THE INFLUENCE OF TANNIC ACID ON STABILIZATION EFFECT OF THE PERICARDIUM TISSUE

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Abstract: Porcine pericardium (PP) tissues for bioprostheses manufacture are modified owing to cross-linking of proteins, mainly of collagen in the tissue. Tannic acid (TA) may be used for obtaining stable biomaterial. In the present work, changes in PP tissue stability have been investigated using electrophoretic and histological methods. Infrared (IR) spectroscopy and atomic force microscopy (AFM) were used for study of the tissue structure and chemical bonds involved in TA-collagen cross-linking. Results of electrophoretic studies show that TA-crosslinked PP was more resistant to proteins extraction as compared with the native one, confirming higher stability of modified tissue. Histological images did not show essential changes in the tissue morphology, apart from straightening of fibrous structure. Considerable differences between collagen topography in native and cross-linked tissues were documented by AFM-studies that show the fibres widening. IR studies show shifting of the bands representing amide I and amide II in the collagen spectra obtained for modified tissues as compared with the native ones. In conclusion it may be stated that the pericardium tissue modification with TA causes its stabilization due to formation of cross-links between TA and tissular collagen, leading to changes in structure of the collagen fibers present in the tissue.

Introduction

Some animal connective tissues including bovine and porcine pericardium (PP) are widely used for bioprostheses manufacture. These tissues are composed mainly of collagen, although other proteins (for example elastin) and glycosaminoglycans (GAGs) are also present in their structure.

The tissue stabilization using various chemical and/or physical methods is necessary for obtaining stable biomaterial [1]. Glutaraldehyde (GA) is the chemical reagent which is most often used for this purpose due to cross-linking of proteins (especially of collagen) in the tissue. However, the tissue GA-fixation introduces *in vivo* premature degradation and cytotoxicity of bioprostheses [2-4]. Many alternative procedures for preserving tissues are being explored to reduce or eliminate the undesired side-effects of GA-treatment of tissues. Some chemical compounds of natural origin like tannic acid (TA) may also be used for this purpose [5-7].

The aim of the present work was to study changes in the PP tissue stability (using electrophoretic and histological methods) as well as changes in structure and chemical bonds involved in TA-collagen cross-linking, using infrared (IR) spectroscopy and atomic force microscopy (AFM).

Materials and Methods

Pericardium tissues: Investigations were performed for the porcine pericardium (PP) tissues obtained from the local abattoir directly after animal slaughtering. The tissues were immediately rinsed in cooled phosphate-buffered saline (PBS; pH 7.4) at 4°C. Tissular fat, heavy vasculatures and ligaments were removed before modification. This pre-treatment procedure did not cause any significant changes in the tissue structure. Modification of the PP tissue was carried out in the presence of 2% TA (Sigma) solution in H_2O, at 4°C, for 4, 24 or 48 hours, at dark.

Electrophoresis: Before electrophoresis, native and modified samples (1 g) were homogenised in 50 ml of water (Polytron ® PT 2100 - Kinematica AG). Aliquots of 1.5 ml of tissular homogenates were collected and concentrated by centrifugation (14000 x g) for 10 minutes to obtain samples of 0.5 ml volume. Native and TA-modified tissues were subjected to the SDS/NaCl extraction procedure performed according to Laemmli [8]. The tissue samples (15µl) were stiffened in 4% gel (voltage 70 V) and then peptides were separated in 10% gel (140 V) (Minipol, Kucharczyk T.E.). Peptides in electrophoregrams stained with Coomassie Brillant Blue were analyzed using Scangel 1.45 program (Kucharczyk T.E.). For destaining, gels were incubated in the same solution without dye. The qualitative analyses of the peptides were performed using Biotec Fischer System.

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Histology: Tissue samples for histological studies were dehydrated in absolute ethanol, and then embedded in paraffin wax. Six micron samples were stained routinely with Harris hematoxylin (background stained with 1% erythrosine solution) or aldehyde fuchsine. Histological observations were carried out under optical microscope Polyvar 2 (Leica), at magnitude 400×. Preparations were documented in Quantimet 500 Plus system.

Atomic force microscopy (AFM): The tissue surfaces were studied using atomic force microscope NanoScope E (Digital Instruments, USA), working in the contact mode and equipped with OTR8 probe (Veeco NanoProbe™). The length and the spring constant of the applied V-shaped cantilever were 200 μm and 0.15 N/m, respectively. The constant forces used were about 10 nN. All measurements were performed in air, at room temperature. Two standard AFM signals were registered: the signal corresponding to the topography of the sample (height) and the differential signal (deflection) that is sometimes more useful in direct observations [9, 10]. Obtained images were processed using the software package WSxM (Nanotec Electronica).

Infrared (IR) spectroscopy: IR spectra were recorded by spectrometer BIORAD FTS 6000, with resolution of 2,0 cm^{-1}, in frequency range of 700-4500 cm^{-1}.

Results and discussion

Results of electrophoretic studies have been presented in Table 1 as molecular weights of proteins extracted from the pericardium tissues, both native and treated with TA during 4, 24 or 48 hours. It has been found that the tissues treatment with SDS/NaCl leads to release of peptides in the molecular weight range of 34-217 kDa. The TA-crosslinked PP tissues were more resistant to proteins extraction as compared with the native ones, confirming higher stability of modified tissues.

Structure of the native tissue stained with hematoxylin and erythrosine has been presented in Figure 1. Structure of TA-modified tissue after staining in the same manner has been revealed in Figure 2. Both the images did not show essential changes in morphology of the PP tissue being treated with TA, apart from straightening of fibrous structure.

Table 1: Molecular weights of proteins extracted from the porcine pericardium tissues: native (N) and treated with TA during 4, 24 or 48 hours (TA 4, TA 24, TA 48).

Tissue	**Molecular weights of peptides extracted from porcine pericardium [kDa]**								
N	207	185	124	106	93	64	63	57	34
TA 4	214	195	135		95	64	63	53	34
TA 24	217	192	131			64	63	57	
TA 48	205	180	127			64		57	

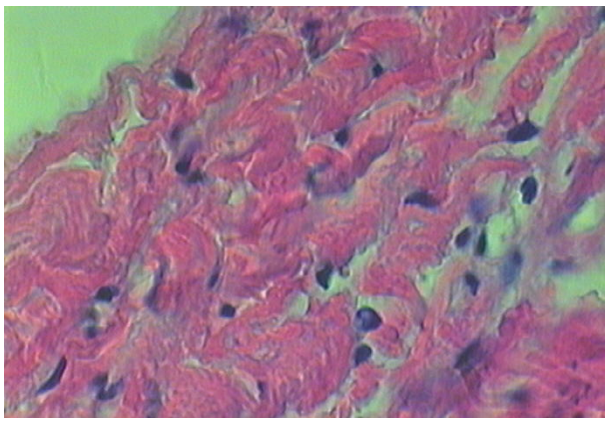

Figure 1: Native PP tissue stained with hematoxylin and erythrosine (magnitude 400×). Tight structure of the tissue. Evident slits and discrete swelling of extracellular matrix. Presence of fibroblasts.

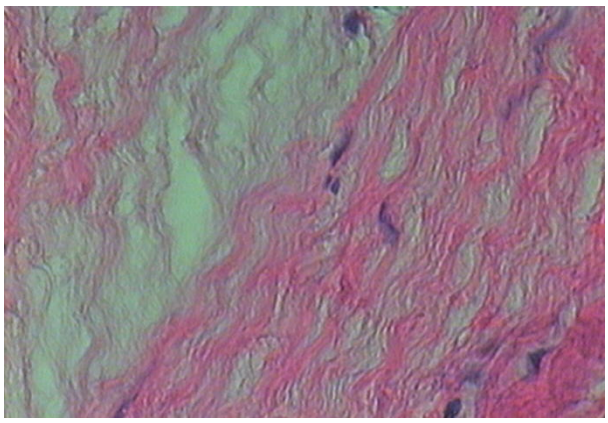

Figure 2: The PP tissue modified with TA for 4 h, stained with hematoxylin and erythrosine (magnitude 400×). Tight and regular structure of the tissue. Evident slits of extracellular matrix. Presence of fibroblasts.

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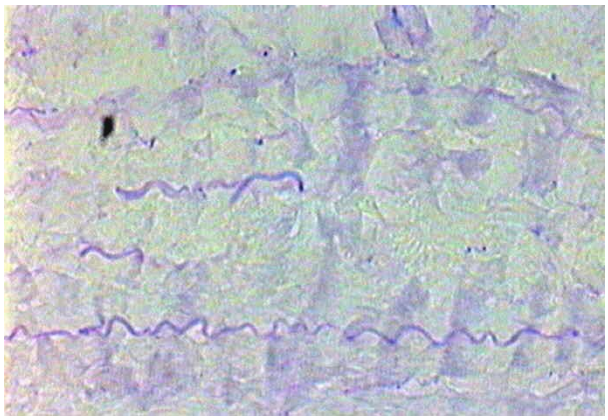

Figure 3: Native PP tissue stained with aldehyde fuchsine (magnitude 400×). Tight structure of the tissue. Visible undulated elastic fibres. Fibroblasts invisible.

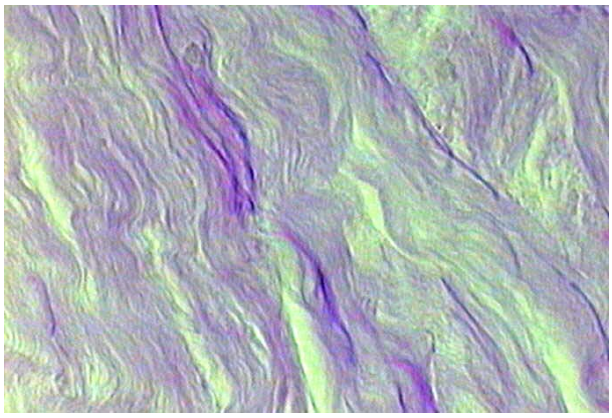

Figure 4: The PP tissue modified with TA for 4 h, stained with aldehyde fuchsine (magnitude 400×). Regular structure of the tissue. Disappearance of the elastic fibres undulation.

Use of two methods of the PP tissues staining enabled us to obtain different images showing different components of the PP tissues. Primary structure of the tissue is visible after its staining with hematoxylin and erythrosine (Figures 1 and 2). Images presented in Figures 3 and 4 show structural components, mainly elastic fibres, which become visible after the tissues staining with aldehyde fuchsine (AF). Fibroblasts are invisible in AF-stained tissues, both native (Figure 3) and modified with TA (Figure 4). Besides, differences have been shown between the PP tissues: native composed of undulated elastic fibres (Figure 3) and stabilized with TA, in which disappearance of the elastic fibres undulation has been shown (Figure 4).

The AFM deflection images show changes in topography of the collagen fibrils present in PP tissue after its modification with TA (Figure 5).

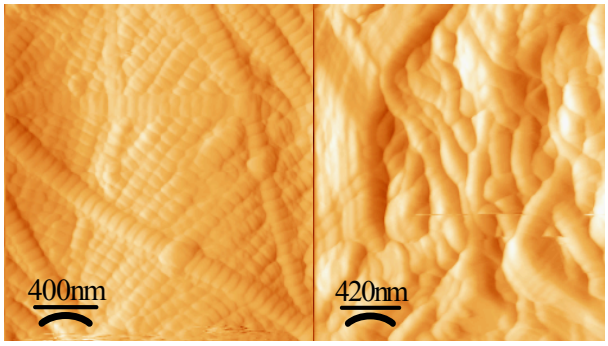

Figure 5: AFM deflection images for PP tissues: native (left) and modified with TA for 4 h (right).

The crimp structure of regular collagen fibrils was distinctly seen for the native tissue, whereas broadening of fibres was observed in the TA-modified tissue.

The results of IR studies have been shown in Figure 6, as IR spectra of collagen present in native and TA-modified PP tissue. IR absorption is sensitive to vibrations in hydrogen bonding and can be used to asses protein conformation influenced by H-bonds formation. The absorption profiles are typical for tissue infrared spectra which are dominated by protein amide I, amide II and amide III absorptions in the 1000-1700 cm^{-1} range. Differences in the spectra of native and modified tissues have been found in amide I vibrations attributed to the carbonyl C=O mode. Amide I frequency for modified pericardium are localized at higher frequencies (from 1654 to 1658 cm^{-1}) in comparison with native material. The shift suggests that intramolecular hydrogen bonds in the pericardium tissue after modification with tannin are stronger and shorter as compared with the native tissue. In the region 2000-4000 cm^{-1}, we observed around 3500 cm^{-1} peak from free water vibration after TA stabilization. These changes suggest increase in stability of collagen helical conformation with hydrogen bonds between collagen, water and TA.

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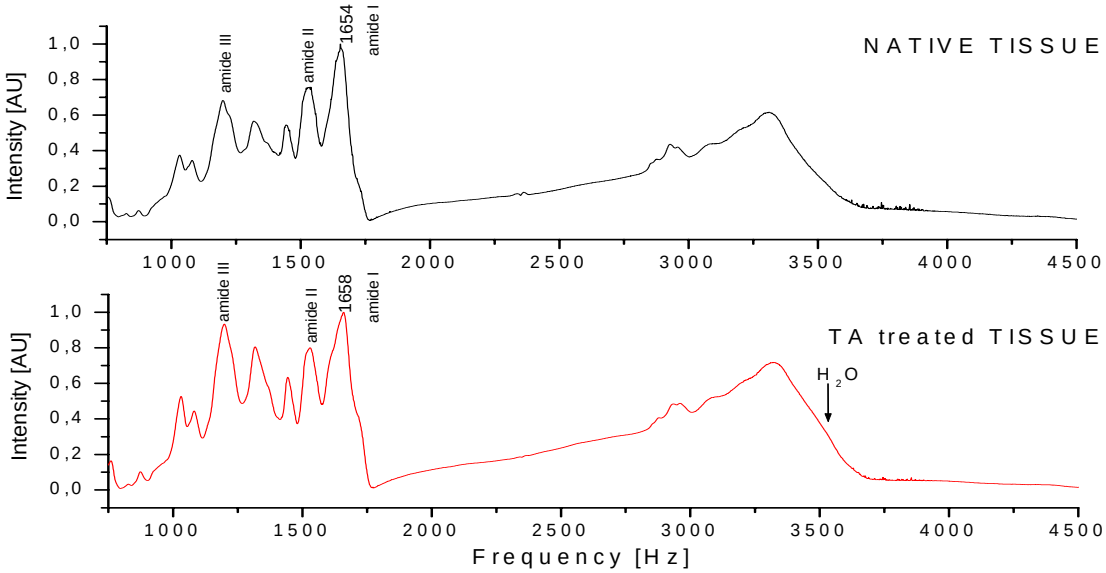

Figure 6: IR spectra of collagen present in PP tissues: native and TA-modified for 4 h.

Conclusions

1. The PP tissues: native and modified with the tannic acid (TA) for 4 h, examined under optical microscope did not show essential changes in morphology after the tissue treatment with TA.
2. Straightening of the PP tissue fibrous structure and disappearance of the elastic fibres undulation are main results of the tissue treatment with TA.
3. Electrophoretic investigations show that TA-treated PP tissue was less susceptible to protein extraction as compared with the native tissue.
4. Widening of the collagen fibres was shown in AFM studies as a result of PP tissue treatment with TA.

Acknowledgements

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