RESEARCH ARTICLE

Preparation and Characterization of Decellularized Caprine Periosteum Scaffolds for Fracture Gap Healing

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Abstract

Certain chemical and enzymes like sodium deoxycholate, sodium dodecyl sulphate and Triton X-100 have been used as biological detergents but are responsible for residual cytotoxicity in the decellularized extracellular matrix. The periosteum plays a key role in bone regeneration. We aimed to prepare decellularized caprine periosteum scaffold by exploring the decellularization property of Sapindus mukorossi fruit pericarp extract (SPE). We developed decellularization protocols to completely remove the periosteum cellular components and for good maintenance of the hierarchical multilayer structures and components of the extracellular matrix (ECM) with no cytotoxicity. Histological analysis of hematoxylin and eosin and Masson's trichrome stained tissue samples decellularized by 5% SPE extract confirmed decellularization with preservation of extracellular matrix microarchitecture. DAPI stained decellularized tissues revealed complete removal of nuclear components, verified by DNA content measurement. It was concluded that 5% SPE is ideal for preparation of decellularized caprine periosteum scaffold and these scaffolds can be used for bone regeneration.

Keywords: Caprine periosteum; DAPI staining; Decellularization, Sapindus mukorossi.

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Introduction

Tissue engineering is a promising approach for bone regeneration. Decellularization of tissues is becoming a common technique to obtain extracellular matrix (Gangwar, 2006). It should be understood that every cell removal agent and method will alter ECM composition and cause some degree of ultra structure disruption. Minimization of these undesirable effects rather than complete avoidance is the objective of decellularization. Several researchers successfully decellularized periosteum and used it as a bio-scaffold to restore long bone defects of large size in rabbits (Chen *et al*., 2015; He *et al*., 2020; Zhao *et al*., 2021). Several methods of decellularization using toxic biological detergents and other chemicals have been described. But there is a need to replace the use of these cytotoxic detergents and other agents used for decellularization of tissues. The saponins present in Sapindus mukorossi fruit pericarp, an herbal detergent, have been used for decellularization of caprine aorta (Goyal *et al*., 2021). Critical micelle concentration (CMC) of Sapindus saponin is between 0.04 and 0.05 % wt/wt, much lesser than synthetic surfactants like, Triton X100 (0.13) and SDS (0.224) (Balakrishnan *et al*., 2006). Due to the low CMC value of Sapindus saponin, it could be further explored for potential applications as biodegradable surfactant in preparation of biological scaffolds. Classification standard of toxicity in ''Hygienic Standard for Cosmetics'', Ministry of Public Health of China, (2002) classified the sample as nontoxic and non irritant to dermal tissue. Caprine cadaver tissues may be considered as a safer source for preparation of collagen matrices (Goyal *et al*., 2021).

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Therefore, the present study was planned with the aim to analyze the efficacy of aqueous extract of the fruit pericarp of Sapindus mukorossi (an herbal detergent) for decellularization of periosteum tissues of caprine origin.

Materials and methods

Preparation of Sapindus mukorossi (Soap nut) fruit pericarp extract

Sapindus mukorossi fruit pericarp extract (5 %) was prepared from the fruit pericarp according to the protocol developed by Goyal *et al.* (2021).

Procurement and cleaning of caprine periosteum tissue sample.

The caprine femur bones were procured from the local slaughter house in chilled sterile phosphate buffered saline (PBS) containing antibiotic amikacin (0.1mg/mL) and proteolytic inhibitor (0.2025% EDTA). The periosteum was separated from these bones in the laboratory using periosteal elevator. The samples were rinsed and washed thoroughly with sterile phosphate buffered saline (PBS, pH 7.4) to remove all adherent muscle fibres, blood and debris.

Decellularization of native caprine periosteum tissue

The caprine periosteal tissues were cut into two pieces of 3.5 x 3 cm and processed in100 mL of 5% extract of soap nut pericarp over orbital stirrer at 4 g for 120 h. The tissue samples were collected for characterization at 24, 48, 72, 96 and 120 h time intervals and thoroughly washed in phosphate buffer saline (PBS) solution. The prepared decellularized biomaterial was stored in PBS solution containing 0.1% amikacin solution at 4°C to analyze the decellularization efficiency of soap nut pericarp extract.

Characterization of decellularized periosteum

Gross observations

Gross observations of samples (n = 6) were done by observing change in color, change in consistency, swelling and stiffness of washed samples as described by Gangwar *et al.* (2015).

Histology

For histological observations, tissue samples (n = 6) were collected in neutral buffered 10% formalin saline. Thereafter, the tissues were dehydrated in graded ethanol, transferred in xylene, embedded in paraffin and 5 micron thick paraffin sections were cut. All tissue sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome for microscopic evaluation as per the scoring system developed by Goyal *et al.* (2021).

DAPI (4, 6-diamidino-2-phenylindole 2Hcl) fluorescent staining

Native and decellularized periosteal samples were collected in neutral buffered 10% formalin saline and processed as for histological study. The sectioned tissues were loaded on the amino propyl triethoxy silane (APTES) coated slides, dewaxed in xylene, rehydrated in graded ethanol. The sections were stained with DAPI as per the method described by Goyal *et al.* (2022).

Scanning electron microscopy (SEM)

The native and decellularised periosteum samples were collected and fixed in freshly prepared karnovasky fixative at 4 °C. SEM was performed as per the procedure described by Goyal *et al.* (2022).

DNA extraction and quantification

Deoxyribo nucleic acid was extracted from native and decellularized caprine periosteum as per the protocol explained by Green and Sambrook (2017).

Estimation of collagen contents

The collagen content of native and decellularized periosteum was estimated as per the procedure of Reddy and Enwemeka (1996).

Statistical analysis

DNA and collagen content of native and decellularized samples was compared by independent sample t-test.

Results and discussion

Gross evaluation

Decellularization of periosteum tissues was evaluated by macroscopic examination. The native periosteum was non-transparent and reddish white in color (Fig.1a). The consistency of native caprine periosteum was soft and firm. When processed for 24 hours with 5% aqueous solution of soap nut pericarp extract (SPE), the tissues were slightly become loose and pale white in color. At 48 h the samples were found swollen due to absorption of water. The processed tissues start changing into milky white at 72 h interval. Thereafter, the consistency of the sample was slightly changed up to 120 hinterval (Fig.1b). At the end, the samples turned completely into milky white color and loose in consistency. It looks thicker and smaller after the decellularization processes. Macroscopic examination of decellularized periosteum tissues revealed milky white appearance which might be attributed to complete removal of cells. He *et al.* (2020) found that complete decellularization of periosteum, changes appearance of tissue from translucent white to milky white which is very much similar to our gross observations. Similar change in appearance was evidenced in aorta and esophagus after decellularization with 5% SPE (Goyal *et al.*, 2021; 2022). Zhang *et al.* (2017) also found that the color of the NTP (native tibial periosteum) and NCP (native calvarial periosteum) samples changed from red to milky white after the decellularization treatement with Triton X 100 (1% v/v) for 12 h and with 10 g/L sodium dodecyl sulfate solution for 2 h

Histology

Microscopic evaluation of native tissues and 5% SPE processed caprine periosteum scaffolds at different time intervals is presented in fig. 2 and histological scoring of decellularized periosteal scaffolds is shown in table 1. H&E stained native periosteal tissue showed the apparent layers composed of fibrous and cambium layer. The native periosteum showed abundant nucleus and collagen fibers. The collagen fibers were thick and compactly arranged in the

Table 1: Histological observations scoring after decellularization of caprine periosteum using 5% aqueous extract of soapnut pericarp.

S. No.	Parameters	Duration					
		Native	24h	48h	72h	96h	120h
1.	Cellular contents	+++	++	+	+	+	-
2.	Cellular debris	-	++	+	+	+	-
3.	Collagen fibers Arrangement	+++	++	++	+	+	-
4.	Porosity	-	+	+	++	++	+++

Table 2: Mean ± SE of DNA (µg/mg of wet tissue) in native and decellularized caprine femur periosteum

Tissue samples	DNA content (µg/mg of wet tissue)	
Caprine periosteum	Native	746.8 ±39.20a
	Decellularized	40.90 ±1.36 b

abdiffer significantly (P<0.05) from the decellularized tissues

Table 3: Mean±SE of collagen (µg/mg of wet tissue) present in native and decellularized caprine periosteum

Tissue samples	Collagen content (µg/mg wet tissue)	
Caprine periosteum	Native	4.82±0.24
	Decellularized	5.16±0.48

native tissue. At 24 h interval, the 5% SPE processed caprine periosteum scaffolds showed 70% to 80% decrease in the cellular contents. Cellular debris was moderately reduced. Collagen fibers were compact, and porosity of scaffold was moderate. At 48 h interval 80% to 85% of decellularization was taken place. Cellular debris was moderately reduced. Collagen fibers were moderately loose and moderate porosity was present. At 72 h interval 85% to 90% of decellularization was taken place. Cellular debris was mildly reduced. Collagen fiber was moderately loose with moderate porosity of the scaffold. Cellularity decreased up to 90-95% at 96 h interval cellular debris was mildly reduced. Collagen fibers were moderately loose, and porosity was also mild. At 120 h the scaffolds, showed distinct cambium layer and fibrous layer of the periosteum, this was well preserved at this time interval. Nucleus were absent in the tissues and there were no evidence of tissue cellularity and cellular debris which confirmed that complete decellularization has been achieved. The scaffolds were highly porous. The Masson trichrome staining of the native femur periosteum indicated that the ECM of periosteal was primarily made up of collagen fibers and bundles. In the fibrous layer, fibrocytes were distributed among the dense and thick fiber bundles. All the layers of periosteum were intact. Hematoxylin and eosin

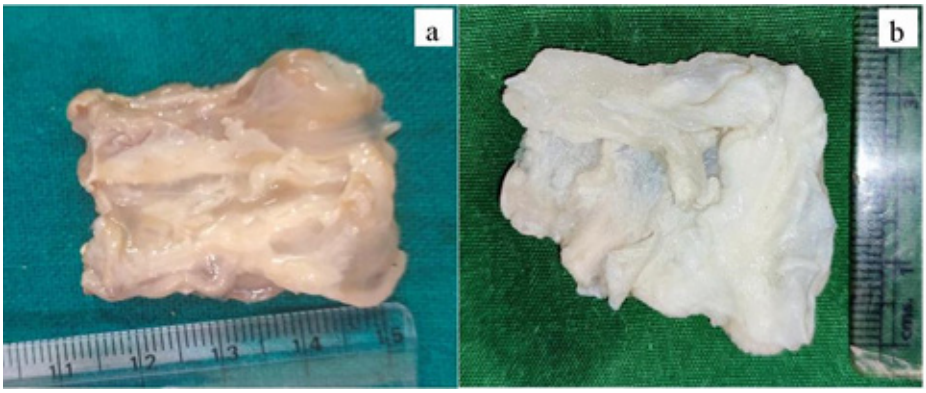

Fig. 1: Macroscopic observations of (a) native (b) decellularized caprine periosteum after processing in 5% aqueous extract of fruit pericarp of Sapindus mukorossi for 120 h

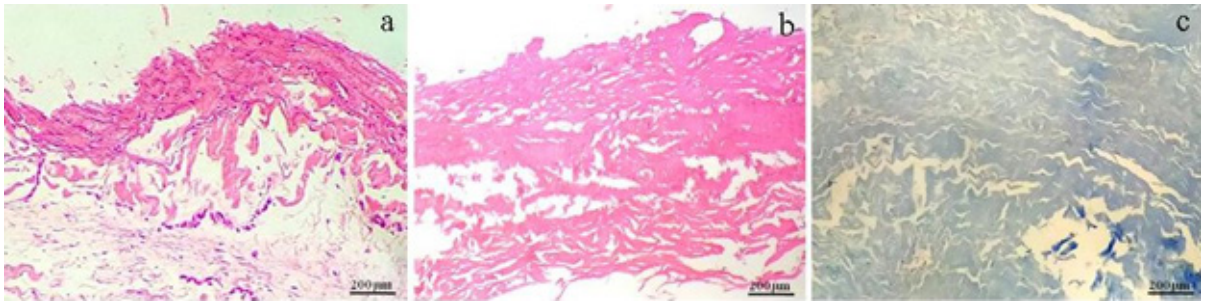

Fig. 2: Micrograph of (a) native (b) decellularized caprine periosteum after processing in 5% aqueous extract of fruit pericarp of Sapindus mukorossi for 120 h (H&E) and (c) decellularized caprine periosteum (Massons trichrome). Scale bar 200µm.

(H&E) staining of decellularized periosteum demonstrated a well reserving pink-staining collagen and a well reserving blue-staining collagen was noticed in Masson's trichrome stained decellularized periosteal tissues (Lin *et al.*, 2018). The integrity and continuity of collagen fibers in the decellularized tibial periosteum (DTP) were well arranged (Zhang *et al.*, 2017). There was no obvious difference in the ECM collagen structure and, distribution between FP (fresh periosteum) and AP (acellular periosteum) indicates that the decellularization process does not remarkably destroy the

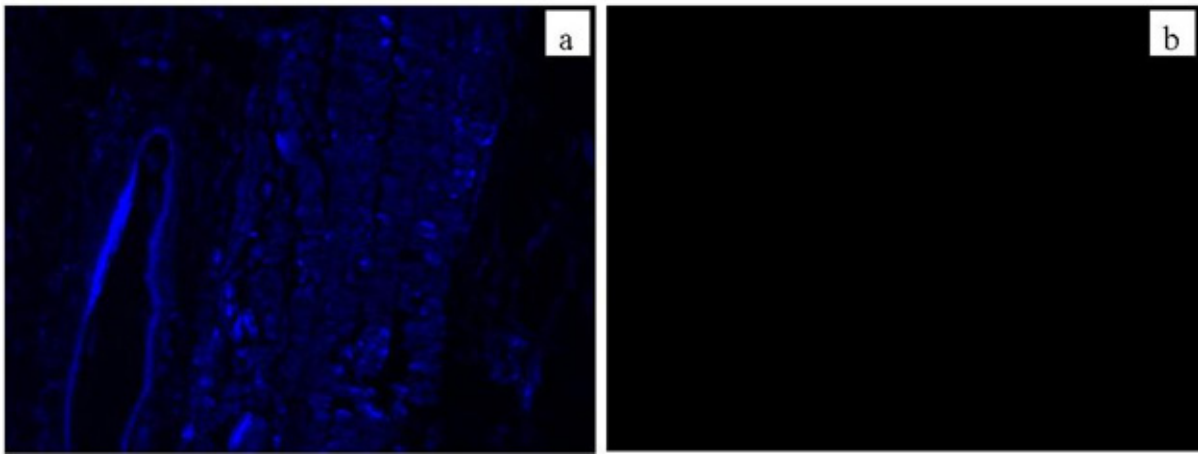

Fig.3: 4′,6-Diamidino-2-phenylindole (DAPI) staining of caprine periosteum (a) native and (b) decellularized after processing in 5% aqueous extract of fruit pericarp of Sapindus mukorossi for 120 h

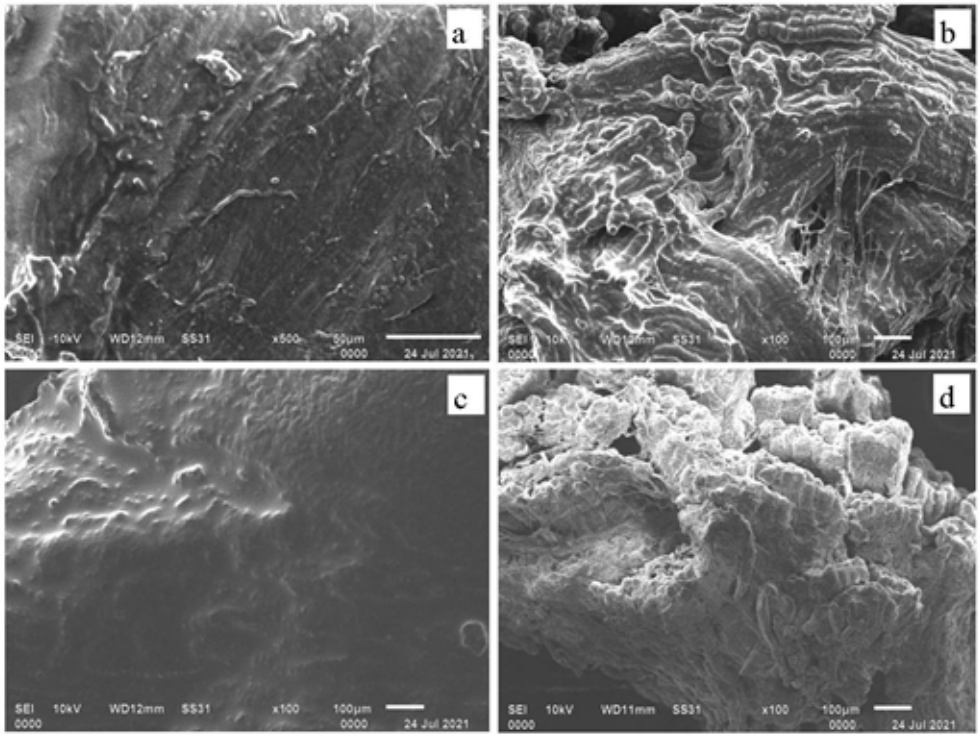

Fig4: Scanning electron microscopic ultrastructure of (a) cambium (inner) layer of native caprine femur periosteum (b) fibrous (outer) layer of native caprine femur periosteum (c) cambium (inner) layer of decellularized caprine femur periosteum and (d) fibrous (outer) layer of decellularized caprine femur periosteum.

collagen structure (He *et al.*, 2020).

DAPI fluorescent staining

Complete removal of nuclear components in periosteum matrices was also confirmed by DAPI fluorescent staining. DAPI stained native periosteum samples showed cold blue fluorescence which depicts the nuclear DNA (Fig. 3a). The soap nut pericarp extract processed tissues showed gradual decrease in cold blue fluorescence up to 96 h interval. However, the samples collected at 120 h were devoid of nuclear components (Fig. 3b). No fluorescence was observed in decellularized samples which confirmed that all nuclei material had washed away successfully to its maximum extent. DAPI staining of the tissues identify the presence of any residual intact nuclei because lack of visible nuclear material confirms the tissue decellularization. An absence of staining indicated the absence of DNA and thus cells in decellularized periosteum (Chen *et al.*, 2015). DAPI staining of decellularized tibial periosteum (DTP) also showed hardly no nuclear material remained after decellularization (Zhang *et al.*, 2017)

Scanning electron microscopy (SEM)

Representative microstructural images showing the cambium and fibrous layer of the native and decellularized periosteum are shown in fig. 4. In native periosteum outer

fibrous layer showed dense massive collagen bundles. The inner cambium layer was smoother. Decellularized layer of the periosteum had increased porosity as the gaps between the collagenous fiber bundles became a bit wider in fibrous layer of periosteum which was similar to the finding of He *et al.* (2020). Dense massive bundles of collagen were noted in the fibrous layer of the tibial periosteum at native stage (Zhang *et al.*, 2017). Increased porosity was due to washing out of most of cell components from their compartment leaving behind the collagen material. The assignment of collagenous fibers in periosteum became irregular in decellularized tissue. It may be probably due to the soaking effect of soap nut fruit pericarp extract. Optimal decellularization process should not harm the three dimensional ultrastructure and content of bioscaffold (Chalikias *et al* ., 2015). After decellularization, cells were removed and the loose and porous collagen scaffold remained. Chen *et al.* (2015) analyzed the ultrastructure of native and decellularized rabbit periosteum. The decellularized periosteum had an uneven and highly porous surface. It consists of long bundles of fibrils with a network showing collagen material. Decellularized periosteum had an irregular fibrous surface three dimensional architecture similar to the native architecture, and the collagen integrity remained unharmed. After decellularization, the collagen bundles became slightly loose (Zhang *et al.*, 2017).

DNA conetent

The Mean ± SE of DNA content (µg/mg of wet tissue) in native and decellularized caprine periosteum is showed in Table 2. Quantitative assessment of DNA in the decellularized tissues by spectrophotometry demonstrated a significant reduction of DNA content. Quantity of dsDNA in native and decellularized caprine periosteum was 746.8 ±39.20 and 40.90 ± 1.36 µg/mg of wet tissue, respectively. Therefore, the protocol adopted in present study fulfill the criteria of elimination of nuclear content and debris from the caprine femur periosteum by reducing the DNA below 50 µg/mg dry tissue (Crapo *et al.*, 2011). Decellularization process minimizes the DNA from the native tissues and acellular scaffolds can be implanted successfully in the recipients without any immune response (Syed *et al.*, 2014). Processing in SDS and Triton X-100 followed by treatment with DNAse and RNAse eliminated more than 95% of the nuclear material tibial periosteum ECM scaffolds (Zhang *et al.*, 2017). Decellularization of rabbit periosteum by three Freeze-thaw cycle followed by triton X-100, SDS and DNAse leads to significant elimination of the cellular structures and DNA content (Chen *et al.*, 2015). Decellularization of rabbit periosteum using freeze-thaw cycle followed by triton X-100, SDS and DNAse leads to reduction in the DNA content by more than 97% (Qiu *et al.*, 2020).

Estimation of collagen contents

The consequences of 5% SPE solution on collagen content was quantified in the decellularized periosteum scaffolds. The collagen contents of native and acellular periosteum is presented in Table 3. The collagen content in native and acellular periosteum was 4.82 ± 0.24 µg/ mg wet tissue and 5.16± 0.48 µg/mg wet tissues, respectively. Collagen quantification assay indicate no significant (P>0.05) difference in its content between native and decellularized caprine periosteum. Our observations corroborate with the reports of Totonelli *et al.* (2013). Slight increase in collagen content in decellularized periosteum tissue might be a relative increase because of removal of cellular content from the native tissues.

Conclusions

It was concluded that the 5% Sapindus mukorossi fruit pericarp extract decellularized the caprine femur periosteum at 120 h interval as evidenced by histological examination (H&E, fluorescent staining, SEM examination and DNA quantification of decellularized scaffold. These scaffolds could serve as a xenogenic biomaterial for reconstruction of critical size bone defects.

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