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Mechanical properties of the porcine pericardium extracellular matrix cross-linked with glutaraldehyde and tannic acid

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Purpose: The aim of this study was to determine the influence of factors such as temperature and glutaraldehyde (GA) concentration on the mechanical properties of porcine pericardia, in order to propose the recommended optimal conditions of a cross-linking process. It was also to verify whether tannic acid (TA), a natural cross-linking agent that stabilizes collagenous tissues by a different mechanism than GA, may positively influence the strength of pericardium. *Methods*: The samples were incubated at various temperatures (4, 22, and 37 °C) and GA concentration solutions (0.6, 1.5 and 3%) for 7 days. Three series were selected and additionally cross-linked with 0.3% TA for another 7 days. Mechanical properties of cross-linked pericardium samples, i.e., ultimate tensile strength (UTS) and elastic modulus (E) were measured in uniaxial tensile testing. The hyperelastic model for incompressible materials – isotropic by Ogden [24] and anisotropic by Fung [7] were utilized to describe the mechanical behaviour of treated pericardium. *Results*: The temperature has an influence on cross-linking effects; the lowest values of UTS were reported for specimens cross-linked at 22 °C, while the mechanical properties of series treated at 4 °C or 37 °C were comparable. At a particular temperature of incubation, the GA concentrations have not affected the mechanical properties of tissues. The dependence between mechanical parameters and agent concentration was only observed for specimens treated with GA at 37 °C. *Conclusions*: The conditions of the cross-linking process affect the mechanical properties of the porcine pericardium. Room temperature (22 °C) and the concentration of 1.5% GA occurred to be ineffective. The mechanical properties of GA-treated pericardium were improved by an additional TA cross-linking.

Key words: *porcine pericardium, collagen, glutaraldehyde, tannic acid, mechanical properties, uniaxial tensile testing*

1. Introduction

Because of its mechanical and surface properties, the pericardium is considered a material full of potential in various medical applications, especially as a candidate to form leaflets of biological heart valves (BHVs). The pericardium is mainly composed of fibrous collagen type I, approximately 77 ± 2%, and around 4 ± 0.5% of elastin fibres [9]. Although the application of bovine pericardium is more common [17], [31], the porcine pericardium possesses a higher regional uniformity in its different parts [5] while being thinner and stiffer [1], [23]. The advantage of the use of thinner tissues, i.e., porcine pericardium, is that BHVs might be implanted in a less invasive way during transcatheter aortic valve replacement (TAVR) [6].

To prolong BHVs' durability, collagenous tissues are chemically or physically cross-linked [22]. Glutaraldehyde (GA) is a chemical agent used commercially to

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stabilize the structure of collagenous tissues. Unfortunately, GA cross-linked biomaterial might be exposed to premature calcification [14], [27] and cytotoxicity [4], [34]. Moreover, GA-treatment introduces changes in the organization of collagen fibrils and decreases their alignment [17], [33]. The studies on the influence of the GA cross-linking process on pericardium mechanical behaviour have been contradictory. According to Arbeiter et al. [2], tissue extensibility excesses and its behaviour shifts to more elastic as a result of GA cross-linking. The longer the process lasts, the more pericardium stiffness decreases [1]. On the other hand, according to Ferrans et al. [13], a higher concentration of cross-linking aldehyde may result in tissue stiffening. Therefore, the concentration of commercially utilised GA has been recently limited to the necessary minimum of 0.2%.

The application of GA with another cross-linking agent, which stabilizes collagenous tissue by different mechanism, may be an alternative solution. It was suggested to use both GA and tannic acid (TA) as stabilizing agents [15], [16]. Aldehyde groups react with free amine groups of lysine and hydroxylysine amino acid residues of the polypeptide chains, forming the covalent bonds [25], while TA cross-links collagen molecules through multiple hydrogen bonds [11], [19]. Moreover, TA is a biocompatible natural cross-linking agent which mitigates the calcification process [37] and is said to inhibit the enzymatic degradation of elastin [16]. GA-TA cross-linking might be potentially useful in the stabilization of extracellular matrix components of the pericardium.

It must be stated that porcine pericardium has been studied insufficiently to define its behaviour after GA-TA modification. The aim of this study was to determine the influence of chemical collagen stabilization with glutaraldehyde (GA) and then both GA and tannic acid (TA, 0.3%) on the mechanical properties of the porcine pericardium. Effects of the factors such as temperature (4, 22 and 37 °C) or cross-linking agent's concentration (0.6, 1.5 and 3% GA) on pericardium strength were verified. Hyperelastic constitutive models by Fung and Ogden were fitted to experimental data obtained for cross-linked pericardium.

2. Materials and methods

2.1. Tissue preparation

Fresh porcine hearts (n = 7) from domestic pigs aged 6−8 months were obtained from local abattoirs in pericardial sacs. Tissues were collected according to the protocol of multiple organ procurement adopted from the human model [26]. The whole hearts were transported to the laboratory in the sterile phosphate-buffered saline solution (PBS, pH 6.5; Sigma-Aldrich), cooled to 4 °C. After the manual separation of pericardial sacs from hearts, the tissue was processed following the procedures described by Simionescu and co-workers [30]. The fibrous layer of the pericardium was separated from the serous layer without causing any damage to tissue structure. Adipose tissue was removed mechanically by hand. The pericardium was rinsed in sodium chloride (0.9% w/v NaCl; Fresenius Kabi, Poland) and stored in the same solution at 4 °C afterwards.

Glutaraldehyde (GA) obtained as 25% aqueous solution (Chempur, Poland) was diluted into 0.6, 1.5 and 3% solutions. The samples were incubated at three different temperatures (4, 22, and 37 °C) and GA concentration solutions (0.6, 1.5 and 3%) for 7 days (Table 1). Three previously selected series incubated in a 0.6% GA solution were additionally cross-linked with 0.3% tannic acid (TA; Pol-Aura, Poland) for another 7 days (series X-XII). All chemicals were used without further purification.

Table 1. The treatment process of specimens in 12 series

Series (number of specimens)	Cross-linking agent	Temperature [°C]
I (n = 7)	0.6% GA	4
II (n = 6)	1.5% GA	4
III (n = 5)	3% GA	4
IV (n = 5)	0.6% GA	22
V (n = 5)	1.5% GA	22
VI (n = 5)	3% GA	22
VII (n = 5)	0.6% GA	37
VIII (n = 5)	1.5% GA	37
IX (n = 5)	3% GA	37
X (n = 5)	0.6% GA and 0.3% TA	4
XI (n = 5)	1.5% GA and 0.3% TA	4
XII (n = 5)	3% GA and 0.3% TA	4

2.2. Mechanical testing

Before the chemical treatment, tissue was cut into rectangles of approximate dimensions of 5 × 30 mm, with the long axis taken parallel to collagen fibres orientation. The samples were taken from different sacs and were randomly assigned into XII series, with at least 5 samples in each group (Table 1). Dimensions of every sample were measured after the cross-linking process.

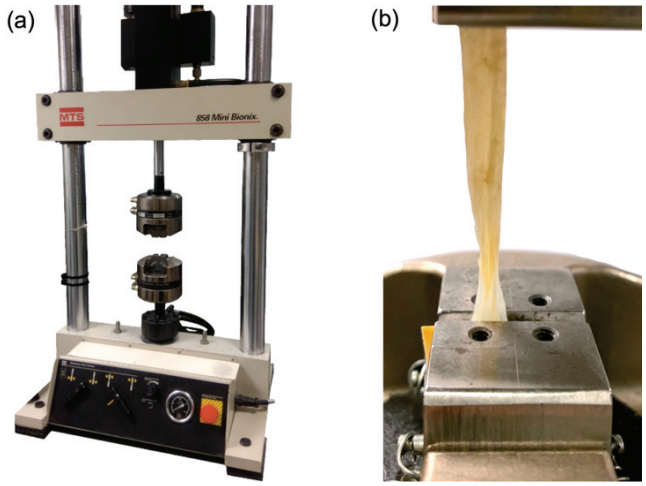

Fig. 1. Uniaxial testing machine MTS 858 MiniBionix® (a) and the specimen between grips during the test (b)

Uniaxial tensile testing (MTS 858 MiniBionix®, Fig. 1) was used to determine the mechanical properties of the cross-linked pericardium. The specimens were loaded to failure at a 10 mm/min displacement rate. Collagen fibres were oriented in parallel to the axis of tension. Both elastic modulus (E) in the linear region and ultimate tensile strength (UTS) were calculated (Fig. 2).

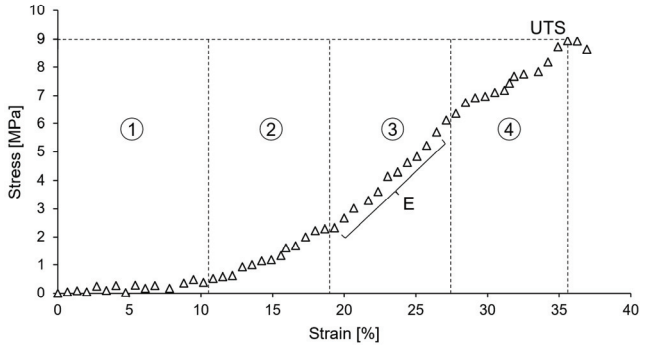

Fig. 2. Stress–strain curve for sample cross-linked with 1.5% GA at 4 °C.
(1) In the *toe* region, collagen fibres become straightening or unfolded by small stresses. (2) At the *transition area (heel),* fibres are deformed and start to shear. (3) Elastic modulus is calculated in the linear part of the curve.
(4) In the *failure area* sample begins to yield and breaks

UTS is determined by the highest obtained value of stress [1], [25], [28].

2.3. Statistics

The values of calculated mechanical properties were averaged over the number of specimens in each series. Therefore, numeric data were expressed as mean ± standard deviation (SD), median (Me) and a range of values to show the distribution of obtained data. Due to low numbers of samples in particular groups, non-parametric Mann–Whitney tests were performed for statistical differences between: i) solution concentrations and ii) temperatures of process and iii) additional influence of TA treatments.

2.4. Constitutive models

The hyperelastic model for incompressible materials, isotropic proposed by Ogden [24] and anisotropic by Fung [7], was utilized to describe the mechanical behaviour of treated pericardium. The Ogden model is mainly used for the stress–strain description of rubber-like solids [24]. The 1-term model formula of strain energy density was utilized as follows:

$$\Psi = \frac{\mu}{\alpha}(\lambda_1^{\alpha} + \lambda_2^{\alpha} + \lambda_3^{\alpha} - 3), \tag{1}$$

where μ is a stress-like material parameter describing the stiffness of the materials, α is the dimension-less material parameter, and λ_1, λ_2, and λ_3 are the principal stretches.

The Fung hyperelastic anisotropic material model was originally defined for a stress–strain description of the arterial wall [7]. The Fung hyperelastic anisotropic material model was originally designated for a stress–strain description of the arterial wall. The model is fully phenomenological, and it is nowadays widely used to describe the load-bearing behaviour of other tissue structures. The strain energy density function for the Fung model is characterized by exponential stress–strain behaviour:

$$\Psi = \frac{c}{2}(e^{Q} - 1), \tag{2}$$

where c is a stress-like material parameter, and Q is described as follows:

$$Q = b_1 E_1^2 + b_2 E_2^2 + b_3 E_3^2$$

$$+ 2b_4 E_1 E_2 + 2b_5 E_2 E_3 + 2b_6 E_1 E_2, \tag{3}$$

where E_{ij} are components of Green–Lagrange strain tensor $\mathbf{E}$, b_1, ..., b_6 are dimensionless material parameters.

The constitutive parameters were determined using a nonlinear least-squares *lsqnonlin* function in the Matlab (ver. 2017b) according to method described in [20]. The standard nonlinear Levenberg–Marquardt algorithm was utilized during the fitting of the curves.

The goodness of fit was evaluated by the coefficient of determination (R^2).

3. Results

3.1. Mechanical properties – uniaxial tensile testing

On the basis of stress–strain curves, the mechanical properties for twelve (I–XII) tested series, such as ultimate tensile strength (UTS) and elastic modulus (E) were calculated and presented in Table 2.

Exemplary stress–strain curves for one sample of each series were presented in Fig. 3. The curves were grouped into nine plots (a–i); curves (a–c) were gathered due to the concentration of GA and (d–f) to the temperature during the treatment. To show the impact of an additional TA treatment, the curves for samples cross-linked with GA or both GA and TA were presented in plots (g–i).

GA concentration

The typical S-shaped curve was observed for series I-III cross-linked at 4 °C (Fig. 3a). The section recognized as the toe region was noticeable. In comparison with series II (1.5% GA, 4 °C) and III (3% GA, 4 °C), series I (0.6% GA, 4°C) was able to carry higher stresses with the lower deformation, which resulted in a shorter toe region and lower strains at the specimen's rupture. There was no significant difference between the mechanical properties of series I and III (Table 2), although series III was treated with a 5× higher concentration of GA. For series I and III the highest values of E were obtained, but the wide variance should be taken under consideration. Variations in values may be caused by the structural differences of the acquired tissue.

The lowest values of both mechanical properties have been reported for specimens cross-linked at room temperature (22 °C), especially for the higher concentrations of the cross-linking agent (Fig. 3b). Mean values of UTS and E were comparable for series V (1.5% GA, 22 °C) and VI (3% GA, 22 °C), although the concentration of GA was twice higher for series VI. It has to be noted that, although the highest values of mechanical parameters were obtained for series IV (0.6% GA, 22 °C), the importance of SD cannot be neglected for both UTS and E.

As shown in Fig. 3c, cross-linking of series VIII (1.5% GA, 37 °C) and IX (3% GA, 37 °C) resulted in an elongated transition phase (heel region) after the relatively short toe region on the stress-strain curves. By the analysis of numerical values (Table 2) for series VII–IX cross-linked at 37 °C, the correlation between increased GA concentration and higher UTS values was observed. For series I–VI, such a dependence between the agent's concentration and improvement of mechanical parameters was not noted.

Temperature

In Figure 3d, an atypical behaviour for collagenous tissue was noted, independently of GA con-

Table 2. Mechanical properties of cross-linked tissue according to different processing protocols (series I–XII); $\overline{X}$ (SD) – mean (standard deviation), Me – median

Series	Cross-linking agent(s)	Temperature [°C]	Ultimate tensile strength UTS [MPa]			Elastic modulus E [MPa]		
			$\overline{UTS}$ ± SD	Me	Range	$\overline{E}$ ± SD	Me	Range
I	0.6% GA	4	11.7 ± 3.3	12.2	6.6–16.1	55 ± 20	48.9	31.3–89.4
II	1.5% GA		8.2 ± 2.3	7.7	5.3–11.6	34 ± 15	32.9	16.1–53.8
III	3% GA		12.1 ± 3.0	12.6	6.8–16.1	57 ± 18	58.3	31.9–84
IV	0.6% GA	22	9.1 ± 4.6	12.1	3.3–13.3	43 ± 25	34.7	17.4–73.6
V	1.5% GA		6.4 ± 1.8	5.9	4.7–9.7	31 ± 12	34.8	13–47.2
VI	3% GA		6.4 ± 1.9	6.8	5.3–9.3	30.7 ± 6.5	31.4	21.5–41.3
VII	0.6% GA	37	7.7 ± 2.2	7.6	5.2–10.6	38.0 ± 8.8	39.7	22–47.2
VIII	1.5% GA		9.1 ± 2.2	8.8	6–12	40 ± 11	36.5	28.5–55
IX	3% GA		14.0 ± 6.3	14.5	6.6–23.5	50 ± 17	44.9	27.1–70.5
X	0.6% GA 0.3% TA	4	13.7 ± 2.9	13.6	9.3–17.5	51 ± 15	45.9	38–80.2
XI	1.5% GA 0.3% TA		10.6 ± 2.5	9.3	8.3–14.9	45 ± 17	46.7	14.5–66
XII	3% GA 0.3% TA		15.6 ± 4.5	14.8	10.2–20.7	78 ± 26	85.7	35.5–106.3

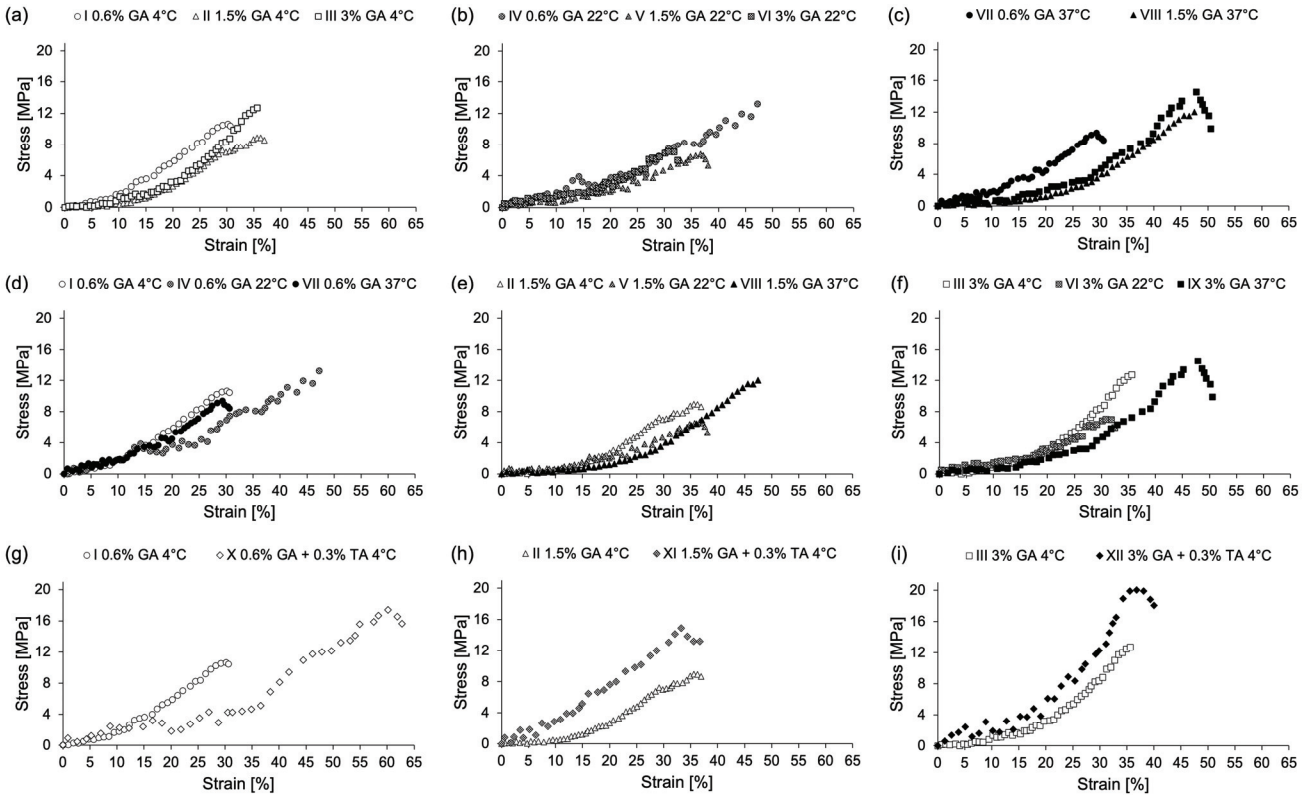

Fig. 3. The stress–strain curves for specimens cross-linked in: (a) 4 °C; (b) 22 °C; (c) 37 °C; (d) 0.6% GA; (e) 1.5% GA; (f) 3% GA; (g) 0.6% GA + 0.3% TA; (h) 1.5% GA + 0.3% TA; (i) 3% GA + 0.3% TA

centration. For all 0.6% GA cross-linked specimens, a steeper and shortened transition area begins with the immediate increase of stress. The toe region that is typical for native tissue is invisible. The stress values increase immediately, accompanied by the slow growth of strain values. Especially for series VII (0.6% GA, 37 °C) the relationship seems to be more linear, instead of standard S-shaped [22] or J-shaped [8] stress–strain curves. The behaviour of specimens from series IV (0.6% GA, 22 °C) slightly differs. After reaching a local maximum of stress values, the tested tissue strengthens.

For 0.6% treatment, it might be noted that the higher the temperature used during the process, the lower the mechanical properties obtained (Fig. 3d). For series cross-linked with 1.5% (Fig. 3e) or 3% (Fig. 3f), such dependence was not observed; the temperature had no remarkable influence on the cross-linking effects. Especially for all series cross-linked with 1.5% GA (series II, V, VIII), the values of UTS and *E* were comparable.

TA treatment

It was noted that the behaviour of TA cross-linked samples changed (Figs. 3g–i). Comparison of

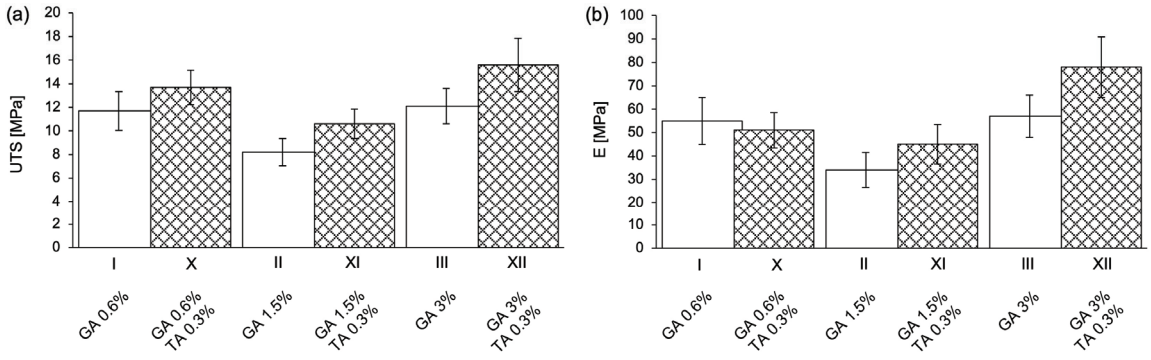

Fig. 4. Increase in (a) UTS values by 17% to 29% and (b) *E* values by 32% (series XI) and 37% (XII) noted after TA additional cross-linking

results obtained for samples cross-linked by GA alone and GA + TA shows a marked change in mechanical properties (Fig. 4). The samples were stiffer under low stresses, resulting in lower strains values before the transition phase. The specimens underwent failures at higher values of both stress and strain as well. The values of elongation at break also increased in comparison with GA cross-linked tissue.

Additional TA cross-linking increases pericardium mechanical properties according to the UTS or E values. As presented in Fig. 4, for series X (0.6% GA and 0.3% TA, 4°C), a slight decrease in the E value was noted after TA cross-linking. The concentration of used TA was relatively low (0.3%). It is possible that higher concentration of this agent would further increase the mechanical properties of the pericardium.

3.2. Constitutive models

Stress–strain curve for native collagenous tissue such as porcine pericardium is typically S-shaped [22] or J-shaped [8]. Chemically cross-linked tissue samples were fitted by two hyperelastic models, anisotropic (Fung's model) and isotropic (Ogden's model), with very high reproducibility; R^2 over 90% (Tables 3 and 4). Although Fung's model has more parameters than Ogden's, almost all cases fitted the stress-strain curves with comparable accuracy independently to the used models. Moreover, for Fung's model for series IX (3% GA, 37 °C, Fig. 5i), fitting of curves with extended toe region and transition area rapidly increased was problematic. Due to the lower number of parameters, good capture of tissue behaviour under load conditions regardless of the stress–strain curve

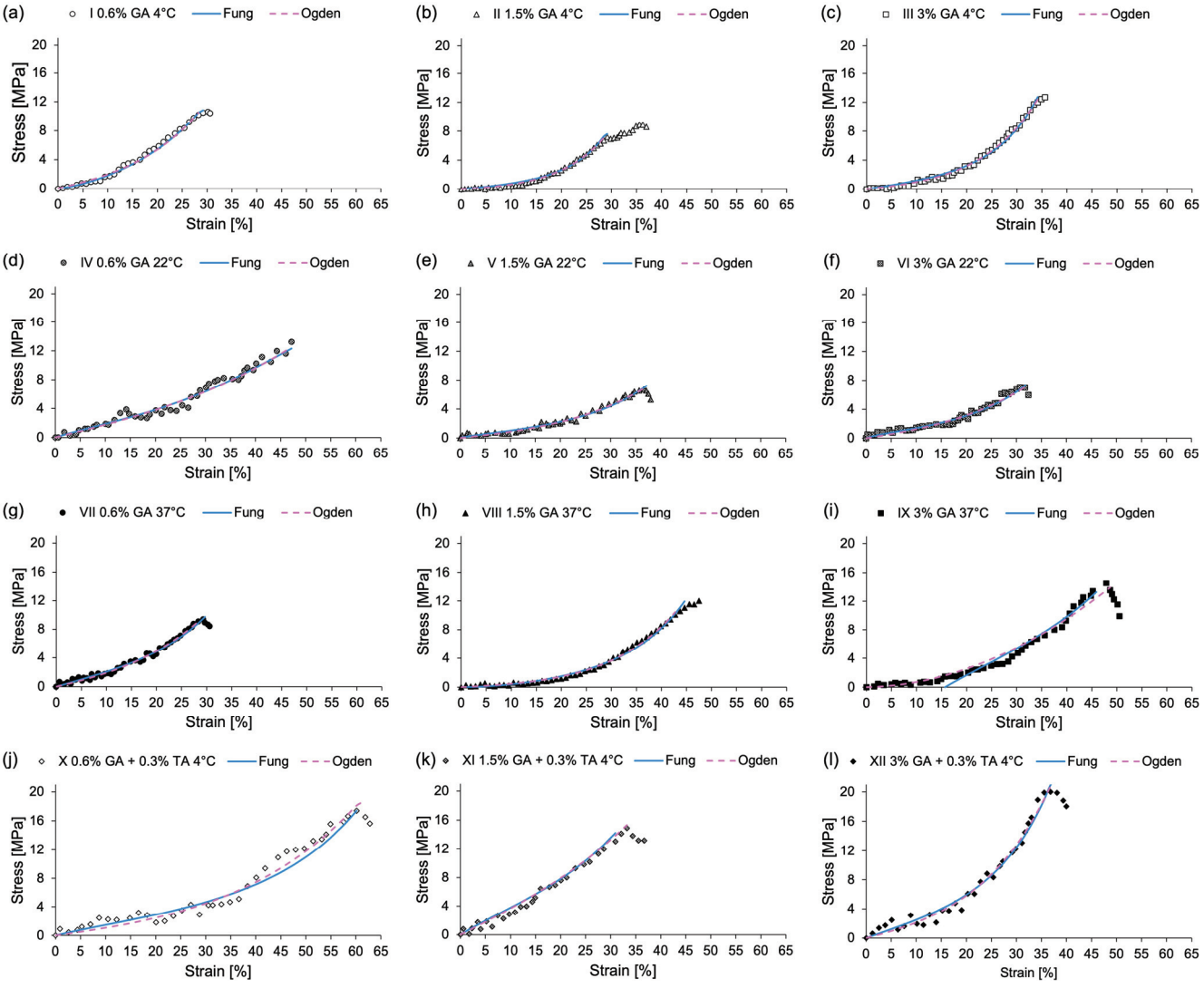

Fig. 5. Stress–strain curves obtained for cross-linked samples of pericardium and fitted by Fung's model and Ogden's model

Table 3. Model parameters determined for Fung's model

Series	Cross-linking agent(s)	$\overline{c}$ ± SD [kPa]	$\overline{b_1}$ ± SD	$\overline{b_2}$ ± SD	$\overline{b_3}$ ± SD	$\overline{b_4}$ ± SD	$\overline{b_5}$ ± SD	$\overline{b_6}$ ± SD	$\overline{R^2}$ ± SD
I	0.6% GA	5.0 ± 3.3	3.6 ± 2.3	–1.2 ± 2.9	–5.6 ± 13	1.9 ± 5.3	3.5 ± 8.5	–1.3 ± 3.5	0.9767 ± 0.008
II	1.5% GA	3.5 ± 2.6	4.0 ± 3.8	–4.2 ± 5.9	–1.2 ± 4.8	–0.01 ± 0.64	2.7 ± 4.4	0.3 ± 2.4	0.97 ± 0.01
III	3% GA	2.7 ± 1.2	0.4 ± 4.7	0.2 ± 1.4	1.0 ± 2.0	–0.6 ± 4.6	4.5 ± 4.9	–3.3 ± 4.8	0.959 ± 0.026
IV	0.6% GA	2.7 ± 1.4	3.1 ± 3.1	1.2 ± 5.4	–0.3 ± 1.6	0.2 ± 2.5	1.9 ± 3.4	–1.8 ± 2.5	0.941 ± 0.027
V	1.5% GA	2.7 ± 1.7	1.5 ± 2.1	3.7 ± 2.2	–0.9 ± 1.8	–0.6 ± 1.1	3.4 ± 2.1	–3.0 ± 2.1	0.935 ± 0.044
VI	3% GA	2.77 ± 0.90	3.4 ± 1.6	–1.5 ± 3.2	0.4 ± 1.5	–0.02 ± 0.99	1.7 ± 2.7	0.5 ± 2.7	0.957 ± 0.015
VII	0.6% GA	4.1 ± 5.3	3.5 ± 2.2	–1.4 ± 4.1	–2.2 ± 4.1	–2.6 ± 1.7	3.0 ± 2.9	–1.8 ± 2.6	0.963 ± 0.018
VIII	1.5% GA	8.0 ± 12.6	3.5 ± 3.0	–10.5 ± 23.9	–0.7 ± 2.7	–1.6 ± 3.6	1.3 ± 1.6	1.25 ± 0.98	0.970 ± 0.022
IX	3% GA	8.7 ± 7.2	1.3 ± 2.1	0.9 ± 2.6	0.4 ± 1.4	–1.9 ± 2.0	1.5 ± 1.2	–1.8 ± 1.2	0.952 ± 0.036
X	0.6% GA 0.3% TA	3.6 ± 1.3	1.9 ± 1.2	1.0 ± 1.3	1.0 ± 1.4	–0.33 ± 0.44	2.5 ± 1.8	–0.7 ± 1.2	0.957 ± 0.006
XI	1.5% GA 0.3% TA	644 ± 1432	1.7 ± 3.1	2.9 ± 3.0	1.2 ± 1.6	–2.5 ± 3.1	6.2 ± 2.6	–3.5 ± 2.7	0.904 ± 0.073
XII	3% GA 0.3% TA	3.18 ± 0.54	2.9 ± 1.7	1.7 ± 1.3	1.3 ± 1.1	–2.11 ± 0.60	1.69 ± 0.75	–2.2 ± 1.2	0.957 ± 0.014

progression, and very good compliance of the model fit with the experimental data, the use of Ogden's model is justified and satisfactory.

Table 4. Model parameters determined for Ogden's model

Series	Cross-linking agent(s)	$\overline{\alpha_1}$ ± SD	$\overline{\mu_1}$ ± SD	$\overline{R^2}$ ± SD
I	0.6% GA	10.6 ± 1.3	0.96 ± 0.64	0.9799 ± 0.009
II	1.5% GA	10.2 ± 3.5	0.84 ± 0.80	0.975 ± 0.014
III	3% GA	9.2 ± 2.3	1.8 ± 1.1	0.966 ± 0.024
IV	0.6% GA	9.9 ± 2.5	1.41 ± 0.90	0.951 ± 0.019
V	1.5% GA	8.1 ± 3.6	1.31 ± 0.90	0.937 ± 0.043
VI	3% GA	9.6 ± 1.1	0.80 ± 0.39	0.955 ± 0.014
VII	0.6% GA	10.5 ± 1.9	1.1 ± 0.8	0.962 ± 0.019
VIII	1.5% GA	11.3 ± 2.5	1.1 ± 1.7	0.966 ± 0.026
IX	3% GA	7.4 ± 4.2	4.2 ± 3.5	0.962 ± 0.034
X	0.6% GA 0.3% TA	8.5 ± 1.1	1.07 ± 0.31	0.9606 ± 0.005
XI	1.5% GA 0.3% TA	7.0 ± 3.0	3.3 ± 1.4	0.91 ± 0.071
XII	3% GA 0.3% TA	11.4 ± 3.1	1.2 ± 0.4	0.963 ± 0.011

4. Discussion

Stress–strain curve for native collagenous tissue such as porcine pericardium is typically S-shaped [22] or J-shaped [8], but chemically cross-linked tissue might act differently. At the beginning (toe region), collagen fibres oriented parallel to the axis of tension are lengthened, and those randomly oriented are strengthened. At some point, fibres become taut, what results in the increase of stress (heel region) [25]. In the linear region of the stress–strain curve, some of the fibres slide against each other or tear [22]. As Olde Damink et al. [25] stated for sheep skin, GA modification affects the formation of cross-links within the fibre rather than between several fibres. Due to that, the sliding will not be affected by GA modification, but additional bonds inside the fibres might reduce the tearing of fibres. This hypothesis would be proven in this study by observing a decrease in stiffness of GA cross-linked tissue and an increase in UTS values, caused by formed cross-links within collagen fibres. According to the results of uniaxial tensile tests of porcine pericardium by Arbeiter et al. [2], the mechanical properties of tested tissue shifted to a more elastic behaviour as a result of cross-linking (4% GA, 24 h), even though the process was significantly shorter than in this research. Elastic modulus for 4% GA cross-linked tissue decreased by 65% in comparison to the native tissue (133 ± 15 MPa). No differences were noted between UTS values for native and GA-treated pericardium. On the other hand, due to Zouhair et al. [40], GA cross-linking significantly influenced the collagen-phase modulus of bovine pericardium causing its increase. Also a slight increase of the UTS values and decrease of the failure strain were noted.

GA concentration

This study found that the strength of tissue cross-linked with the lowest used concentration of GA (0.6%) was similar to that treated with 3% GA. Mechanical properties of series II, V and XI treated with 1.5% GA were lower than the values of these parameters for series treated with 0.6% (I, IV, X). In

comparison with other concentrations, 1.5% GA was not as effective as expected.

No general relationship between the increase in the concentration of the cross-linking solution and the improvement in tissue strength was found. Bodnar and Frater [13] stated that the use of higher aldehyde concentrations might cause excessive tissue stiffening. In contrast, Zilla et al. [38], [39] reported that the optimal concentrations of GA during aortas cross-linking process should be higher than 1%, because of the increased susceptibility to calcification of tissue cross-linked with low GA concentrations. During the cross-linking procedure, the balance between mechanical properties and a tendency for calcium-binding should be kept.

Temperature

The lowest values of E and UTS have been reported for series IV–VI fixed at room temperature (22 °C). Similar conclusions were published by Zilla et al. [39] for aortic walls. It was suggested that the best results are expected by cross-linking at 4 °C, which is confirmed by values shown in Table 2. *E* for tissue cross--linked at 4 °C with 0.6% GA (series I) and 3% GA (series III) was higher than for series VII–IX treated at 37 °C. The highest UTS values were reported for tissue treated with GA at 4°C and 37 °C, and the values were comparable. Focusing on stress–strain relationships, the standard S-shaped curves were preserved (Fig. 3a) for specimens cross-linked at 4 °C (series I–III). In comparison, series cross-linked at 37 °C (VIII, 1.5% GA and IX, 3% GA) have shown an elongated transition phase (Fig. 3c). Thereby, more time was required to align fibres and begin the process of stress transmission along the sample [25].

Due to the important role of water in keeping the mechanical properties of collagen and the fact that the cross-linking agents were water solutions, the influence of water on cross-linked tissue behaviour ought to be considered. It was reported by Jastrzębska et al. [18] that GA cross-linked porcine pericardial tissue is significantly more hydrated than native. Water acts as a plasticiser [10], [12], therefore, the decrease in values of elastic modulus after GA cross-linking was predicted. In this study, *E* was lower even up to 77% for series V (1.5% GA, 22 °C) and VI (3% GA, 22 °C) in comparison to native tissue [2].

As observed by Jastrzębska et al. [18], the increase in the hydration level is much higher between triple helices than in extra-fibrillar or extra-fibre spaces. At low temperatures, water molecules may form pentagonal structures between neighbouring collagen triple helices in the form of water bridges. The highest density of water at 4 °C might have led to more efficient cross-linking due to the decrease in space between water molecules. It could have facilitated either GA or water to bind to collagen fibres, introduce additional cross-links and, in effect, improve the durability of the pericardial tissue. The ability to carry higher stresses with lower deformations in the initial regions of stress-strain curves, observed for series I–III cross-linked at 4 °C (Fig. 3a) may suggest that the strength of collagen fibres was improved [25].

As Leikina et al. [21] reported, collagen type I may be unstable even at 36 °C. Collagen in pericardium could have unfolded at 37 °C and exposed additional space for large molecules of GA or water to penetrate the tissue. As claimed by Olde Damink et al. [25], an elongated transition phase observed for series VIII (1.5% GA, 37 °C) and IX (3% GA, 37 °C) (Fig. c) may imply the presence of cross-links between fibre bundles, not only within collagen triple helices. As the water bridges are unexpected to form in spaces between adjacent fibrils or fibres [18], it may be hypothesised that GA covalent bonds were introduced with the highest effectiveness during cross-linking at 37 °C. Covalent bonds are stronger than hydrogen ones [22] and it may be noticed after comparison of higher UTS values for series VIII (1.5% GA, 37 °C) or IX (3% GA, 37 °C) and respectively of series II (1.5% GA, 4 °C) or III (3% GA, 4 °C). Only for tissue cross-linked at 37 °C a dependence between mechanical parameters and agent's concentration was observed. It could be helpful to use this relation while obtaining a biomaterial with specific mechanical properties.

Walrafen and Chu [36] claimed that bulk water pack more efficiently into polyhedral structures at room temperature in comparison to cool water. Due to that, free water between collagen fibrils could have formed pentagonal structures instead bonding to the protein. In addition to that, the interaxial spacing between collagen triple helices in native tissue decreases while heating the collagen, so the molecules bigger in size could not fit into those spaces. According to Jastrzębska et al. [18], the interaxial distance should increase with the formation of collagen-GA cross-links but, nonetheless, bonding with GA could be more difficult at 22 °C what in effect might have caused lower mechanical properties of tested pericardium.

The role of glycosaminoglycans (GAGs) may be diminished in this case due to the reports proving that GA cross-linking is unable to stabilize GAGs within the collagenous tissue [29].

TA additional treatment

Additional TA cross-linking had a positive influence on pericardium strength, which may be gathered from the UTS or *E* values (Table 2). The increase in UTS values has been reported for all TA cross-linked specimens and was equal 17, 29 and 29% for series X–XII, respectively. The highest elastic modulus among cross-linked specimens has been reported at 78 ± 26 MPa (series XII). That value is still by 41% lower than for untreated native tissue [2]. In comparison with series II–III treated only with GA, *E* values for series XI–XII increased by 32% (XI) and 37% (XII), as presented in Fig. 4.

Velmurugan et al. [35] investigated the interaction of TA and collagen extracted from rat tail tendon. According to the study, TA has the ability to form hydrogen bonds with bulk water between collagen fibrils, therefore, in the presence of TA, collagenous samples possess excess amount of water. In effect, the mechanical behaviour of tissue may be shifted to more elastic. It may be possible that the water gathered by GA in spaces between triple helices [19], as mentioned before, was bonded by TA during an additional cross-linking process. This hypothesis may be easily tested in a future study by a swap of cross-linking agents' order.

Cwalina et al. reported that prolonged TA modification results in a more cross-linked biomaterial [10] and also slighter looseness of connective fibres [11]. Consequently, the concentration of TA might affect the mechanical properties of tissue. Sionkowska et al. [32] noted a relation between the concentration of TA and material stiffness, for collagen extracted from rat tail tendon. The use of high concentrations of cross-linking agent, i.e., 5, 10, 20 wt.%, increased the ability of tested samples to withstand higher compression loads [3], [32]. As it was noted before, the concentration of TA used in the current study was relatively low (0.3%). It is necessary to examine the relation between the concentration of TA as a cross-linking agent and the mechanical properties of the pericardium.

Wang et al. [37] reported that TA treatment effectively mitigated the calcification of bovine pericardium treated with GA at first. What is more, the shrinkage temperature of GA-TA cross-linked tissue was higher and tissue resistance to enzyme degradation was also improved. Those advantages and higher values of mechanical properties in comparison to GA cross-linked issue may be key features considered while choosing the combined cross-linking method. Even though, according to Zilla et al. [38], the tissue cross-linked with a low concentration of GA may tend to calcify, an additional TA post-treatment may mitigate the unwanted process and, in effect, prolong the process lifespan of implanted BHVs. The combination of GA and TA may be a solution for seeking a balance between the durability and strength of a cross-linked biomaterial, cytotoxicity and tendency to calcify.

5. Conclusions

Both the temperature and the concentration of cross-linking agent affect the mechanical properties of the porcine pericardium. Physical properties of water and collagen structure vary with temperature, determining the effectiveness of cross-linking process. Room temperature (22 °C) occurred to be ineffective as a cross-linking environment. As the mechanical properties for series treated at 4 °C and 37 °C were comparable, both temperatures may be recommended for setting the conditions of cross-linking procedure. Introducing the temperature as an important factor during pericardium cross-linking process may positively influence the tissue properties, so that the required treatment duration could be reduced in the future proceedings. The use of low GA concentrations may be satisfactory, as 0.6% GA resulted in similar mechanical properties as tissue modified with the highest tested concentration (3%). Considering only the mechanical properties tested in this study, the concentration of GA may be reduced without the significant reduce in strength or durability of porcine pericardium, especially during cross-linking at 4 °C. The resistance of GA-treated pericardial tissue may be further improved by an additional TA treatment, which resulted in an increase of mechanical properties of specimens tested in this study, even if the concentration of agent was relatively low (0.3%). As a non-toxic natural compound, TA may be applied as an additional cross-linking agent during the processing of porcine pericardium used for the creation of BHVs' leaflets.

Limitations

The study has a few limitations. First, native pericardial tissue was not tested in this study therefore, the mechanical properties of native pericardium were obtained from the literature [2] to compare. All tested samples were cut out in parallel to the collagen main fibres orientation. Confirmed anisotropy of pericar-

dium by other Authors [28] was not tested mainly due to proven GA treatment mechanism leading to cross-linking collagen fibres on triple helix level while the alteration in architecture of matrix (between fibers) is not sufficient. The numbers of samples in particular series are low, unable to reduce the variation within obtained values of mechanical properties and grasp the difference on a significant level. Moreover, increasing the number of samples could minimize the meaning of the influence of anatomical differences of tissue on mechanical properties. Uniaxial tensile testing provided the basic mechanical properties of cross-linked pericardial tissue, necessary to compare the strength of multiple series and determine the direction for further research. It must be noted that performed tests are insufficient to establish how the material would work under the multi-modal loading pattern that BHV's leaflet exhibits after implantation. However, it provides fundamental materials data necessary for finite element analysis of complex behaviour of BHVs under hydrodynamical loadings conditions.

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