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Research Article

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2025.56.2.332-337

Comparative evaluation of mechanical properties of decellularised non-cross-linked and cross-linked porcine tunica vaginalis scaffolds

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Citation: Abin, J. P., Dhanush, K. B., George, A. J., Pradeep, M., Vasudevan, V. N., and Pooja, K. 2025. Comparative evaluation of mechanical properties of decellularised non-cross-linked and cross-linked porcine tunica vaginalis. *J. Vet. Anim. Sci.* **56** (2):332-337

Received: 20.02.2025 Accepted: 21.03.2025 Published: 30.06.2025

Abstract

Biomaterial implants for soft tissue repairs often lack biomechanical properties, leading to deformation and failure in integration and regeneration. Extracellular matrix-based collagen implants are potential candidates, but their mechanical properties vary by extraction method and source. Cross-linking, a common process, is used to modify these properties. Hence, the present study aims to investigate the biomechanical properties of chemically and enzymatically decellularised porcine tunica vaginalis scaffolds (DPTV) and glutaraldehyde cross-linked decellularised porcine tunica vaginalis scaffolds (DGPTV). Biomechanical properties of Ultimate tensile strength, Young's modulus, elongation at break, and strain at maximum load were estimated. The materials did not differ in tensile strength. However, a significant increase in Young's modulus was observed in DPTV implants. A significant increase in elongation at break and strain at maximum load was observed in DGPTV implants. This increase in biomechanical properties of cross-linked materials is attributed to the formation of new bonds between the collagen bundles, and the increase in fibre crimp of collagen fibres. These properties could translate to better flexibility of cross-linked materials while retaining sufficient tensile strength to support surgical implantation.

Keywords: *Biomaterials, scaffolds, biomechanical properties, glutaraldehyde cross-linking, porcine tunica vaginalis*

In the last few decades, biomaterials have become more and more significant in the design of biomedical devices as well as in the creation of tissue engineering solutions in the process of replacing damaged or lost animal and human tissues, and other applications (Balakrishnan-Nair *et al*., 2018). To increase the biocompatibility and biodegradability of supplied materials, tissue engineering is increasingly using natural substrates, such as macromolecules found in plants and animals. These materials are also thought to be biologically inert and have advantageous mechanical qualities. More significantly, the distinct chemical makeup and structure of these macromolecules give them inherent functionalities and characteristics that boost their bioactivity and therapeutic potential across a variety of applications (Ashna *et al*., 2019). Although incorporating these materials into these devices through a variety of crosslinking techniques has received a lot of attention, resultant biomechanical properties which is frequently hindered throughout the different processes of isolation, purification, and manufacturing, receives little attention (Joyce *et al*., 2021). Therefore, developing effective methods

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to retain its structural components without alteration is of important clinical significance and application value.

Biomaterials are substances that may be well suited to the human body and can be transplanted into the body to replace a sick part or it can be utilised to repair damaged human body parts. Biomaterial implants can be natural or synthetic with varying physio-chemical and biomechanical properties and it affects their applications and durability. Collagen is the most abundant fibrous protein in the extracellular matrix (ECM) and is associated with other ECM fibres and together give mechanical properties such as elasticity to the tissue (Frantz *et al*., 2010). Mechanical properties of ECM-based scaffolds vary by the method of extraction and source of tissue. Compared to hard tissue, soft tissue scaffolds are subjected to a high degree of deformation *in-vivo* post-implantation. These differences in properties could possibly be important in the functional restoration of the damaged tissue and are an important criterion for material selection (He *et al*., 2021). There are different methods used to modify the mechanical properties of scaffolds in biomaterial engineering. Cross-linking is the creation of chemical bonds between polymer chains and can alter the structural properties of the material. Glutaraldehyde is the most commonly used cross-linking agent. It forms links between the aldehyde group and amine groups of lysine or hydroxylysine, though it causes cytotoxic effects on cells (Sheehy *et al*., 2018). Glutaraldehyde cross-linking reduces the immunogenicity of the material by masking antigenic domains while increasing its resistance to enzymes and metalloproteinases, slowing its degradation and absorption. Short-duration cross-linking with 0.2 per cent glutaraldehyde produces favourable cellular responses in bovine pericardial tissue (Umashankar *et al*., 2011). Strong mechanical qualities are thought to be crucial for the fabrication of soft tissues like skin and cartilage since ability of these tissues to support cell proliferation and the production of extracellular matrix depends heavily on their mechanical characteristics (Omiyale *et al*., 2022). For organs or tissue to be repaired and regenerated, the implant needs to offer enough mechanical support to withstand load-bearing cycles and *in vivo* stressors. Hence, our study focussed on the biomechanical properties such as ultimate tensile strength, Young's modulus, elongation at break, and strain at maximum load of short duration 0.2 per cent glutaraldehyde cross-liked and uncross-linked decellularised porcine tunica vaginalis scaffolds.

Materials and methods

Preparation of scaffolds

Tunica vaginalis was separated from the testes of three adult healthy Large White Yorkshire pigs weighing around 90 kg. The samples were cleaned using normal saline. The excess fat and connective tissues were removed by manual stripping. The scaffolds were then defatted in chloroform-methanol (2:1 v/v) solution overnight for 12 hours. The defatted scaffolds were further processed as follows.

Decellularised porcine tunica vaginalis (DPTV)

The defatted porcine tunica vaginalis was subjected to detergent and enzymatic decellularisation for producing the decellularised scaffolds. The procedure for decellularisation was followed according to Arathy (2023). The defatted material was rinsed with deionised water in an incubator shaker at 120 revolutions per minute (rpm) for one hour. An initial detergent wash was done using one per cent Triton-X-100 solution for 30 minutes at 120 rpm in an incubator shaker at room temperature to remove the solvents. The materials were then incubated with 0.05 per cent Trypsin and 0.02 per cent EDTA for 4 hours in an incubator shaker at 150 rpm at room temperature and rinsed in deionised water for one hour at 150 rpm. The samples were treated with one per cent SDC for 4 hours at 150 rpm. The samples were then incubated in 1kU/mL DNase for hours at room temperature to remove DNA remnants (Fig. 1). A final rinsing was done using 1 X PBS and the scaffolds were freeze-dried and stored until further processing.

Glutaraldehyde crosslinked porcine tunica vaginalis (GPTV)

DPTV was further processed by treating it with a glutaraldehyde solution according to Megha (2021). DPTV was cross-linked by immersing the material in 0.2 per cent glutaraldehyde in PBS in a shaking incubator at 120 rpm for 10 minutes at room temperature (Fig.2). The material was then rinsed in 1 X PBS and the scaffolds were freeze-dried and stored until further processing.

Efficiency of decellularisation

The efficiency of decellularisation was tested using haematoxylin and eosin-stained sections of scaffolds. The absence of cellular or nuclear remnants with the retention of wavy collagenous structure of ECM can confirm the efficacy of decellularisation (Megha *et al*., 2022)

Alcian blue staining

Sections from each scaffold were stained with alcian blue to detect the presence of glycosaminoglycans (GAGs). GAG contents in the scaffolds will be visualised as blue in colour under microscopic examination.

Biomechanical characterisation

Biomechanical characteristics like uniaxial tensile strength, elongation at break, strain at maximum load and Young's modulus were tested. Six strips of 5 cm

Table 1. Biomechanical properties

Group	Tensile Strength [MPa]	Young's Modulus [MPa]	Elongation at Break (Standard) [mm]	Strain at maximum load [%]
DPTV	$27.65^{a} \pm 3.10$	$193.79^{a} \pm 23.57$	$5.33^{b} \pm 0.40$	$4.70^{c} \pm 0.34$
DGPTV	$25.16^{a} \pm 1.17$	$55.69^{b} \pm 4.23$	$14.56^{a} \pm 0.88$	$14.28^{a} \pm 0.89$
t-value (p-value)	0.751^{ns} (0.480)	5.244** (0.001)	10.159** (<0.001)	10.838** (<0.001)

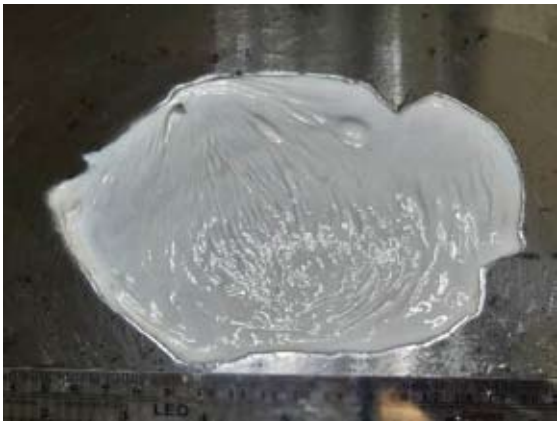

Fig. 1. DPTV scaffolds

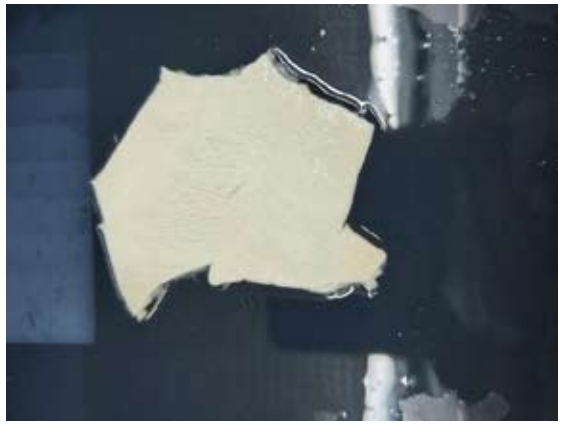

Fig. 2. DGPTV scaffolds

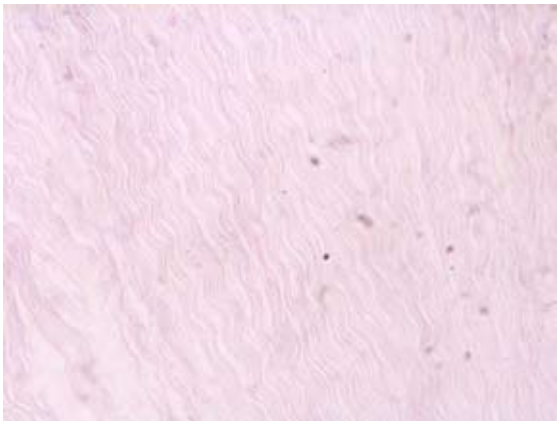

Fig. 3. DPTV scaffolds, showing wavy collagenous matrix with no cellular remnants (H&E, x400)

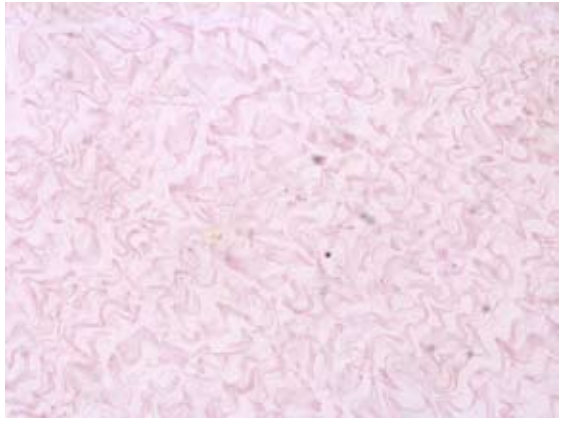

Fig. 4. DGPTV scaffolds, showing crimps in collagen bundles (H&E, x400)

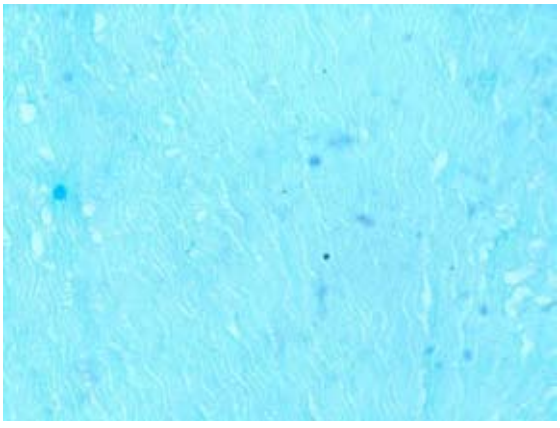

Fig. 5. DPTV implants with bright blue colouration indicating presence of GAGs (Alcian blue, x400)

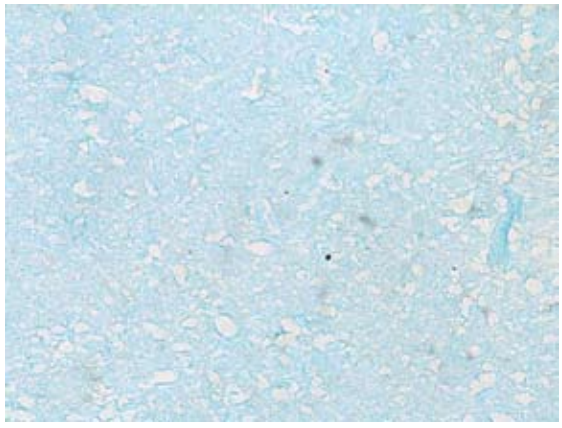

Fig. 6. DGPTV implants with blue coloured inclusions indicating presence of GAGs, (Alcian blue, x400)

x 1 cm samples were cut randomly from each group. The thickness of the material was measured on five points using gauge and mean thickness was calculated. Uniaxial tensile strength was measured using tensile testing machine (Instron 3345) to determine tensile strength in Mega Pascal (MPa). Young's modulus (MPa), elongation at break (mm) and strain at maximum load were also determined in the machine.

Statistical analysis

Biomechanical properties were compared between groups using the independent t test. Alpha level was taken at 5% (0.05).

Results and discussion

No cellular or nuclear contents were observed in H&E-stained sections of the scaffolds indicating sufficient decellularisation. DPTV scaffolds showed wavy collagenous matrix and crimping of collagenous matrix was observed in DGPTV scaffolds (Fig. 3 & 4). Alcian blue-stained sections revealed the presence of GAGs in both scaffolds (Fig. 5 & 6), with a higher concentration on DPTV implants.

The tensile strength of decellularised porcine tunica vaginalis (DPTV) and decellularised glutaraldehyde cross-linked porcine tunica vaginalis (DGPTV) was accessed by stretching the material with a constant force of 100 Newton. The mean tensile strength of implants was 27.65 ± 3.10 (DPTV) and 25.16 ± 1.17 (DGPTV) respectively. DPTV and DGPTV scaffolds didn't significantly differ in tensile strength. The mean values for Young's modulus of DPTV and DGPTV implants are 193.79 ± 23.57 and 55.69 ± 4.23. Young's modulus of DPTV scaffolds was significantly higher than DGPTV implants. The mean values for elongation at break for DPTV and DGPTV implants are 5.33 ± 0.40 and 14.56 ± 0.88. The values for DGPTV scaffolds were significantly higher than those of DPTV. The mean values of Strain at maximum load for DPTV and DGPTV implants are 4.70 ± 0.34 and 14.28 ± 0.89. DGPTV scaffolds showed significantly higher values than DPTV implants. The mean values of Tensile Strength, Young's modulus, elongation at break and strain at maximum load are given in the Table. 1. DPTV implants showed higher glycosaminoglycan contents than DGPTV scaffolds based on staining intensity by alcian blue.

Because of their quick degradation and poor mechanical qualities, biomaterials for soft tissue surgery are frequently limited. Although synthetic materials are constrained by their poor biocompatibility, but they frequently have higher durability. ECM-based biomaterials repair injured tissue with collagen while giving cells a backbone to migrate and multiply (Balakrishnan-Nair *et al.,* 2019). Soft tissues made of collagen exhibit a variety of biomechanical characteristics. To enhance the material's biomechanical qualities and increase its durability over time after implantation, chemical cross-linking is used (Umashankar *et al.,* 2011). Protein polymers are stabilised and rendered resistant to breakdown by glutaraldehyde's reaction with amine and hydroxyl groups. According to Gao *et al.* (2017), the process of cross-linking occurs when amino acid moieties in collagen fibres provide functional groups that link to generate new links between collagen fibres, enhancing mechanical qualities. Cross-linking can enhance a material's mechanical qualities, but at high quantities, it can be hazardous. Therefore, for the material to be accepted in clinical settings, ideal reagent concentrations are needed. The in-vivo cellular and remodelling response of bovine pericardial scaffolds has been demonstrated to be enhanced by chemical cross-linking with 0.2 per cent glutaraldehyde (Umashankar *et al*., 2013). Hence, 0.2 per cent glutaraldehyde was used to cross-link decellularised porcine tunica vaginalis in this study and tensile strength, Young's modulus, elongation at break, and strain at maximum load of uncross-linked and cross-linked scaffolds were measured.

In our observation, tensile strength, though lower in DGPTV scaffolds, did not vary significantly from DPTV scaffolds, while Young's modulus of DPTV scaffolds was significantly higher than cross-linked scaffolds. DGPTV scaffolds showed higher elongation at break and strain at maximum load. A similar reduction in tensile strength and Young's modulus of cross-linked ECM scaffold was noticed by Megha (2021) in omental scaffolds. Vesely (1996) proposed that cross-linked collagen matrices show lower elastic modulus caused by the formation of crimps in collagen bundles during cross-linkage, which causes greater deformation at a given tension. A similar reduction in tensile strength and increased elasticity was observed in glutaraldehyde cross-linked bovine pericardium by Steitz *et al*. (2023) who attributed the reduction in ultimate tensile strength of the scaffolds to the increase in cross-linkages, which reduces its ability to shear and the increase in elongation at the break of cross-linked scaffolds to the fibre crimping due to cross-linking. The mechanical strength and biocompatibility of biomaterials are essential for a range of skeletal repair applications for implants and natural tissue materials. To improve the mechanical integrity of the manufactured pieces, more study is needed to determine the best materials and production processes for the scaffolds. Most of the glycosaminoglycans are lost during decellularisation. GAGs play a role in the cellular migration and remodelling of ECM, interacting with cells by acting as an anchorage for attachment (Uhl *et al.,*2020). For the creation of tissue structure, it is necessary to ascertain how processing parameters affect the biochemical and biophysical properties of biomaterials. Some of the most important characteristics of biomaterials that should be thoroughly inspected and enhanced prior to implantation are their ultimate tensile strength, Young's modulus, elongation at break, and strain at maximum load.

Conclusion

The tensile strength of the materials was the same. DPTV implants, however, showed a markedly elevated Young's modulus. DGPTV implants showed a substantial increase in strain at maximum load and elongation at break. The creation of new linkages between collagen bundles and an increase in collagen fibre crimp are responsible for the biomechanical features of cross-linked materials.

Acknowledgments

The authors acknowledge Dean, CVAS, KVASU, Pookode, for the facilities provided for the successful completion of this work and Biomedical Wing, SCTIMST, Trivandrum for the facilities provided for biomechanical testing. The authors also acknowledge Dr Sunanda C, PhD, for interpreting statistical data.

Conflict of interest

The authors declare that they have no conflict of interest.

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