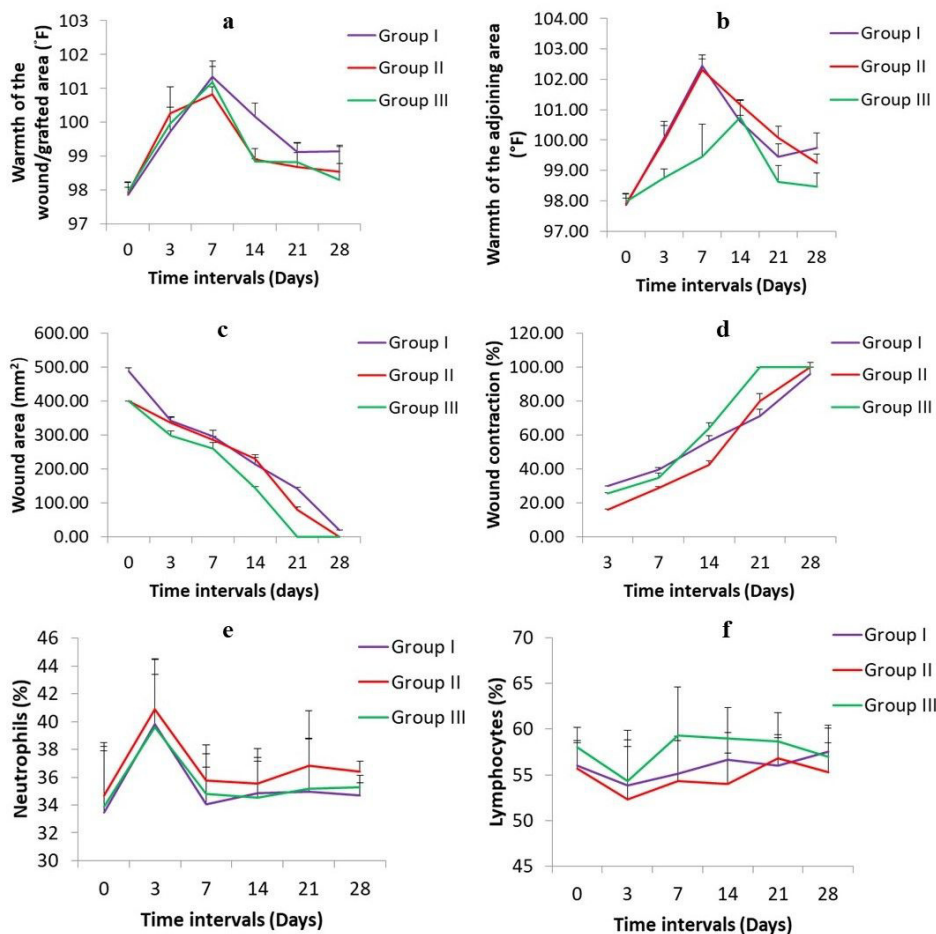


Figure 4: Evaluation of decellularized fish swim bladder for regeneration of full thickness skin defects in sham (I), autograft transplanted (II) and decellularized fish swim bladder transplanted (III) groups on days 0, 3, 7, 14, 21, and 28. Data are expressed as the mean $\pm$ standard error: Warmth at the wound / grafted area ($^{\circ}$ F) (a); Warmth at the adjoining area ($^{\circ}$ F) (b); Wound area (mm^2) (c); Wound contraction (%) (d) and Leukocyte count; Data are expressed as average percentage $\pm$ standard error (e,f)

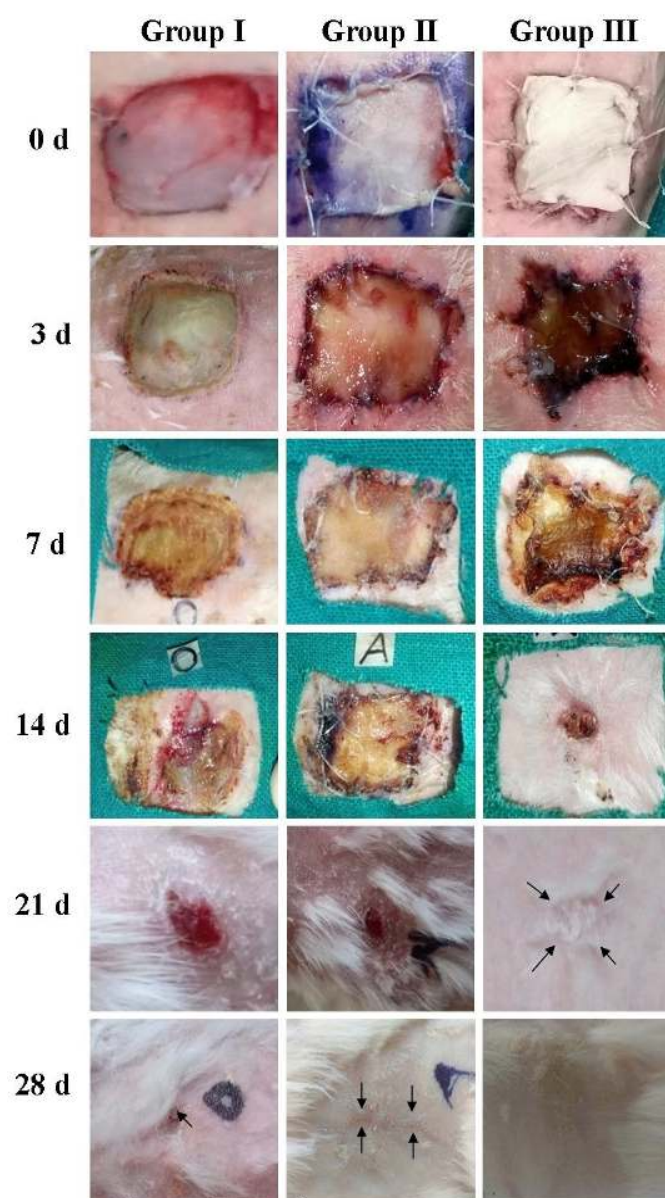


Figure 5: Representative images of full thickness skin wounds of sham (I), autograft transplanted (II), and decellularized fish swim bladder transplanted (III) groups on days 0, 3, 7, 14, 21 and 28

DNA quantification

Quantitative analysis of DNA content in DFSB (39.00 ± 2.74 ng/mg) revealed a significant decrease when compared to NFSB (460.17 ± 44.63 ng/mg) (figure 3a) which confirmed the histological and scanning electron microscopical observations. Adequate decellularization requires a DNA content of less than 50 ng/mg tissue [24]. Traces of DNA in the scaffold elicit an immune reaction in the recipient's body [25]. If the scaffold contains any residual antigens, such as DNA, MHC-I and the Gal epitope, it may lead to rejection of transplanted tissue [26].

Hydroxyproline contents

The free hydroxyproline contents in DFSB tissues decreased ($P > 0.05$) as compared to NFSB (figure 3b). This could be due to

removal of nuclear components from the native tissues during the process of decellularization. The hydroxyproline values indicate that the collagen obtained from the FSB is of high purity [27].

Molecular weight analysis

The electrophoretic pattern of the protein bands of NFSB and DFSB are shown in figure 3c. After decellularization process, the soluble protein decreased as revealed in SDS-PAGE of acellular matrix of samples. The protein band pattern of collagen in DFSB did not show any high protein band. The occurrence of the pattern of α , β and γ isomers of the fish collagen and no other major protein bands were observed indicating the nativity of the extracted fish collagen. Two α bands corresponding to $\alpha 1$ and $\alpha 2$, which were the unfolding polypeptide chains of the triple helix of the fish collagen [27]. The pattern of α , β and γ isomers of the fish collagen compares favorably with the electrophoretic pattern reported earlier in rohu fish [28].

Hemocompatibility determination

The extent to which erythrocytes are damaged when they come into contact with the scaffold is represented by the hemolysis ratio (HR). The hemolysis ratio of the NFSB and DFSB was 7.68 ± 0.92 and 0.84 ± 0.18 percent, respectively (figure 1d). A higher degree of hemolysis demonstrates that the biomaterial is not hemocompatible [29]. Hemolysis ratio of less than 5% for a scaffold indicates that it is highly hemocompatible [30]. Decellularized FSB induced very low level of hemolysis which corresponded to the basic requirement for implantable scaffold, indicating excellent hemocompatibility. The percent hemolysis caused by the decellularized FSB scaffolds prepared with 5% SPE was less than 1% as proposed by Li and coworkers [23]. Extremely low hemolysis by DFSB might be due to less immunogenicity of fish-sourced materials because fish do not contain many immunogens, such as alpha-gal [31].

Evaluation of DFSB scaffolds for regeneration of full thickness skin defects

Decellularized scaffolds are the common biomaterial used for tissue engineering purpose because they effectively promote tissue healing and regeneration [32]. Acellular swim bladder (ASB) is the fascinating biological membrane that could be used as a replacement material [23]. In the present study the DFSB scaffolds developed by treating in 5% SPE solution were evaluated for regeneration of full thickness skin wounds in New Zealand white rabbits.

Warmth

Mean $\pm$ SE values of the warmth ($^{\circ}$ F) at the site of wound/graft implanted site and adjoining areas at different time intervals in all the groups are presented in figure 4a and 4b. The warmth of the wound/graft area was significantly ($P < 0.05$) higher on day 28, 21 and 14 interval in group I, II and III, respectively. However, warmth of the adjoining area was significantly ($P < 0.05$) higher on day 21 interval in group I and II, and up to day 14 interval in group III animals. This increase in warmth may be either due to imbibition of angiogenic tissue within the graft or inflammation caused by surgical trauma. The vasodilation beneath the grafted tissue leads to increased blood flow resulting in increase in the temperature of the graft [33]. Contrary to this, significantly ($P < 0.05$) lower warmth after reconstruction of skin defects in rabbits may be due to a thick crust formation over the wound surface [34].

Wound area and percent contraction

Mean $\pm$ SE values of the wound area (mm^2) and total wound contraction (%) at different time intervals in various groups are

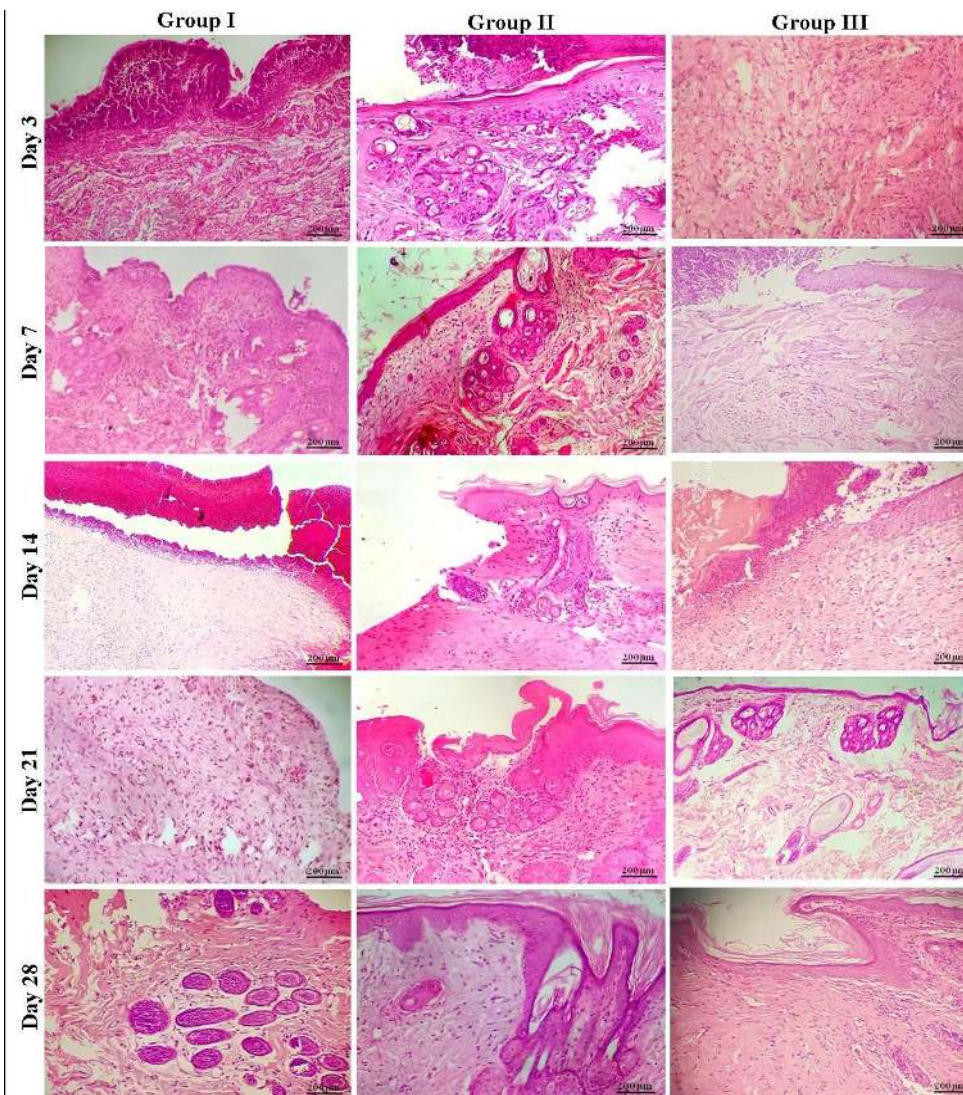


Figure 6: Representative micrographs of hematoxylin and eosine stained histologic wound sections of sham (I), autograft transplanted (II), and decellularized fish swim bladder transplanted (III) groups on days 3, 7, 14, 21 and 28 (x 40 magnification and scale bar 200µm)

presented in figure 4c and 4d, respectively. Surgical wounds appeared apparently healthy in all the animals of different groups and none of the wound showed any complication like infection or pus formation. On day 14 wound area was significantly ($P < 0.05$) decreased in all the groups but decrease was highest in group III animals and complete healing was observed on day 21 in group III. However, complete healing was recorded on day 28 in group II and the wounds of group I were still in the process of healing. Significant ($P < 0.05$) increase in percent contraction was observed in all the groups during the observation period. On day 14, maximum wound contraction (87.22%) was recorded in group III animals where decellularized fish swim bladder matrix was used. Wound contraction is caused by myofibroblasts that contain α -actin and is associated with contractile forces developed by granulation tissue [35]. Double layered skin construct showed significantly less wound contraction than the untreated wounds [36]. The fibroblasts embedded collagen scaffolds not only provide mechanical enforcement counteracting dermal contraction and thus prevented wound contraction [37]. Fibroblasts growth factor-2

(FGF₂) inhibited the change of fibroblasts to myofibroblasts, which is likely involved in contraction [38]. When cells are randomly attached to the scaffold the sum of the contracting forces are near zero [39]. Decreased contraction in group III might be due to acceptance of decellularized FSB matrix by the wound bed. The fibroblasts reduce the contraction of the wound and support collagen synthesis and neovascularization [40].

Hematological observations

A significant ($P < 0.05$) increase in neutrophil count and decrease in lymphocyte count was observed on postoperative day 3 in all the animals of different groups (figure 4e and f). The changes observed in the eosinophil and monocyte count were non-significant ($P > 0.05$). The values of DLC were found within physiological ranges on day 28 in all groups. The neutrophils act as the initial cellular defense of the body and increased neutrophil count is generally occurring during inflammation [41]. Neutrophilia for short duration revealed the impact of surgical trauma rather than the implant provoked response [42].

Table 1: Gross observation scores in animals of different groups at different time intervals

Parameter	Post-operative days														
	3			7			14			21			28		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Color of graft	3	2	4	3	4	4	2	2	1	1	1	-	1	-	-
Dryness of graft	3	2	2	4	2	1	4	3	1	1	1	-	2	1	1
Wound margins	2	1	1	2	2	1	2	2	1	1	1	1	-	-	-
Sloughing of graft	1	1	1	1	2	2	1	2	1	3	2	1	-	-	-
Surface granulation	3	1	1	3	1	1	2	2	1	1	1	1	-	-	-
Scar	1	1	1	1	1	1	1	1	1	1	1	1	4	2	1
Total	13	8	10	14	12	10	12	12	6	8	7	4	7	3	2

Color of graft: 1-Pink, 2-pinkish pale 3-Pale, 4-light brown, 5-Dark brown, 6-Black

Desiccation of graft: 1-Resembling normal skin, 2-slight, 3-Moderate, 4-Severe

Wound margins: 1-pink, 2-brown, 3-Black

Sloughing of graft: 1-Not sloughed, 2-Partially sloughed, 3-complete sloughed

Surface/marginal granulation: 1 -Mild, 2-Moderate, 3-Severe

Scar: 1-No scar, 2-Mild, 3-Moderate, 4-Severe

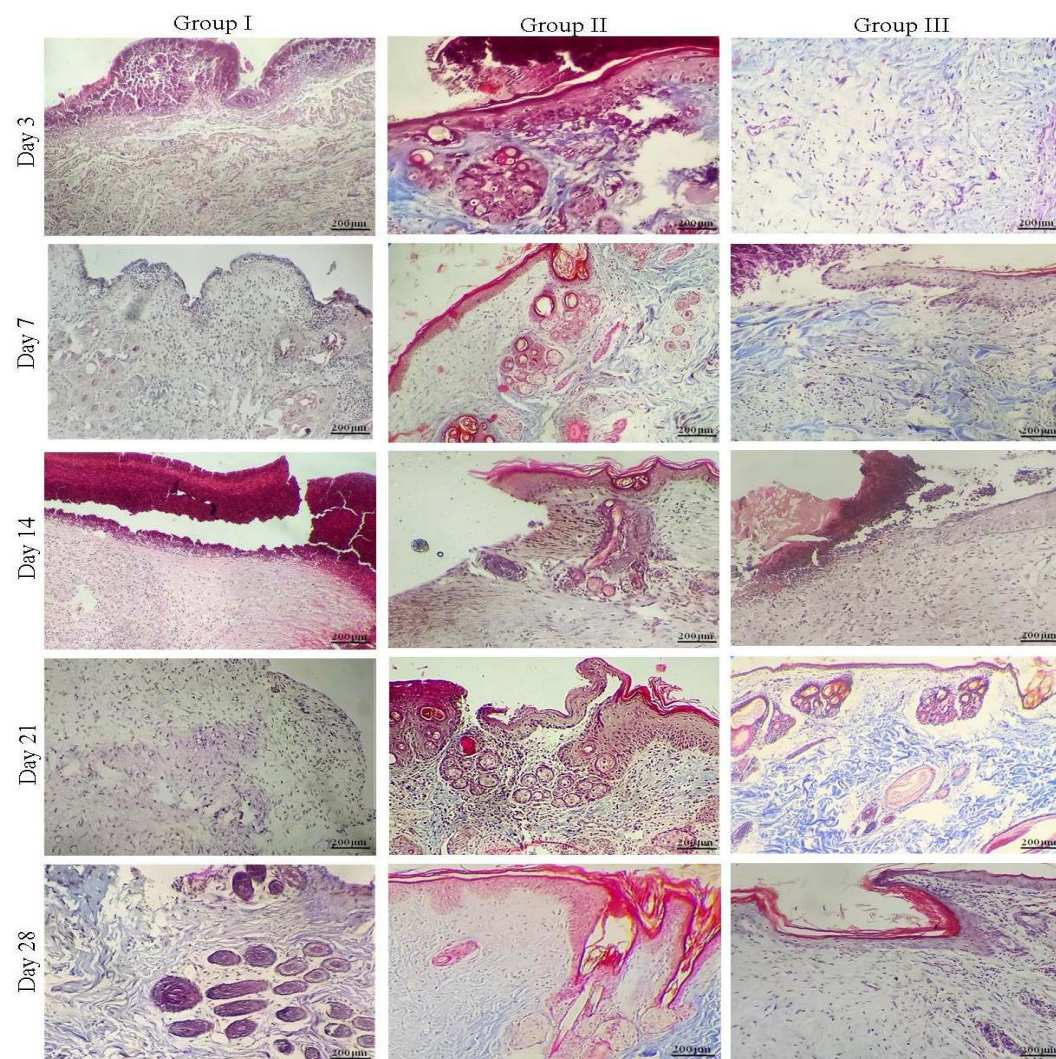


Figure 7: Representative micrographs of Masson's trichrome stained histologic wound sections of sham (I), autograft transplanted (II), and decellularized fish swim bladder transplanted (III) groups on days 3, 7, 14, 21 and 28 (x 40 magnification and scale bar 200µm)

Table 2: Histopathological scores of grafted sites in different groups of animals at different time intervals

Parameter	Post-operative days														
	3			7			14			21			28		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Epithelization	2	2	3	2	2	3	3	2	2	2	2	1	1	1	1
Inflammation	4	3	3	3	3	2	3	3	2	4	3	1	1	1	1
Fibroplasia	4	3	4	2	3	2	3	3	2	4	3	1	2	2	1
Neovascularization	3	3	3	1	2	2	2	2	1	4	3	1	1	1	1
Collagen fiber Density	2	2	2	3	2	2	2	2	1	2	1	1	1	1	1
Collagen fiber Thickness	2	3	3	3	2	2	2	2	1	2	3	2	2	2	2
Collagen fiber arrangement	2	2	3	4	3	1	2	2	2	2	2	1	2	1	1
Total	19	18	21	18	17	14	17	16	11	20	17	8	10	9	8

Epithelization: 1-Present, 2-Partially present, 3-Absent

Inflammation: 1-Resembling normal skin, 2-Mild, 3-Moderate, 4-Severe

Fibroplasia: 1-Resembling normal skin, 2-Mild, 3-Moderate, 4-Severe

Neovascularization: 1-Resembling normal skin (0-1 new blood vessel), 2-Mild (2-5), 3-Moderate (6-10), 4-Severe (> 10)

Collagen fiber density: 1-Denser, 2-Dense, 3-Less dense

Collagen fiber thickness: 1-Thicker, 2-Thick, 3-Thin

Collagen fiber arrangement: 1- Best arranged, 2-Better arranged, 3-Worse arranged, 4-Worst arranged

Gross observations

The representative images of different groups wound and gross scores are presented in figure 5 and in table 1. In group I, on day 3, the wounds were covered with soft and fragile yellowish white mass having mildly desiccated top surface. On day 7, the surface became yellow, more desiccated and necrosed. By day 14, thick crust developed with sloughing of some part. On day 21, the crust detached off leaving a raw granular pinkish tissue. On day 28, severe contraction was observed leaving a small unhealed area. In group II on day 3, the autograft appeared pinkish brown in colour and on day 7, the graft showed retraction of its margins. On day 14, the graft was firmly adhered with the underlying pinkish granulation tissue. On day 21, the autograft adhered more firmly with underneath tissue and the wound healed completely on day 28. In group III on day 3, the top layer of decellularized fish swim bladder graft became light brown in colour and the interface was marked with reddened border. On day 7, it got shrink slightly and seemed merged with the granulation tissue. On day 14, most of the part of the graft was absorbed exposing granulation tissue. By day 21, the wound healed completely and on day 28, the grafted area seemed similar to the normal skin with no scar at the site. Evaporation of moisture contents from the scaffold lead to its colour change and desiccation in due course of time [33]. The scaffold provides an environment that promotes neovascularization of the graft and fibroblasts play an active role in the replacement of matrix [43].

Histopathological observations

Histopathological observations of different groups at different time intervals are shown in figure 6 and 7 and their scores are depicted in table 2. On day 3, in group I, the upper layer showed dead and necrotic debris infiltrated with polymorph nuclear cells (PMNs). The underlying tissue showed fibroblasts proliferation with spilled neutrophils along with neovascularization. In group II, moderate inflammation and moderate fibroplasia with epithelium regeneration from the margins was evident. In group

III, the underlying acellular fish swim bladder scaffold showed infiltration of fibroblasts with angiogenesis. On day 7, in group I, the necrosed surface was detached from the underlying tissue. The granulation tissue had less dense, thin and worst arranged collagen fibers with infiltration of neutrophils. In group II, the changes were similar as on day 3. In group III, the collagen fibers were better arranged with moderate neovascularization. On days 14 in group I, epithelization was seen at the margins. In group II, inflammation was reduced and moderate numbers of new blood capillaries were noted. The collagen fibers were better arranged. In group III, the collagen of the graft was under the process of resorption with organization of granulation tissue. On day 21, in group I the denser collagen fibers were dense. In group II, the epithelization, started in skin adenexa and collagen fibers were sparsely arranged. In group III, epithelization was complete and fibroplasia resembled normal skin with mild neovascularization. On day 28 in group I, there was incomplete regeneration of the superficial epithelium. In group II, epithelization was complete with denser, thick and best arranged collagen fibers. Full thickness skin defect with more than 1 cm in diameter needs skin grafting or other alternative techniques to prevent excessive scar formation [44]. Collagen scaffolds enabled faster migration of cells involved in skin wound healing. It readily integrates with the wound tissue and facilitates the attachment, migration and proliferation of cells on the wound site [45]. On day 3, necrosis of the superficial surface and proliferation of fibroblasts in deep layer of acellular grafts was noted in group III. Acute inflammatory response showed infiltration of polymorphonuclear cells and subsequent loss of the graft material [46]. Degradation of extracellular matrix occurs by the enzymes namely matrixmetalloproteinases (MMPs) and through phagocytosis by macrophages [47]. The release of growth factors and cytokines by the neutrophils, macrophages and mast cells increases inflammation at the wound site which may slow down the regeneration process [48]. The proliferation and migration of epithelial cells are dependent on an adequate supply of oxygen. Vascularization of the scaffolds from the patient's wound bed normally takes about 3 weeks [49].

Conclusions

The results of the present study demonstrate that the FSB can be successfully decellularized using 5% *Sapindus mukorossi* fruit pericarp extract (SPE) while maintaining extracellular matrix integrity and microarchitecture. The decellularized scaffolds were found to be hemocompatible. Implantation of these scaffolds at the site of full-thickness skin wounds in rabbits provided early epithelialization, neovascularization, and collagen maturation. Overall, the results show that the decellularized FSB scaffold prepared using SPE has good potential for the reconstruction of full-thickness skin wounds.

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