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Research Article

EVALUATION OF DECELLULARISATION PROTOCOLS FOR DEVELOPING EXTRACELLULAR MATRIX BASED PORCINE TUNICA VAGINALIS SCAFFOLDS FOR TISSUE ENGINEERING APPLICATIONS

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ABSTRACT: The relevance of biomaterials to successfully restore the function of lost tissues to improve human health is increasing. There is a need to develop new biocompatible, low-cost biomaterial with better strength that can perform the intended purpose. Porcine tunica vaginalis, - a readily available abattoir bio-waste from slaughterhouses and being an expansion of the peritoneum into the scrotum, can be considered a candidate scaffold. A study was carried out to examine the *in-vitro* characteristics of variably decellularised porcine tunica vaginalis. The native or non-decellularised tissue was maintained as a control group (PTV). The varied protocols for decellularisation included (a) Trypsin-EDTA-Triton-X-100 treated tunica vaginalis (DTV1) (b) Sodium deoxycholate (SDC) and DNase treated porcine tunica vaginalis (DTV2) and (c) a combined protocol (DTV3) for decellularising porcine tunica vaginalis. The efficiency of decellularisation was evaluated by histological examination, DNA quantification, and 4, 6- diamidino-2-phenylindole (DAPI) nuclear staining. The biomechanical properties of the materials were examined by analyzing tensile strength, maximum load at the break, and Young's modulus. Further, the surface and other structural characteristics were analyzed using scanning electron microscopy and special staining techniques. The concentration of DNA, nuclear staining, and surface characters was in an acceptable range after decellularization for all three scaffolds. All decellularisation protocols reduced DNA concentration significantly compared to the non-decellularised control and retained collagen and other extracellular matrices (ECM) components. However, the DTV3 scaffold revealed minimal nuclear remnants, acceptable DNA concentration as well as good surface topography. Comparison of biomechanical characters of the decellularised scaffolds with non-decellularised tissue evidenced better tensile strength and Young's modulus for DTV3. This work highlights the influence of different decellularisation protocols on the final scaffolds which could pave the way for developing a novel scaffold that can address various clinical problems in the medical field.

Key words: Biomaterials, Biocompatibility, Biomechanical, Porcine tunica vaginalis, Decellularisation.

INTRODUCTION

The extracellular matrix-derived bio-scaffolds gain significant interest in tissue regeneration due to their versatile structural and functional components over synthetic scaffolds in recent years (Yi *et al.* 2017). Synthetic materials fail to provide a biocompatible microenvironment with similar biomechanical properties to native tissue. Additionally, organ transplantation poses serious health risks associated with foreign body responses elicited by the host immune system (Anastasya *et al.* 2022). Moreover, there is a deficit between donors and patients requiring a new organ over the past few years (Balakrishnan-Nair *et al.* 2019). Decellularisation is a process where the cellular as well as nuclear remnants of the tissue/ organ of interest are removed using a variety of methods such as chemical, physical, or biological to obtain a decellularised extracellular matrix, a framework that contained extracellular macromolecules such as collagen, elastin, fibronectin, laminin, and matricellular proteins (Zhang *et al.* 2022, Singh *et al.* 2022). Properly decellularised scaffolds can act as a three-dimensional framework for host immune cells to infiltrate, proliferate, attach, and migrate to create an optimal environment for regaining tissue and organ loss and function. Additionally,

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there is a lack of better-standardized protocols to obtain decellularised scaffolds for regenerative medical applications. Extensive research has been continuing to optimize the decellularisation protocols for cell removal (Tomlins 2016). Meanwhile, the biochemical and biomechanical properties of the decellularised tissue can be altered after the process. Therefore, before being used therapeutically, these decellularised scaffolds must first be tested for various *in-vitro* parameters. The choice of a decellularisation method depends on the nature of the tissue and the efficiency of which need to be analyzed in terms of DNA content, morphology, and mechanical properties (Badylak and Gilbert 2008).

Porcine tunica vaginalis being an extension of the peritoneal layer into the scrotum through the inguinal canal can be considered as a candidate scaffold in regenerative medicine. Decellularised extracellular matrices are being used for rebuilding tissue and organ loss. The decellularisation process is a relatively new technique where most of the procedures are largely non-standardized (Laker *et al.* 2020). However, tissue engineering using ECM-based tissues has shown great clinical potential for organ reconstruction, but efficient removal of cellular remnants from tissues and maintaining normal structure and strength remain great challenges. Therefore, this study aimed to differentially decellularise porcine tunica vaginalis tissue and evaluate the efficiency of decellularisation protocols in removing cellular remnants as well as testing their biomechanical properties and structural characterization to translate this biomaterial for future medical applications.

MATERIALS AND METHODS

Preparation of scaffolds

Fresh porcine tunica vaginalis were collected from Meat Technology Unit, Kerala Veterinary and Animal Sciences University, Mannuthy, India at the time of slaughter, after careful separation from the testes of three adult healthy pigs of the Large White Yorkshire breed weighing around 90-150 kgs. The samples were cleaned with normal saline and the adhering connective tissue and fat were removed by manual stripping for developing the scaffolds. Chemical defatting was performed by immersing the samples in chloroform and methanol (2:1 v/v) overnight for 12 hours. The materials used in the study are as follows;

Non-decllularised control/native porcine tunica vaginalis (PTV)

The defatted parietal tunica vaginalis were rinsed in 1X phosphate-buffered saline (PBS) and dried under laminar air flow overnight. This scaffold was then stored at four degrees (Fig.1a) and categorized as native porcine tunica vaginalis (PTV).

Decellularisation using Trypsin-EDTA-Triton-X-100 (DTV1)

The procedure of decellularisation was followed according to Abhin (2021). The defatted materials were rinsed with deionized water in an incubator shaker at 120 rpm for an hour. Initial detergent washes were applied using one percent Triton-X-100 solution for 30 minutes at room temperature to remove the solvent. Samples were then incubated at room temperature in a solution containing 0.05 percent Trypsin and 0.02 percent EDTA for 6 h in an incubator shaker at 150 rpm for decellularisation. Final rinsing was done with deionized water continuously to remove the enzyme followed by drying under laminar airflow and stored at four degrees until further use (Fig.1b).

Decellularisation using sodium deoxycholate (SDC) and deoxyribonuclease (DNase) (DTV2)

The defatted materials were rinsed with deionized water in an incubator shaker at 120 rpm for an hour. Decellularisation was done using one percent of the detergent sodium deoxycholate (SDC) for 24 hrs in an incubator shaker (Simsa *et al.* 2021). The SDC solution was changed twice daily. The tissue was washed for 4–5 hrs in water and 125 mL 5kU/mL DNase was added for 12 hrs without shaking. The final rinse was done with phosphate buffer saline (PBS) (1X concentration) and stored at four degrees until further use (Fig.1c).

Combined decellularisation protocol (DTV3)

The defatted materials were rinsed with deionized water in an incubator shaker at 120 rpm for an hour. The initial detergent wash was done using one percent Triton-X-100 solution for 30 minutes at room temperature to remove solvents. Samples were then incubated at room temperature in a solution containing 0.05 percent Trypsin and 0.02 percent EDTA for 4hrs in an incubator shaker at 150 rpm. After rinsing with deionized water at 150 rpm for an hour, the tissue samples were treated with one percent SDC for 4 hrs at 150 rpm. Finally, the tissue was incubated in 1kU/mL DNase for 6 hrs at room temperature, was rinsed with 1X PBS, and stored at four degrees until further use (Fig.1d).

Efficiency of decellularisation

Histological analysis using hematoxylin and eosin (H&E) method

Representative samples of PTV, DTV1, DTV2, and

DTV3 were fixed, dehydrated, paraffin-embedded, and sectioned at 5 µm thickness. Sections were stained with H&E according to Suvarna *et al.* (2018).

Quantification of genomic DNA

The samples of native tissue and decellularised scaffolds were subjected to DNA extraction.

Genomic DNA content of tunica vaginalis, before and after decellularisation was quantified by using DNeasy Blood & Tissue Kit (QIAGEN, Germany. Catalog number: 69504). Total DNA concentration was determined with a spectrophotometer at λ =260 nm (Nanodrop 1000, Techno Scientific, CELBIO, Milan, Italy), and the mean value recorded for each sample was expressed as ng/mg of dry tissue.

4, 6- diamidino-2-phenylindole (DAPI) - nuclear staining

The efficiency of decellularisation was further validated using a modified DAPI staining procedure (Xing *et al.* 2015). DAPI stock solution at a concentration of 5 mg/mL was prepared by mixing 10 mg DAPI in 2 mL distilled water. The working solution was prepared by diluting 4 µL of the stock solution into 1 mL of distilled water. The decellularised tissue sections were deparaffinized using xylene and rehydrated through descending grades of alcohol. The sections were then washed in distilled water for 10 min. The sections were incubated in Mcllvaines buffer for 5 min. Mcllvaines buffer was prepared using citric acid and disodium hydrogen phosphate at pH 2.5. Subsequently, a DAPI working solution was added and allowed to act for 20 min. The excess solution was removed by dabbing the slide and drying it. The slide was mounted using glycerol and viewed under a fluorescent microscope with excitation/emission at 358/461 nm.

Biomaterial characterization

Mechanical properties

The biomechanical characterization of scaffolds was analyzed for uniaxial tensile strength. For this purpose, six strips of 5 mm x 50 mm samples were cut randomly from PTV, DTV1, DTV2, and DTV3. The thickness of the material at five different points was determined using a digital thickness gauge (Mitutoyo, Japan), and the mean thickness was calculated. The scaffold was subjected to a uniaxial tensile test using the tensile testing machine (Instron 3345, USA) to determine the tensile strength in Mega Pascal (MPa).

Young's modulus (MPa) and maximum load at break (N) were also determined using Instron 3345, (USA) at a full-scale load of 100 Newton (N) and at the cross-head speed of 10mm/min and calculated in Blue Hill-3 software. Tensile testing was done by stretching the tissues against a maximum force after specimens were fastened to grips (100 N). The tissues were stretched to the point where the fibers could no longer be stretched and eventually broken (Ashna *et al.* 2019).

Scanning Electron microscopy (SEM)

Surface characterization of the scaffold materials was carried out by observing the collagen fiber arrangement and pore size. For this, representative samples of PTV, DTV1, DTV2, and DTV3 scaffolds were fixed in a 2.5 percent glutaraldehyde solution for 48 hours (Ashna *et al.* 2019). The samples were then washed thrice with buffer (0.1 M PBS) for 10 min each. The samples were then dehydrated through ascending grades of alcohol (30%, 50%, 60%, 70%, 80%, 90%, and three changes of 100 % alcohol) for 45 min each. The samples were mounted on a metal stub having double-sided carbon adhesive tape after the completion of air drying of the samples. Later, the samples were sputter coated with a thin layer of gold and examined using a scanning electron microscope (Jeol JSM 6390LA) at an accelerated voltage of 20 kV.

Special staining

a) Picrosirius red staining method for collagen

Picrosirius red staining was done to observe the collagen staining pattern in the scaffolds as per Suvarna *et al.* (2018).

b) Masson's trichrome stain for collagen

Masson's trichrome staining for muscles and collagen fibers was done on formalin-fixed, paraffin-embedded sections (Suvarna *et al.* 2018).

Statistical analysis

Statistical analyses were performed with Statistical Product and Service Solutions software, version 24 (SPSS 24.0). Results of DNA quantification, tensile strength, strain at maximum load, and Young's modulus were analyzed by one-way ANOVA. A p-value less than 0.05 was considered statistically significant ($p<0.05$).

RESULTS AND DISCUSSION

The decellularised scaffolds grossly appeared as thin mild transparent membrane sheets with minute visible fibers (Fig.1). The histological features of differently decellularised tunica vaginalisscaffolds were obtained in H and E-stained sections. Megha *et al*. (2022) proposed that the most fundamental and simplest criterion for evaluating the effectiveness of decellularisation methods

Fig. 1. Porcine tunica vaginalis scaffolds developed under different protocols.

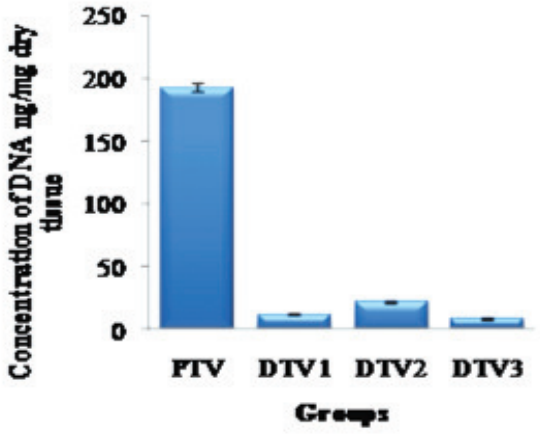


Fig. 2. Comparison of concentration of DNA in the PTV, DTV1, DTV2 and DTV3 scaffolds.

be the histological and ultrastructural characterization of ECM bioscaffolds. In the present study, the lack of cell nuclei with H&E confirmed the effectiveness of the decellularisation process (He *et al.* 2020). Nuclear remnants appeared blue and wavy collagen appeared pink in color. In comparison, nuclear remnants were highest in PTV. Traces of nuclear remnants were observed in DTV1 and DTV2 but not with the DTV3 scaffold (Fig. 3A). Trypsin can break the peptide bonds on the carboxyl side of arginine and lysine while Triton-X - 100, a non-ionic detergent is gentle and interfere with the interactions between lipids and lipid and protein aiding in nuclear removal (Zhang *et al.* 2022). The traces of nuclear remnants with the DTV1 scaffold could be attributed to the non-ionic detergents which are safer to the ECM, but their efficiency to remove nuclear remnants is limited and hence requiring supporting agents like enzymes are required for proper decellularisation. Further, our results could not confirm the findings of Simsa *et al.* (2021) but are similar to Laker *et al.* (2020) and highlight that not all detergent-nuclease mixtures could remove the traces of cellular remnants completely. The residual cellular components are primarily responsible for the adverse immune response that the xenogenic ECM scaffold can induce.

The samples of native tissue and decellularised scaffolds were further subjected to DNA quantification using a NanoDrop spectrophotometer. The obtained values of PTV, DTV1, DTV2, and DTV3 are compared in Fig. 2. The values between the decellularised groups were significantly different from each other. Higher DNA concentration was observed with PTV (192.62 ± 3.07 ng/mg). Among the decellularised matrices, the mean DNA concentration was found lowest with DTV3 (7.82 ± 0.37 ng/mg), followed by DTV1 (11.5 ± 0.62 ng/mg) and DTV2 (21.37 ± 0.77 ng/mg). Methods for assessing the effectiveness of the decellularisation process on tissues and entire organs were suggested by (Badylak and Gilbert 2008), wherein the concentration of ds DNA per mg of dry ECM weight is less than 50 ng which is considered an ideal decellularisation protocol. All three decellularization protocols were able to achieve the mentioned criteria indicating efficient decellularisation. The outcomes might not be unexpected given that trypsin is an enzyme that selectively cleaves cell-adhesive proteins to release cells from the tissue surface and Triton X-100 is a mild non-ionic detergent. SDC, on the other hand, is a harsher ionic detergent that can damage protein covalent bonds.

Furthermore, DAPI nuclear staining pattern validated these results. As per Xing *et al.* (2015), decellularisation efficiency could be assessed by DNA immunofluorescence (DAPI) labeling and DNA quantification, and it was found that the DAPI staining was compatible with the DNA quantification data. In the present study, the cellular debris appeared as fluorescent bright blue dots (Fig. 4). While PTV showed an abundance of nuclear fluorescence, traces of blue fluorescence were observed with DTV1 and DTV2 scaffolds. The specific fluorescence was absent or minimal with the DTV3 scaffold, indicating a successful decellularisation.

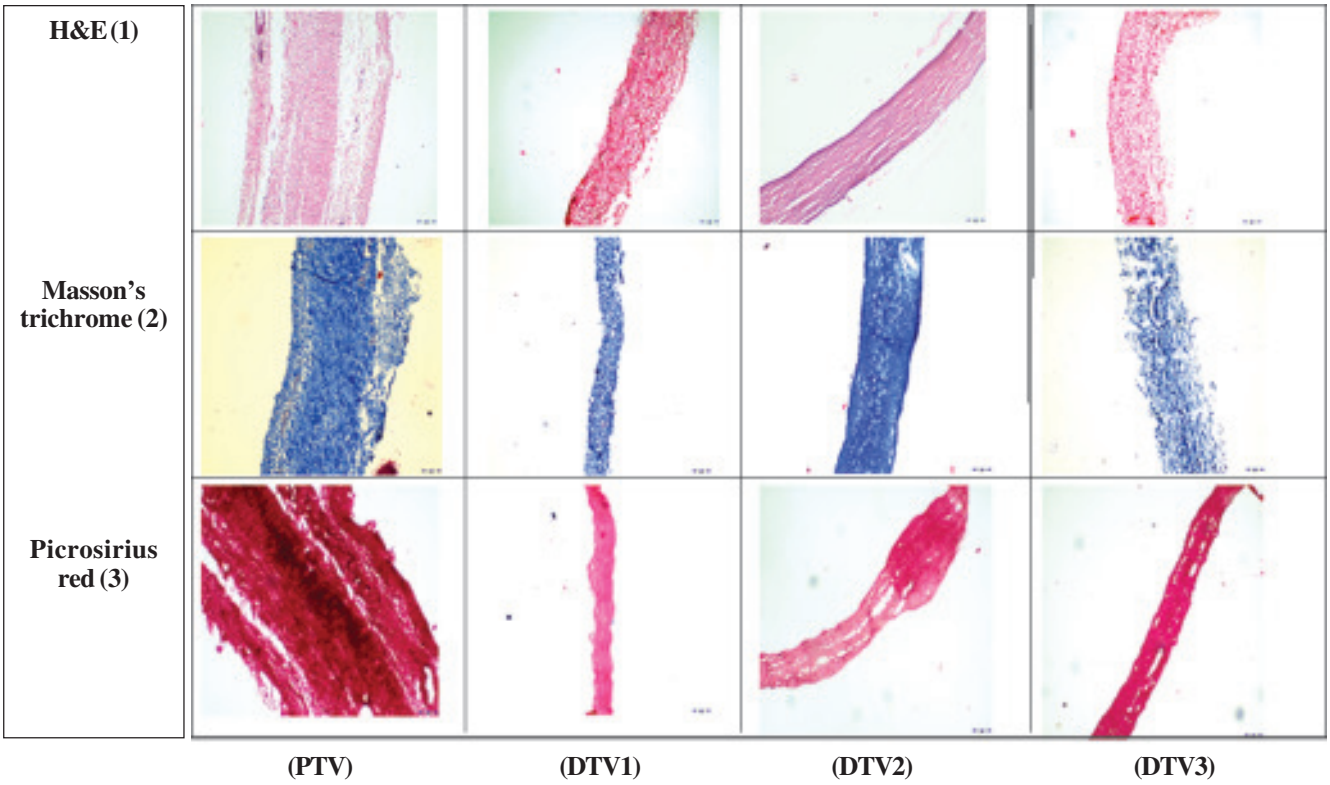


Fig. 3. Representative images of porcine tunica vaginalis scaffolds.
(1) Scaffolds in H&E-stained sections at x100. Abundance of cell contents could be observed in PTV. Traces of nuclear remnants noticed with DTV1 and DTV2 which was absent in DTV3. Scaffolds stained with Masson's trichrome stain, (2) Picrosirius red stain (3) at x100 magnification showing presence of collagen fibres.

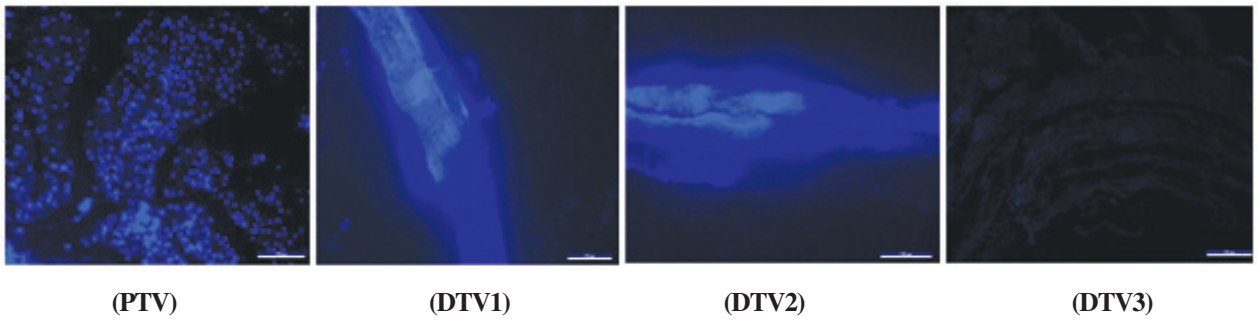


Fig. 4. Representative DAPI nuclear staining images.
(a)The extracellular matrix cell nuclei in PTV exhibited normal round shape and bright blue DAPI staining; Mild degree fluorescence of cellular debris in the DTV1 scaffold and DTV2; Complete absence of nuclear fluorescence in the DTV3 scaffold (DAPI x200).

The efficiency of decellularisation in the present study was evaluated by a battery of parameters because of the lack of universal standards to obtain complete decellularisation. The DNA content present within the decellularised scaffold forms a major parameter to be assessed (Choudhury *et al*. 2020). Given that cellular removal has been confirmed, the effects of decellularisation on the mechanical characteristics or morphology are to be analyzed.

Tensile strength represents the ability of the material to withstand a force and elongate (Ashna *et al.* 2019). For analyzing the tensile strength of PTV, DTV1, DTV2, and DTV3, the scaffolds were subjected to stretching by applying a constant force of 100 N. The tensile strength of PTV, DTV1, DTV2, and DTV3 scaffolds were 25.13 ± 3.16 MPa, 43.07 ± 12.72 MPa, 28.98 ± 12.79 MPa, and 67.95 ± 7.85 MPa respectively. The tensile strength of DTV3 and DTV1 scaffolds showed significantly higher

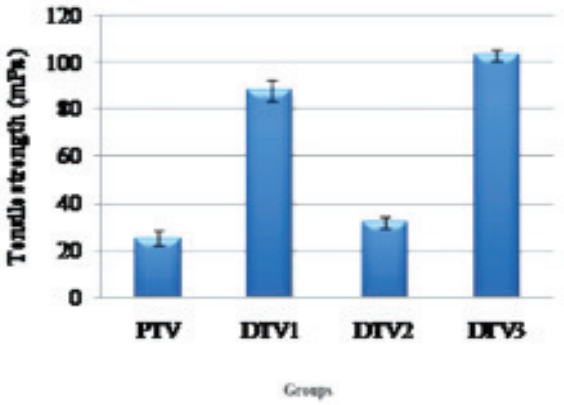


Fig. 5. Comparison of tensile strength of differently decellularised tunica vaginalis scaffolds.

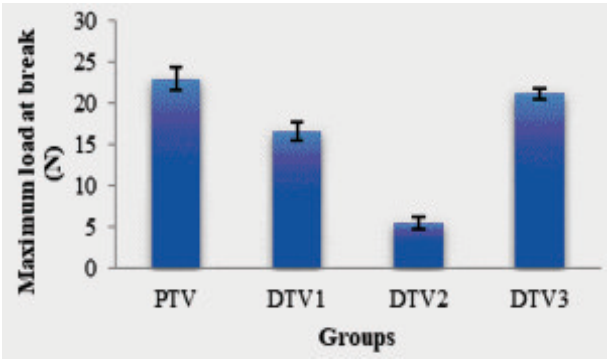


Fig. 6. Comparison of mean maximum load at break (N) of differentially decellularised tunica vaginalis scaffolds.

values than PTV and DTV2 (Fig. 5). A reduced tensile strength with the DTV2 scaffold could be attributed to the harsh detergent action of sodium deoxycholate on the elastin fibers by reducing their mechanical ability to withstand external force (Zhang *et al.* 2022).

The maximum load at the break of each scaffold was compared between groups (Fig. 4). However, PTV had a significant load-bearing capacity (23.02 ± 1.33 N). Among the decellularised groups, DTV3, DTV1, and DTV2 showed the load at break of 21.24 ± 1.68 N, 16.62 ± 7.08 N, and 5.56 ± 0.75 N respectively. From the data, it was clear that DTV3 could take up a maximum force followed by DTV1 and DTV2 (Fig. 6). A reduced load-bearing capacity of decellularised tissues from the native tissue was noticed which is similar to the findings of Williamse *et al.* (2020). Moreover, the findings of Hulsmann *et al.* (2012) showed that there was a significant reduction in the tensile strength of trypsin-EDTA-treated bovine pericardium from that of native tissue. Further, Suvaneeth *et al.* (2016) also concluded that deoxycholic acid-treated bovine pericardium had a higher tensile strength compared to the enzymatically treated pericardium.

The stiffness of the material is an essential criterion to evaluate the ability of a material to stretch under load (Ashna *et al.* 2019). The stiffness of materials was analyzed by finding the value of Young's modulus of PTV, DTV1, DTV2, and DTV3 scaffolds. The values of Young's modulus corresponding to the scaffolds were 106.83 ± 31.56MPa, 517.73 ± 82.34MPa, 85.22 ± 21.24 MPa, and 548.25 ± 61.74 MPa respectively. Young's modulus for DTV1 and DTV3 was significantly higher compared to PTV and DTV2 (Fig. 7). The findings were in accordance with the findings of Suvaneeth *et al.* (2016) and Hulsmann *et al.* (2012) who performed decellularisation with trypsin-EDTA and deoxycholic acid agents respectively.

Scanning electron microscopy was employed to study the surface morphology of the scaffolds which provided a better understanding of each of the decellularised materials (Fig. 8). The surface of PTV showed higher matrix density with an abundance of cells (Fig. 8a). There were visible differences in the surface topography of the material from the native tissue. The alignment of the fibers as well as the pore size in each scaffold could be visualized. Surface porosity was noticed with DTV1 (Fig. 8b) and with DTV3 (Fig. 8d) with the absence of cellular remnants. The fiber arrangement of DTV2 (Fig. 8c) resembled that of PTV with a few cellular remnants and porosity in between fibers.

The complex network of ECM is composed of different types of collagen fibers (Balakrishnan-Nair *et al.* 2018). Masson's trichrome and picrosirius staining showed a dense collagen pattern across decellularised scaffolds as bright blue and red stained patterns respectively (Fig. 3B and 3C). Decellularised matrix scaffolds pose several challenges including a sensible detection of residual toxic agents, and selection of organ/tissue sources due to the

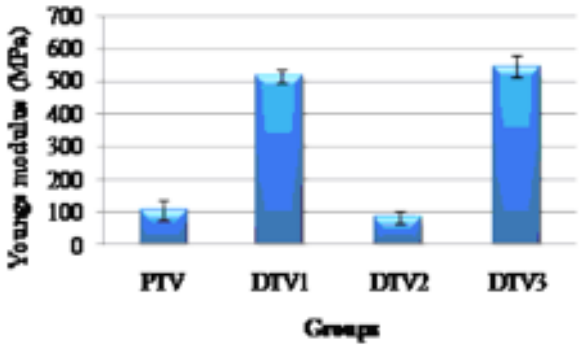
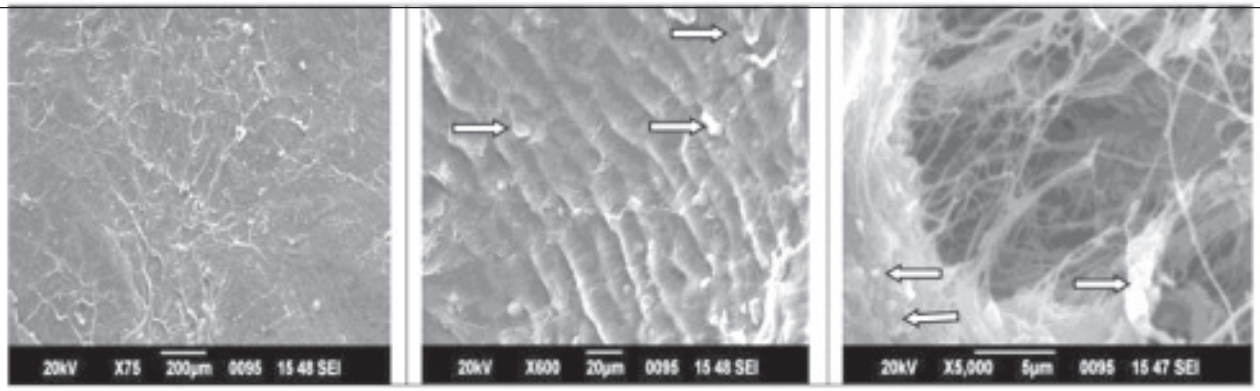
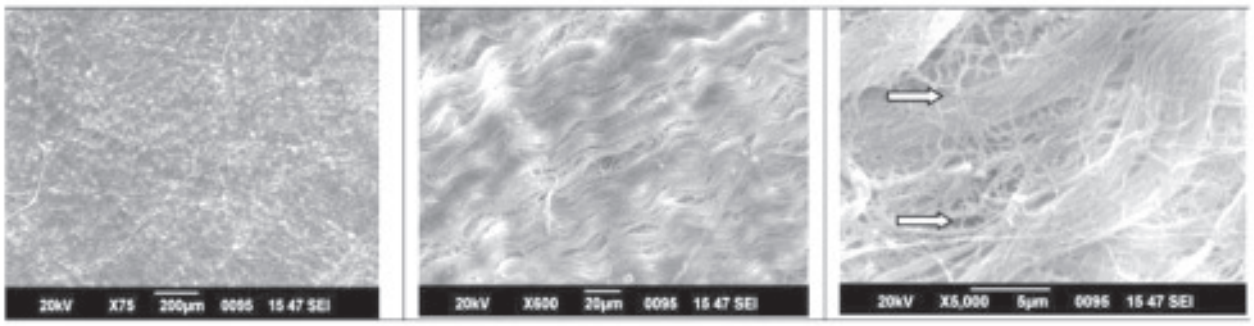


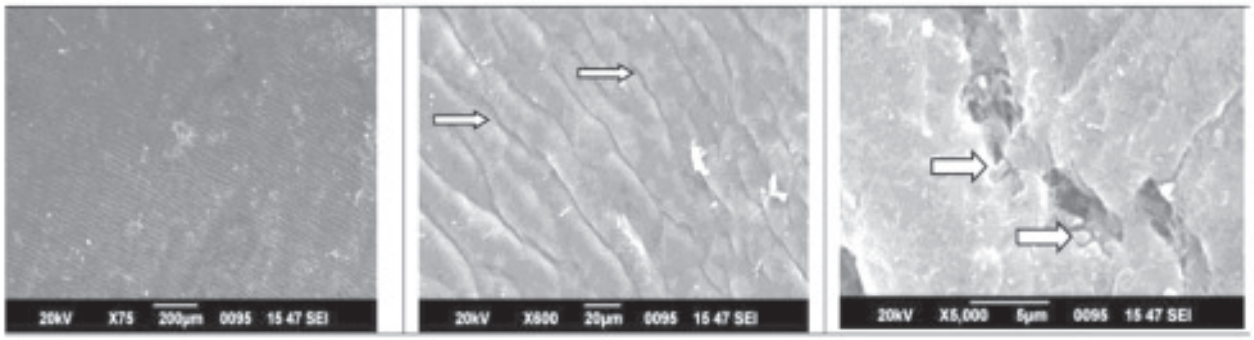
Fig. 7. Comparison of Young's modulus of differently decellularised tunica vaginalis scaffolds.



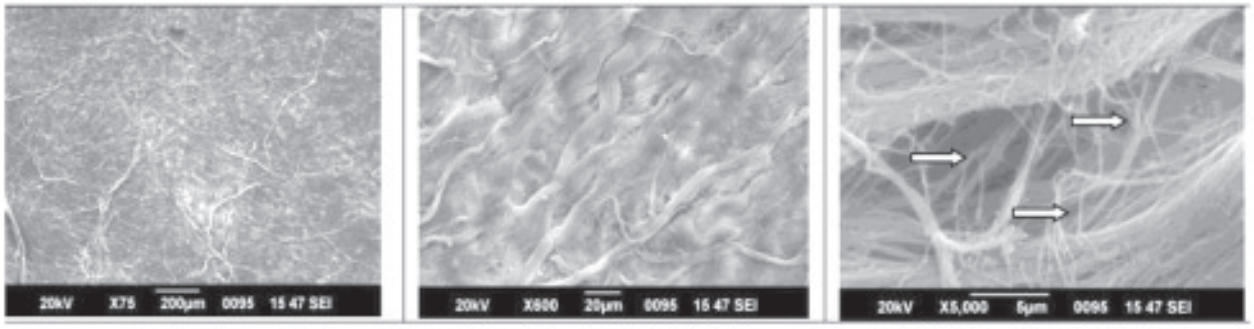
A. Scanning electron microscopic images of the PTV. A higher density matrix fibre arrangement could be observed. At higher magnification presence of cells could be visible (SEM x75, SEMx600, SEM x 5000).



B. Representative scanning electron microscopic images of the DTV1. Porous structures were visible in between the fibres (arrows) (SEM x75, SEM x600, SEM x 5000).



C. Representative scanning electron microscopic images of the DTV2. Thin arrows showed the presence of pores between the fibres. At higher magnification, presence of cellular remnants could be observed (thick arrows) (SEM x75, SEM x600, SEM x5000).



D.Representative scanning electron microscopic images of the DTV3. Thin arrow showed presence of the porous structures in between the fibres (SEM x75, SEMx600, SEM x 5000).

Fig. 8. Scanning electron microscopy images.
Surface characters of scaffolds showing fibre arrangement of (A) PTV (B) DTV1 (C) DTV2 (D) DTV3.

risks of transplantation due to disease transmissions, structural affections, and donor shortage (Ma *et al.* 2018). There is currently no gold standard method for decellularisation, which is heavily influenced by the species, age, anatomical location, and size of the source tissue. Ultimately, the present study concluded that all the decellularisation protocols led to a significant reduction in nuclear contents when compared to the non-decellularised control with less altered mechanical strength and morphology.

CONCLUSION

The extracellular matrix-derived scaffold can provide a natural biochemical and structural framework to its surrounding cells. Hence, it is essential to identify the optimal decellularising agent, its concentration, and exposure time to obtain ideal decellularisation. The current investigation concluded that when compared to the non-decellularised control, all three protocols significantly reduced nuclear contents and with less altered mechanical performance and structure. Pre-clinical studies should be conducted to examine the clinical applications of these decellularised scaffolds. Moreover, various protocols need further modifications to maximize yield and quality to improve biological cues that would assist tissue regeneration to improve human life. Future studies are required to assess the suitability of these scaffolds before they can be used for clinical applications. The scaffolds will need to be tested in animal models to evaluate the immunological response and the ability to integrate with the surrounding tissue and perform as a potential scaffold to replace damaged or diseased tissue.

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