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Original Article

Dentistry Section

Decellularised Sheep Tendon Derived Extracellular Matrix: An In-vitro Biochemical and Histological Analysis

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ABSTRACT

Introduction: Demineralised ovine cartilage is derived from sheep cartilage with its mineral content removed, preserving the collagen-rich organic matrix. It provides an osteoconductive scaffold that supports cell attachment, proliferation, and differentiation. Its biocompatibility and structural similarity to human bone Extracellular Matrix (ECM) make it suitable for periodontal, promotes new bone formation and periodontal ligament attachment.

Aim: To fabricate decellularised sheep tendon derived ECM and to analyse Deoxyribonucleic Acid (DNA) and Glycosaminoglycan (GAG) content using histological Haematoxylin & Eosin (H&E), Alizarin Red and Alcian Blue staining and DNA quantification.

Materials and Methods: This in-vitro study was done in July 2024 at Saveetha Dental College and Hospitals in Chennai,Tamil Nadu, India. Fresh ovine/sheep tendon samples were obtained, cleaned of extraneous tissues, and stored at -20°C. They were then cut into small pieces (1.5×1.5 mm) using a 15-number size BP blade scalpel. The tendon fragments were submerged in a decellularisation fluid consisting of 10% Phosphate-Buffered Saline (PBS), Sodium Dodecyl Sulfate (SDS), and Triton-X. The mixture was shaken at 37°C until foam formed, with the froth being removed and the solution replenished every six hours for three days. After decellularisation, the tendon pieces were thoroughly washed with distilled water, freeze-dried, and the resulting ECM was refrigerated. Morphology of one sample was evaluated using a scanning electron microscope. Histological analysis was performed by haematoxylin & eosin, Alizarin Red and Alcian Blue staining to detect GAG content. DNA quantification was done to confirm the remaining DNA percentage.

Results: Scanning Electron Microscopy (SEM) image revealed that the ECM exhibited a fibrous shape with a high density of linked fibers. Haematoxylin and eosin staining revealed 90% decellularisation of the sheep tendon. Alizarin red staining showed that almost no mineral content remained after decellularisation. Alcian blue staining is used to detect the presence of GAG and shows that almost 90% of the GAG has been removed from the ovine sheep/tendon by decellularisation. This result is further substantiated by the DNA quantification which shows only around 10-15% DNA remaining in the decellularised tissue. The GAG quantification also gives similar observations which prove the almost complete removal of cells from the sheep tendon (100% naive tendon and 15% decellularised tendon).

Conclusion: Decellularised ovine/sheep tendon may prove as a useful material for tissue regeneration as it helps in collagen synthesis, combining effective decellularisation with the preservation of key ECM components. Further studies are needed to evaluate its potential use as a graft material and for various clinical applications.

Keywords: Bone regeneration, Periodontal attachment, Tissue engineering, Tissue regeneration

INTRODUCTION

Tissue engineering and regenerative medicine are two interrelated, multidisciplinary specialties that work to improve patients' health by encouraging remodeling and repair. Although the terms are frequently used interchangeably and grouped under the headings of regenerative medicine and tissue engineering, these two fields are not the same [1]. Regenerative medicine is a branch of medicine that uses in-vivo and ex-vivo methods to repair, regenerate, or replace damaged cells, tissues, or organs using endogenous healing [2]. It also makes use of scaffolds, growth factors, other signaling molecules, and/or gene manipulation. Scaffolds emulating genuine tissue microenvironments are essential for tissue engineering applications. The mechanical strength, architecture, and composition of the biomaterial in scaffolds all influence how well cells connect with the host tissue, how well they integrate, and how well they function during regeneration. Tissue engineering relies on three essential elements: scaffolds, signals, and cells. Scaffolds made of native biological tissue and organs are crucial for in-vivo tissue and organ regeneration [3]. Native ECM contains intrinsic signals that may promote cell adhesion, proliferation, and differentiation, as well as naturally occurring 3D scaffold structures that contribute in the process of tissue and organ regeneration [3]. Nevertheless, the synthetic scaffold lacks the cell type specific signal niches and scaffold structures specific to tissues and organs.

Scaffolds used for tissue engineering applications are made using a wide range of biomaterials, including synthetic material, natural derived and composite materials [4]. However, because of their advantages in terms of their biological qualities and other relevant biomechanical characteristics, natural biomaterials with ECM components are favored over artificial or synthetic ones. The biomaterial utilised to build the scaffolds is essential to tissue engineering, where scaffolds play a major role. By acting as a temporary support in lieu of the native ECM, though they are eventually replaced by newly regenerated tissue, these biomaterials, whether natural or synthetic, must be biocompatible, biodegradable, and possess qualities appropriate or favorable for the regeneration of the tissue type [5]. All tissues of living, multicellular animals contain the ECM in some capacity. A combination of proteoglycans, polysaccharides, glycoproteins, and proteins compose the ECM of all animals [6]. Plants employ cellulose, the most prevalent biopolymer on the planet, to construct their cell walls. Fungi and arthropods use chitin for their cell walls and exoskeletons.

Decellularisation of tendon is a promising technique in regenerative medicine, aiming to create scaffold materials that simulate the native ECM while removing cellular components. This process involves the

removal of all cellular content from tendon tissue, leaving behind a structurally intact and bioactive ECM [7]. The decellularised ECM provides an ideal scaffold for tissue regeneration, as it retains the natural composition, mechanical properties, and biological cues necessary for the development of functional cartilage in-vitro. The decellularisation process can be achieved by means of a variety of methods, such as physical technique, chemical methods and enzymatic techniques, each with its advantages and limitations [8]. Physical methods such as freeze-thaw cycles and high pressure treatments disrupt cell membranes through mechanical stress. Chemical methods involve the use of detergents (SDS) or solvents to solubilise cell membranes and intracellular components. Enzymatic methods utilise proteolytic enzymes (e.g., trypsin, dispase) to degrade cellular proteins. Often, a combination of these methods is employed to ensure the complete removal of cellular material while preserving the integrity of the ECM [9].

In periodontal therapy, regenerating the complex anatomy of the periodontium is challenging. Traditional methods like Guided Tissue Regeneration (GTR) are limited in predictability. Decellularised scaffolds, such as those derived from sheep tendons, offer promise for periodontal regeneration by mimicking the native ECM, promoting cell adhesion and differentiation, and reducing immune rejection [10].

This study evaluated decellularised sheep tendon derived ECM using, SEM for morphologic analysis and histological staining and DNA quantification.

MATERIALS AND METHODS

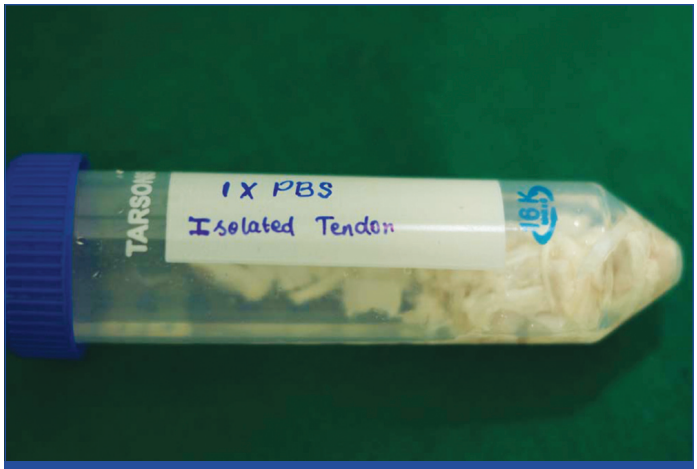
This in-vitro study was conducted at the Department of Biomaterials and Department of periodontology, Saveetha Dental College, Chennai, Tamil Nadu, India. Approval for the conduction of the study was obtained from the Scientific Review Board of Saveetha Dental College (SRB/SDC/PERIO-2201/24/402).

Study Procedure

To evaluate the potential of decellularised ovine/sheep tendon fresh ovine/sheep tendon samples were procured, cleaned of extraneous tissues, and stored at -20°C. The tendons were taken from an ovine/sheep carcass that was purchased from a nearby slaughterhouse when it is still sterile. Next, a 15-number size BP blade scalpel was used to cut the tendon into tiny pieces that are 1.5 mm x 1.5 mm in size [Table/Fig-1].

[Table/Fig-1]: Preparation of tendon fragments.

The tendon fragments were then submerged in decellularisation fluid (20 mL) and 10% PBS. To create the decellularisation solution, one gram of SDS and 200 µL of Triton-X to 100 mL of distilled water were added [Table/Fig-2]. For naive tendon, the decellularisation process is not done. The tendon sample was then mixed with 25 mL of the decellularisation solution and shaken at 37°C until foam forms. Every six hours for three days, the froth was removed and the decellularisation solution was replenished. Post demineralisation,



[Table/Fig-2]: Isolated tendon fragments.

the ovine/sheep tendon pieces were thoroughly washed with distilled water to remove residual agents and then freeze-dried. The resultant decellularised tendon ECM was refrigerated [11].

For an entire night, the ECM sample was fixed in a 4% paraformaldehyde solution. Following that, the ECM sample was dried using progressively higher ethanol baths (10%-100%). Following dehydration, the ECM sample was placed in aluminum stubs and then it was coated with gold using a sputter coater.

Morphology assessment: Next, a field emission scanning electron microscope (JEOL JSM-IT800 Tokyo, Japan) was used to analyse the ECM sample morphology (texture, appearance, porosity and structural integrity) [11]. Haematoxylin & Eosin staining were performed to evaluate the presence of nuclei in the tissues.

Mineralisation measurement: In order to measure matrix mineralisation, cells were frozen for a whole night at -20°C using 100% ethanol. Following a gentle washing with tap water, the fixed cells were stained with 50 µL of 0.5% w/v alizarin red solution (pH 4.0) and allowed to incubate for 30 minutes at room temperature. The cells were cleaned of extra Alizarin Red by running them under tap water. The dyed cells were imaged in a bright field using a microscope (Thermo Fisher Scientific, EVOS FL).

Glycosaminoglycan (GAG) content: The GAG content of the sheep tendon sample was ascertained using Alcian Blue staining (detects acidic epithelial and connective tissue mucins) [12]. Following fixation, the sample was stained for 30 minutes with an Alcian blue solution (Sigma-Aldrich), rinsed with deionised water, and cleaned for two minutes under running tap water. Next, the sample was stained for five minutes with 0.1% nuclear fast red (Sigma-Aldrich) and one minute with tap water.

For GAG content analysis, the freeze-dried sheep tendon was pulverised and digested in a papain digestion buffer at 60°C for 24 hours. The GAG content in the supernatant was quantified using a Dimethyl Methylene Blue (DMMB) dye colorimetric assay, with a chondroitin sulfate calibration curve to determine the concentration.

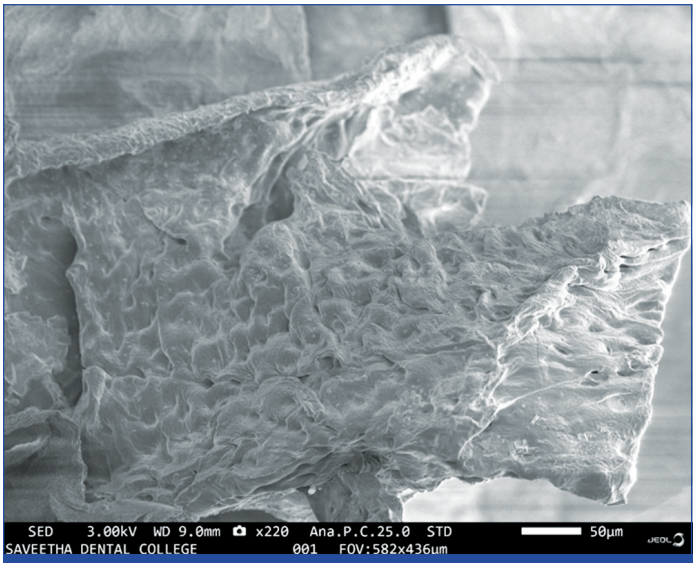
DNA analysis: DNA content analysis, DNA was extracted from the powdered, freeze-dried sheep tendon using a Qiagen DNeasy Blood and Tissue Kit (QIAGEN NV, Venlo, The Netherlands) following standard protocols. The PicoGreen dsDNA reagent was then used to quantify the isolated DNA.

STATISTICAL ANALYSIS

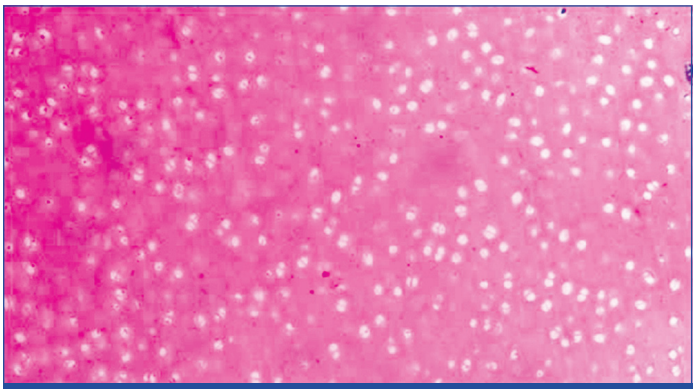
Descriptive statistics were used for analysis of samples.

RESULTS

The evaluation of the SEM image revealed that the ECM exhibited a rough and fibrous shape with a high density of linked fibers [Table/Fig-3]. Haematoxylin eosin staining showed 90% decellularisation of the ovine/sheep tendon [Table/Fig-4].



[Table/Fig-3]: Scanning electron microscope image of the decellularised sheep tendon (fibrous shape with a high density of linked fibers).

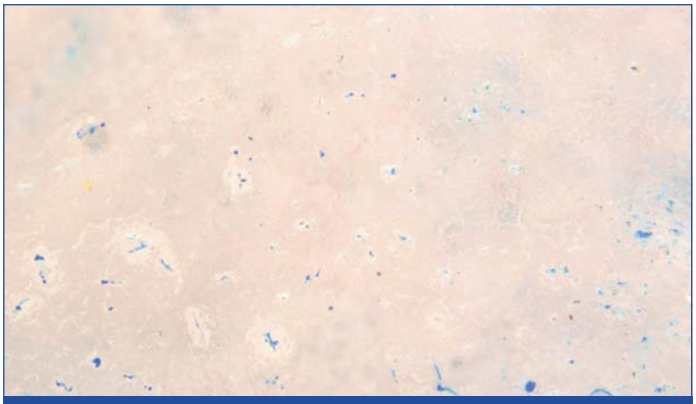


[Table/Fig-4]: Haematoxylin eosin staining (40x magnification), confirms that the decellularisation process has been effective, leaving only about 10% of the original cellular content (faint or sparse nuclei in the tissue).

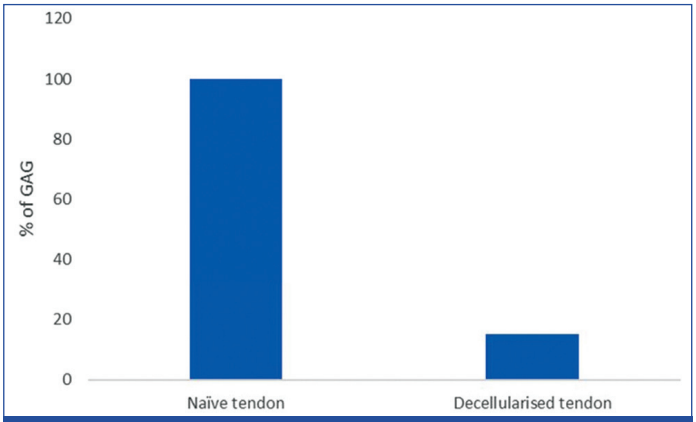
[Table/Fig-5]: Alizarin red staining (40 x magnification), The absence of significant red staining in the image indicates that there is little to no calcium or mineral content remaining in the sheep tendon after the decellularisation process.

Alizarin red staining was used to detect the presence of calcium compounds and showed that there was almost no mineral content remaining in the ovine/sheep tendon after decellularisation [Table/Fig-5].

Alcian blue staining was used to detect the presence of GAG and shows that almost 90% of the GAG has been removed from the ovine sheep/tendon by decellularisation [Table/Fig-6]. According to the results of the Alcian blue staining technique, GAG was eliminated because blue colour was no longer visible in the image following the decellularisation process. This result was further substantiated by the GAG quantification that also gave similar observations [Table/Fig-7] (100% naive tendon and 15% decellularised tendon). With a GAG content of 12.5±1.2 µg/mg, the material retained critical biochemical components. The decellularisation process resulted in a notable reduction in GAG concentrations.

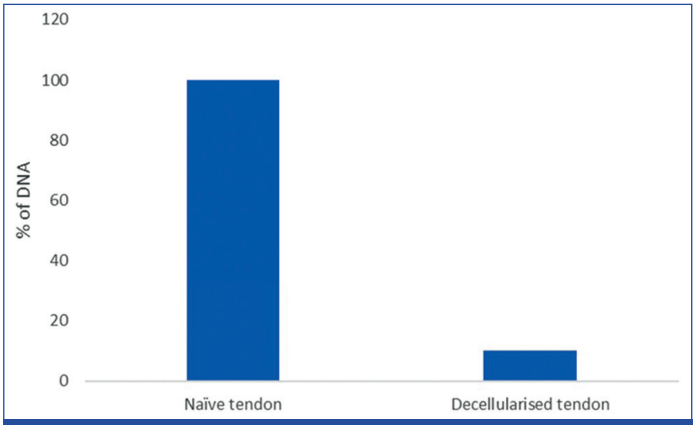


[Table/Fig-6]: Alcian blue staining (40x magnification), The blue staining in the image is relatively sparse, indicating that the majority of the GAG content has been removed from the sheep tendon during the decellularisation process.



[Table/Fig-7]: The decellularisation process effectively reduced the GAG content in the sheep tendon (100% naive tendon and 15% decellularised tendon).

The result was further substantiated by the DNA quantification which showed only around 10-15% DNA remaining in the decellularised tissue [Table/Fig-8].



[Table/Fig-8]: The decellularisation process effectively reduced the DNA content in the sheep tendon, ensuring minimal residual cellular material (0.05% concentration).

DISCUSSION

Periodontitis is a chronic inflammatory disease that results in loss of periodontal attachment. GTR is a popular therapeutic strategy that improves periodontal tissue regeneration utilising biodegradable membranes [13]. It is thought that GTR surgery facilitates the migration of periodontal ligament cells and the healing of the damaged periodontal tissues. The study of dental tissue regeneration and using bioactive dental materials has significantly increased in the last few years. In particular, it has been suggested that ECM can be applied to dental tissue engineering applications as a biologic scaffold, providing a workable solution for a variety of clinical applications. ECM contains a wide range of structural proteins that have been shown to affect cell function, including as collagen type I, GAGs, proteoglycans, and glycoproteins, in addition to cytokines and growth factors [14]. Numerous biologic peptides and

molecular components present in extracellular matrices have been studied for their potential to promote bone repair and epithelial tissue regeneration. Moreover, there is a great deal of species conservation in extracellular matrices. The importance of the ECM in maintaining cell balance and repairing tissues is highlighted by the similarities in ECM composition and conservation across different species [15].

From the H&E staining we see that there is almost 90% decellularisation of the ovine tendon. The Alizarin red stain which is used to detect the presence of calcium compounds shows that there is almost no mineral content remaining in the ovine tendon after decellularisation. The Alcian blue stain which is used to detect the presence of DNA shows that almost 90% of the DNA has been removed from the ovine tendon by decellularisation. As per findings the result is further substantiated by the DNA quantification which shows only around 10-15% DNA remaining in decellularised tissue. The preservation of GAGs indicates maintained structural integrity of the ECM, essential for tissue regeneration. Decellularised ovine tendon offers advantages over traditional graft materials, such as reduced donor site morbidity, consistent supply, biocompatibility, and osteoconductive properties, making it a promising alternative for clinical application. The ECM, produced by eliminating all cells and cellular components, can be utilised in hydrogel scaffolds for regenerative therapies aimed at treating injuries or tissue damage [11].

A previous study by He J et al., aimed to create an artificial sheep periosteal material and investigate its possible use in bone regeneration. A modified decellularisation process was used to collect and decellularise the sheep periosteum. DNA quantification, agarose gel electrophoresis, and haematoxylin and eosin and phenylindole staining were used to demonstrate the efficacy of cell elimination. After decellularisation, the microstructure showed increased porosity, but the collagen fibers remained intact and the levels of GAG and collagen were not significantly decreased. The results of the biomechanical investigation demonstrated a considerable drop in the elastic modulus but no change in the yield stress, most likely as a result of the collagen's integrality. The acellular periosteum not only had no harmful effects on the MC3T3-E1 cells, but also somewhat promoted cell proliferation, according to an in-vitro investigation using the CCK-8 assay. Using a rabbit cranium model, an in-vivo directed bone regeneration experiment was conducted [16]. The periosteum without cells effectively prevented fibrous connective tissues from growing in and might have also enhanced bone healing, according to the micro-CT and histological data. In conclusion, acellular sheep periostea would have a lot of potential for directed bone regeneration around tooth and dental implant site due to its larger sources, lower costs, and easier manufacturing procedure.

Any decellularisation protocol's primary objective is to eliminate all cellular material without negatively impacting the residual ECM's composition, mechanical characteristics, or biological activity. In addition to collagen, the network of elastic fibers that makes up the pericardium's ECM gives the tissue flexibility. In another study, a unique methodology was used wherein numerous phases of heat shock, detergent washes, and alcohol dehydration was done to decellularise and delipidise bovine bone. Compared to the other three techniques examined in this study, the novel protocol exhibited higher biocompatibility and is more successful in removing cellular debris [17]. Moreover, histological and morphological examinations verify the preservation of an entire ECM in bone. Experiments conducted in-vitro and in-vivo demonstrate the osteoinductive and osteoconductive capabilities of the ECM scaffold that was created. Two techniques for decellularising the pericardium of cows are contrasted. Compared to the freeze/thaw procedure, the osmotic shock-based protocol yields superior outcomes in terms of cell component removal, biocompatibility, preservation of original ECM structure, and host tissue reactivity. Overall, the study's findings show how to characterise a novel protocol for decellularising cow bone for use as a bone graft and how to acquire a technique for creating pericardium membranes appropriate for GBR applications. These findings are similar to our present study.

In order to facilitate the healing of all periodontal tissues, biomaterials utilised in periodontal tissue engineering should be able to mimic the complex structure and composition of the periodontium. Alveolar bone regeneration remains extremely challenging, particularly in individuals with unequal socket injury and extensive bone loss. For full periodontal remodeling, new bioactive scaffolds are needed, which will serve as substitutes for current therapies [18]. Recently, ECM generated from a decellularised tendon has been studied as a possible source of complex compounds that promote osteogenic activity and cell proliferation [19]. The potential of CQ's herbal component in combination with cell-derived ECM to support periodontal regeneration has not received significant attention. Hydrogel scaffolds containing hyaluronic acid, cissus quadrangularis and ovine tendon derived ECM was evaluated in a study for periodontal regeneration. Using SEM, the samples' morphology was examined. There was just minimal hydrogel edema seen. The cells were discovered to be alive and exhibited good tenogenesis and differentiation. The produced hydrogels exhibited good characteristics, and optimal compression levels were noted [20]. In another in-vitro study [11], the researchers wanted to see if adding an extract from a natural product Ocimum sanctum to the ECM of the tendon could improve the regenerative ability of PDLSCs grown in hyaluronic acid scaffolds. The tenogenesis assay was employed to verify the existence of developed tendon cells, while SEM, swelling, compatibility, compression, and differentiation analyses were utilised to assess the scaffolds' structural and organisational qualities, as well as their viability and biocompatibility. PDLSCs exposed to Ocimum sanctum showed increased tenogenic activity as indicated by picrosirius red staining, which quantifies absorbance at 540 nm (OD value). The sample contains hyaluronic acid, the tendon's ECM, and Ocimum sanctum, which, with an OD of around 0.4 at 540 nm, produces the highest amount of tendon cells. When they compared the results, they found that the OD value has increased to over 1.5 times due to the inclusion of Ocimum sanctum. This demonstrates increasing the tenogenic properties of the natural substance Ocimum sanctum to the ovine tendon component of the hydrogel scaffold. The findings of these two studies are similar to our novel study.

Periodontal Ligament Stem Cells (PDLSCs) cultivated on ECM based membrane which was derived from urinary bladder of pigs were compared to those cultured on type I collagen membrane, a scaffold that is frequently employed in guided periodontal tissue regeneration, in terms of viability, proliferation, apoptosis, and migration in an in-vitro study by Wang Y et al., [21]. The study evaluated how PDLSCs adhere and differentiate into cementoblasts/osteoblasts, comparing the effects of ECM and type I collagen on their tissue-regenerating abilities. ECM membrane increases PDLSCs' capacity for proliferation and regeneration, suggesting that it may be used as an appropriate scaffold when GTR is applied to treat periodontal disease In this study porcine sources were used but we have used ovine tendon in our novel study, which makes a new hope to treat periodontal diseases. The ECM obtained by the removal of all cells and cellular contents can be employed in hydrogel and lyophilised scaffolds in regenerative periodontal therapy for cases of vertical bone defects and furcation management.

Since this is a preliminary in-vitro study, further investigation and experiments need to be conducted in-vivo to know the safety and efficacy of using ECM ovine/sheep tendon-based scaffolds for periodontal bone regeneration.

Limitation(s)

Biocompatibility assays should be evaluated. More studies are needed to evaluate the stability and the ability to integrate with host tissue.

CONCLUSION(S)

Decellularised ovine/sheep tendon can be a patential grafting material, combining effective decellularisation with the preservation of key ECM components. It helps in collagen synthesis, combining effective decellularisation with the preservation of key ECM components. Future studies are required to evaluate its potential as a graft material and other clinical applications.

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