The Pharma Innovation Journal 2021; SP-10(10): 1342-1346

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(10): 1342-1346

www.thepharmajournal.com
Received: 21-07-2021
Accepted: 08-09-2021

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Physico-chemical properties of decellularised tunica vaginalis of buffalo

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Abstract
The present study was undertaken to assess the physico-chemical properties of decellularised tunica vaginalis of buffalo. For decellularisation, the fresh parietal layer of tunica vaginalis (PTV) procured from testes of ten adult healthy Murrah buffalo between two to five years of age, slaughtered at Meat Technology Unit, College of Veterinary and Animal Sciences, Mannuthy. The samples were cut into 2cm × 2cm square pieces, washed with deionised water and stored in high-density polyethylene pouches at -20 °C. Defatted by immersing overnight in mixture of chloroform and methanol (2:1 v/v) at room temperature. Decellulularisation by Trypsin-EDTA protocol utilising Triton-X-100 as detergent. The physico-chemical properties were analysed and compared to raw parietal layer of tunica vaginalis. The decellularised PTV samples showed acceptable physico-chemical characteristics. Mean thickness, tensile strength and collagen concentration of decellularised samples were significantly reduced while the collagen solubility per cent and percentage weight loss after collagenase degradation were significantly higher in decellularised PTV than raw PTV samples.

Keywords: Decellularised tunica vaginalis, buffalo, physico-chemical properties

Introduction
Natural biomaterials are materials that are of animal or plant origin and are eco-friendly, biocompatible, nontoxic, biodegradable and has remodelling characteristics. Protein-based biomaterials, polysaccharide-based biomaterials and decellularized tissue-derived biomaterials are all examples of naturally derived biomaterials [14]. Collagen-based scaffolds derived from a variety of sources like animals and birds, play an important role in modern medicine, owing to its unique triple helix structure, biocompatibility and potential breakdown by human collagenases [13, 9]. Decellularized collagen matrix preserves the original tissue shape and ECM structure and its various components being extracted, purified and polymerized to generate a functional scaffold. Tunica vaginalis, the serous covering of testis and epididymis, is derived from the peritoneum and imitates the properties of the natural abdominal wall. Since the tunica vaginalis is a bio-waste of meat industry, the present study aim to evaluate the physico-chemical properties of decellularised parietal layer of tunica vaginalis (PTV) for its proper utilization and future research as a promising biomaterial.

Materials and Methods
For the current study, fresh tunica vaginalis removed from ten pairs of adult healthy Murrah buffalo bulls testes, slaughtered at Meat Technology Unit, College of Veterinary and Animal Sciences, Mannuthy. The animals were subjected to ante-mortem inspection prior to slaughter. The fresh parietal layer of tunica vaginalis was separated manually from fascia. For decellularisation, the parietal layer of tunica vaginalis samples were cut into 2 cm × 2 cm square pieces, placed in phosphate buffered saline (PBS) and washed properly with deionized water. The samples were then packed in high-density polyethylene (HDPE) pouches, frozen and stored at -20°C for further processing as per the protocol followed by with appropriate modifications [15]. Frozen samples were removed and thawed to room temperature prior to defatting and decellularisation. Defatted by immersing overnight in a mixture of chloroform and methanol (2:1 v/v) at room temperature. Modified method of decellularisation, Trypsin-EDTA protocaol (0.05 per cent Trypsin and 0.02 per cent EDTA) with sodium dodecyl sulphate replaced by triton-X-100 as detergent was adopted.

i. Defatted tunica vaginalis was rinsed with deionized water in an incubator shaker for 30 minutes twice to eliminate solvents.

ii. To remove solvents, the samples were rinsed in one per cent triton-X-100 solution for 30 minutes at room temperature (15 min x 2 times).
iii. Incubated at room temperature for six hours in an incubator shaker at 250 rpm in a solution containing 0.05 per cent trypsin and 0.02 per cent ethylene diaminetetraacetic acid (EDTA).
iv. Continually rinsed with deionized water to eliminate the enzyme.
v. To remove the cellular components, they were washed with one per cent triton-X-100 solution for two hours at 37 °C in an incubator shaker.
vi. To remove the detergent, thoroughly washed with deionized water (30 min x 4 times).
vii. Excess moisture was removed by drying for two hours in a laminar air flow cabinet. The resulting decellularised parietal tunica vaginalis was packaged in HDPE pouches and stored at -20 °C until further processing [15].

Following physico-chemical properties of developed decellularised and raw PTV were assessed:

a) Developed matrix Thickness were measured at three separate sites using digital screw gauge, the mean thickness was expressed in µm.
b) Tensile Strength was measured using texture analyser (Model-EZ texture analyser, Shimadzu, Japan) and a 500 N load cell. Thin strips of the samples were cut at random from the acellular matrices, each measuring approximately 15 mm x 50 mm, and thickness and width were measured at five separate sites using a digital screw gauge. The strips were fastened to the machine's grips. The grips were initially held at a distance of 25 mm and tensile strength was determined by stretching the tissues at a rate of 3 mm/min while moving the grips. The machine recorded the maximum load applied to the sample before it ruptured.
The strips were attached to the grips of machine. The grips were initially held at a distance of 25 mm and tensile strength was calculated by subjecting the tissues to stretching by moving the grips at a speed of 3 mm/min. Maximum load exerted on the sample before rupture was recorded by the machine. Tensile strength was estimated by dividing the greatest load by the sample's cross-sectional area and was represented in kilograms per square metre (kg/cm^2).
c) Total Collagen Content was calculated from the hydroxyproline content of the sample using the Stegman and Stalder method [12], modified by the International Organisation for Standardization (ISO) [7].
About 70 mg of dried fat-free tissue sample was hydrolyzed in a sealed test tube with 10 ml 6 N HCl for 18-20 hours at 110 °C. The hydrolysate was cooled and filtered into a standard flask using Whatman No. 1 filter paper before being diluted to 100 ml with distilled water. Sodium hydroxide solution was used to neutralise 5 ml of the diluted hydrolysate to pH 6.8 and the volume was increased to 100 ml using distilled water. The neutralised diluted tissue hydrolysate (5 ml) was combined with 2 ml of oxidising agent and allowed to sit at room temperature for 20 minutes. Colour reagent (2 ml) was added and incubated in a water bath at 60°C for 15 minutes, then cooled in running tap water for three minutes before measuring the absorbance of the coloured solution at 560 nm with a single beam spectrophotometer (Systronics, India). At 560 nm, the chromophore's absorbance was measured and a standard graph was drawn (Appendix). The hydroxyproline content of unknown samples was determined using a regression equation built from absorbance readings for known standard quantities.

$$y = 0.2382x + 0.0704$$

where, y is the absorbance and x is the concentration of hydroxyproline

The total collagen content of the sample was calculated by multiplying the hydroxyproline content by 7.46 to get the total collagen content. Total collagen was calculated as a percentage of the dry fat-free weight of the sample.
d) Collagen Solubility was estimated from sample's soluble hydroxyproline content using Hill's method [5]
Raw and processed buffalo tunica vaginalis (70 mg) were lyophilized for 12 hours in a lyophilizer (Operon FDU 7003, Korea), cooked in 12 ml one-fourth strength Ringer's solution in a water bath at 77°C for 63 minutes and centrifuged for 10 minutes at 6000 rpm. The supernatant was removed and the residue was centrifuged again with 8 ml of one-fourth strength Ringer's solution. In a standard volumetric flask, the two supernatants were mixed and the volume was increased to 25 ml. In a sealed test tube at 110°C for 18-20 hours, 5 ml of supernatant was hydrolyzed with 5 ml 6N HCl. The hydrolysate was allowed to cool before being filtered through Whatman No. 4 filter paper. The pH was then adjusted to 6.8 using sodium hydroxide solution and the volume was increased to 50 ml with distilled water.
Five ml of neutralised diluted tissue hydrolysate was combined with 2 ml oxidising agent and stored at room temperature for 20 minutes. The colouring reagent (2 ml) was added and incubated in a water bath at 60°C for 15 minutes, then cooled under running water for three minutes before measuring the absorbance of the coloured solution at 560 nm with a single beam spectrophotometer (Systronics, India). The hydroxyproline content of the supernatant was determined using a regression equation built from the absorbance values of established standard concentrations.
The soluble collagen content of the sample was calculated by multiplying the hydroxyproline concentration by 7.25 to get the collagen content. Collagen solubility was calculated by dividing soluble collagen by collagen content and given as a percentage.
e) Resistance to Collagenase Degradation was assessed using collagenase from *Clostridium histolyticum* (125 µg/mg, Sigma Aldrich, USA) [11].
30 mg of raw and decellularized tunica vaginalis (wet weight, in duplicate) were weighed. 500 ml 0.1M tris chloride (pH 7.4), 0.005 M calcium chloride and 0.05 mg/ml sodium azide were added to these samples. For one hour at 37 °C, the samples were incubated. Collagenase enzyme was produced at 37 °C in 0.1M Tris chloride (pH 7.4) and then added to the vials to achieve a final concentration of 2 U/mg of tissue. The samples were chilled and centrifuged at 12,000 rpm for 20 minutes at 4°C after being incubated at 37 °C for 24 and 48 hours. After discarding the supernatant, remaining tissue samples were collected and blotted with filter paper for five minutes to dry them and weighed. The per cent

weight loss of the tissues was calculated using a matched comparison before and after enzyme treatment

Statistical Analysis of the physico-chemical properties was done using comparison paired t-test to assess the difference between decellularised and raw PTV using computerized software programme SPSS version 24.

Results and Discussion

Developed Matrix Thickness

Statistically significant differences were noticed in the mean thickness of separated parietal layer of tunica vaginalis and decellularized PTV. The separated parietal layer of tunica vaginalis of right and left testes that measured 265.20 ± 35.37 µm and 195.40 ± 14.37 µm respectively, was reduced to 125.25 ± 0.77 µm and 75.10 ± 6.29 µm respectively in the decellularised samples ($p \leq 0.01$) as shown in Table 1. This result is in accordance with the report given by Zou *et al.,* [16] in porcine aortic valve, who opined that using sodium dodecyl sulfate (SDS), triton X-100 and trypsin protocols caused significant decrease in thickness after the decellularisation process. Liao *et al.,* [8] observed that decellularisation of porcine aortic valve by treating with SDS (0.48 ± 0.07 mm to 0.43 ± 0.08 mm) and triton-X (0.31 ± 0.05 mm to 0.16 ± 0.04 mm) caused reduction in tissue area and thickness dimensions.

Tensile Strength

Statistically significant differences were noticed in the mean tensile strength of decellularised and raw buffalo PTV samples (Table 2). The mean tensile strength of decellularised PTV sample was lesser and measured 485.21 ± 7.76 kg/cm² in the right and 474.14 ± 7.79 kg/cm² in the left testis and the corresponding values were 594.49 ± 10.63 kg/cm² and 592.95 ± 14.06 kg/cm² respectively in the raw buffalo PTV of right and left testes. Cartmell *et al.,* [2] stated that treatment of rat tail tendon with triton X-100 caused disruption of collagen fibers, leading to approximate 50 per cent decrease in wet tensile strength and stiffness values. Hülsmann *et al.,* [6] found that tensile strength of trypsin-EDTA treated bovine pericardium was reduced from native tissue. This decreased tensile strength of decellularised samples might be due to reduction of collagen content after prolonged exposure with trypsin-EDTA.

Total Collagen Content

The total collagen concentration was slightly lower in the decellularised PTV sample when compared to the raw buffalo PTV for both pairs of testes ($p \leq 0.05$) (Table 3). The total collagen content in right and left testes was 38.876 ± 0.841 and 37.597 ± 1.108 per cent respectively in the decellularised PTV sample and 41.254 ± 0.078 and 41.102 ± 0.157 per cent respectively in the raw buffalo PTV. These findings corroborated with the previous reports of Gilpin and Yang [4] and Abhin [1] that treating bioscaffolds with trypsin caused modification in the collagen arrangement which led to disruption and loss of collagen.

Collagen Solubility

Statistically significant difference was recorded in the collagen solubility percent of decellularized PTV samples of left (82.89 ± 1.07 per cent) and right testes (84.39 ± 1.08 per cent) and it was higher than that of the raw PTV samples of left (11.88 ± 0.69 per cent) and right testes (13.49 ± 0.72 per cent) ($p \leq 0.01$) as presented in table 4. These results are in agreement with findings of Abhin [1] in boar PTV. Cartmell *et al.* [2] stated that there was significant decrease in the mechanical strength of samples incubated in trypsin which suggested increased collagen denaturation. Reddy *et al.,* [10] reported that the solubility of collagen in acids depends mainly on the extent of disruption of non-covalent bonds as well as covalent bonds.

Resistance to Collagenase Degradation

The resistance of raw and decellularised PTV to enzymatic digestion was assessed after 24 h and 48 h of collagenase enzyme treatment shown in table 5. The percentage weight loss after 48 h of collagenase enzyme treatment in right and left testes was 59.85 ± 1.31 and 59.33 ± 1.47 per cent respectively in the raw buffalo PTV and 86.53 ± 1.75 and 83.72 ± 1.53 per cent respectively in the decellularised PTV sample. The percentage weight loss after collagenase degradation differed significantly and was more for decellularised PTV than the raw PTV in both pairs of the testes ($p \leq 0.01$). Similar to these findings, Ronny [11] reported that complete weight loss of extracellular matrix was seen in porcine dermis matrix after 48 h of collagenase digestion. Eckhard *et al.,* [3] proved that clostridial collagenases were extracellular matrix degraders. The carbon-terminally located collagen binding domains (CBDs) of clostridial collagenases recognized the triple-helical conformation of collagen and allowed binding and localisation by mechanically expelling and unwinding the triple-helical collagen molecules by a "chew and digest" mechanism.

Table 1: Comparison of thickness of separated parietal layer of tunica vaginalis and decellularised parietal tunica vaginalis (PTV) (n=10)

Thickness PTV (µm)	Group		t- value	P-value
	Separated PTV (µm)	Decellularised PTV (µm)		
Left testis	195.40 ± 14.37	75.10 ± 6.29	13.341**	< 0.001
Right testis	265.20 ± 35.37	125.25 ± 0.77	9.991**	< 0.001

**Significant at 0.01 level

Table 2: Comparison of tensile strength of raw and decellularised parietal layer of tunica vaginalis (n=10)

Tensile strength (kg/cm²)	Group		t-value	P-value
	Raw PTV	Decellularised PTV		
Left testis	592.95 ± 14.06	474.14 ± 7.79	7.983**	< 0.001
Right testis	594.49 ± 10.63	485.21 ± 7.76	8.889**	< 0.001

**Significant at 0.01 level

Table 3: Comparison of total collagen content of raw and decellularised parietal layer of tunica vaginalis (n=10)

Collagen content (%)	Group		t-value	P-value
	Raw PTV	Decellularised PTV		
Left testis	41.10 ± 0.16	37.59 ± 1.11	3.380*	0.008
Right testis	41.25 ± 0.08	38.88 ± 0.84	2.945*	0.016

*Significant at 0.05 level

Table 4: Comparison of collagen solubility of raw and decellularised parietal layer of tunica vaginalis (n=10)

Collagen solubility (%)	Group		t-value	P-value
	Raw PTV	Decellularised PTV		
Left testis	11.88 ± 0.69	82.89 ± 1.07	56.316**	< 0.001
Right testis	13.49 ± 0.72	84.39 ± 1.08	75.210**	< 0.001

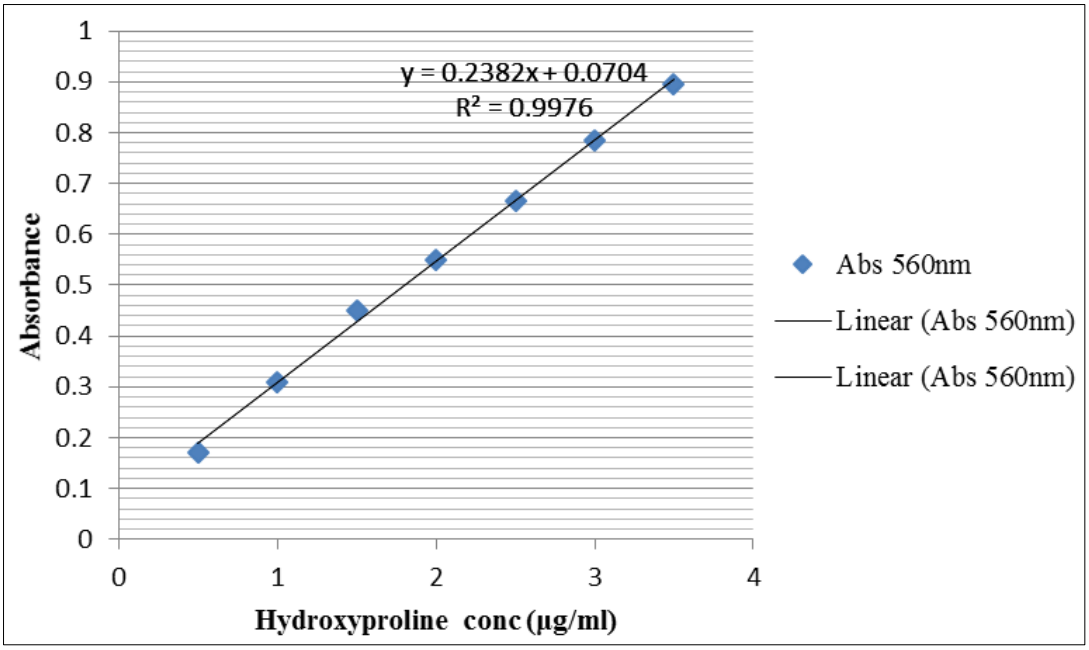
**Significant at 0.01 level

Table 5: Comparison of collagenase enzyme degradation of raw and decellularised parietal layer of tunica vaginalis (n=10)

Treatment	Weight loss (%)	Group		t-value	P-value
		Raw PTV	Decellularised PTV		
After 24 hours	Left testis	33.12 ± 1.58	61 ± 1.65	15.396**	< 0.001
	Right testis	34 ± 1.39	61.94 ± 1.06	18.910**	< 0.001
After 48 hours	Left testis	59.33 ± 1.47	83.72 ± 1.53	14.799**	< 0.001
	Right testis	59.85 ± 1.31	86.53 ± 1.75	10.775**	< 0.001

**Significant at 0.01 level

By using the newly standardised decellularisation technique, strong and flexible novel acellular matrices were developed from the parietal layer of tunica vaginalis, with acceptable physico-chemical properties. Since the tunica vaginalis is a bio-waste of meat industry, the present study will pave way for its proper utilization after extensive clinical screening and for future research as a promising biomaterial in regenerative medicine.



Appendix: Standard graph for hydroxyproline assay

References

1. Abhin RKP. Structural and physicochemical evaluation of raw and decellularised porcine tunica vaginalis as a novel acellular matrix. M.V.Sc. thesis, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad 2021,83p.
2. Cartmell JS, Dunn MG. Effect of chemical treatments on tendon cellularity and mechanical properties. J. of Biomed. Mater. Res 2000;49:134-140.
3. Eckhard U, Huesgen PF, Brandstetter H. Proteomic protease specificity profiling of clostridial collagenases reveals their intrinsic nature as dedicated degraders of collagen. J. Proteomics 2014;100:102-14.
4. Gilpin A, Yang Y. Decellularisation strategies for regenerative medicine:from processing techniques to applications. BioMed Res. Int 2017;4:1-14.
5. Hill F. The solubility of intramuscular collagen in meat animals of various ages. J. Food Sci 1996;31:161-166.
6. Hülsmann J, Grün K, El Amouri S, Barth M, Hornung K, Holzfuß C *et al*. Transplantation material bovine pericardium: biomechanical and immunogenic characteristics after decellularization vs. glutaraldehyde-fixing. Xenotransplantation 2012;19:286-297.
7. ISO. Meat and meat products-Determination of

hydroxyproline content. ISO, Geneva 1994,69p.
8. Liao J, Joyce EM, Sacks MS. Effects of decellularization on the mechanical and structural properties of the porcine aortic valve leaflet. Biomaterials 2008;29:1065-1074.
9. Oladeji OI, Lasisi E, Umoru, Aribo S. A minefield of biomaterials. Rev. Nat. Prod. Article ID 983062 2012,20p.
10. Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem. PMID: 8740508 1996;3:225-9.
11. Ronny S. Effect of gamma irradiation on the characteristics of collagen based bioscaffolds. M.V.Sc. thesis, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad 2015,83p.
12. Stegemann H, Stalder K. Determination of hydroxyproline. Clin. Chim. Acta 1967;18:267-273.
13. Stynes G, Kiroff GK, Morrison WAJ, Kirkland MA. Tissue compability of biomaterials: benefits and problems of skin biointegration. J. Surg. Res 2008;78:654-659.
14. Tran LBH, Minh QT, Doan NV, Minh SD. Naturally derived biomaterials: preparation and application. Biomaterials 2013;10:5772-55668.
15. Vasudevan VN. Development and characterisation of bioscaffolds from abattoir byproducts. Ph. D. thesis, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad 2014,165p.
16. Zou Y, Zhang Y. Mechanical evaluation of decellularized porcine thoracic aorta. J. Surg. Res 2012;175:359-368.