Radioisotopic investigation of crosslinking density in bovine pericardium used as a biomaterial

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Abstract. Stabilized bovine pericardium (BP) belongs to tissues routinely applied in production of heart valves. Commercial products are manufactured from tissues crosslinked by glutaraldehyde (GA). Dye-mediated photooxidation was also proposed as an alternative method, which allows for the elimination of clinical failures of GA-treated tissues. The aim of the study was to investigate the density of BP stabilized by GA and the methylene blue-mediated photooxidation, as compared with a native tissue. Crosslinking density was evaluated based on their ability to accumulate radioactive cobalt ions ($^{60}Co^{2+}$) and the permeability to these ions. Radioactivity was examined using a γ-spectrophotometer (Packard). The results showed the changes in the crosslinking density between the native tissues and photooxidized or GA-crosslinked tissues. Significant decreases in radioactivity were detected only in the thinnest tissues after photooxidation and in filtrates penetrating the same samples. Photooxidized pericardium of a larger thickness did not reveal any significant changes. Weight-dependence for the permeability was observed in the case of filtrates penetrating the GA-treated tissues. However, $^{60}Co^{2+}$-accumulation in these samples remained at the same level. Photooxidation may lead to obtaining biomaterials with advantageous properties, i.e. a decreased calcium-binding capacity. Photooxidation efficiencies were dependent both on compactness and thickness of tissues and on process duration. It should be emphasized that the tissues' structure after photooxidation was characterized by lower density. This may point to the presence of crosslinks of a smaller compactness in comparison with GA treatment. It has been shown that the factor indeed influencing the result of crosslinking is tissue thickness.

Key words: bioprostheses • bovine pericardium (BP) • crosslinking • methylene blue (MB) • photooxidation • radioisotopic investigations

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Background

Stabilized collagenous tissues belong to the biomaterials relatively frequently used in medicine. Under native conditions, both intra- and intermolecular natural crosslinks significantly contribute to the stability of tissues and organs [15, 24].

Since the late 60s of the 20th century, animal tissues have often been used because of the general deficit of autogeneic and allogeneic materials [5, 33]. Bovine pericardium (BP) is one of animal tissues applied in the production of biological heart valves [35]. BP is composed of two distinct elements, i.e. the visceral or serous layer and the parietal multi-layered connective tissue including the fibrous tissue. Commercial products are manufactured from tissues stabilized by glutaraldehyde (GA) treatment. Alternatively, photooxidation processes were proposed [33, 35, 39, 43]. These stabilization processes are achieved by the crosslinking reactions, which result in the introduction of additional crosslinks into the tissue structure. Thereby, crosslinking influences the decrease in their susceptibility to widely understood degradation and a

decrease of immunological response [12, 28, 35]. It is particularly important in the case of xenografts.

In our earlier works we have shown that stabilized tissues demonstrate an increased strength and simultaneously a decreased chemical degradability, including lower susceptibility to enzymatic digestion as well as extraction of polypeptides by sodium dodecyl sulphate [10, 11, 20, 41].

Tissue stability is usually reached via crosslinking reactions with GA [23]. However, in the last decade attention has been paid to some methods that use alternative processes, including irradiation [4, 18, 35, 39], because of clinical failures associated with implantation of GA-treated tissues, i.e. premature calcification, cytotoxicity, and excessive stiffness [17, 19, 27, 31, 38]. The disadvantages of GA-treated tissues are a result of GA polymerization and reaction mechanism. Tissues are modified by various forms of GA which are usually present in aqueous solutions, i.e. monomers, dimers, trimers, products of aldol condensation and polymers [6–8, 16, 17, 30, 44]. The variety of GA forms influences the crosslinking mechanism and effect (Fig. 1a).

The tissues' photooxidation leads to the formation of structures quite different from the GA-induced ones. Mechanisms of these processes are still not completely recognized. However, it was shown that in the tissues exposed to irradiation, generation of free radicals in the residues of aromatic acids [14] and interaction between the residues of amino acids took place (Fig. 1b) [29]. Dye-mediated photooxidation seems to
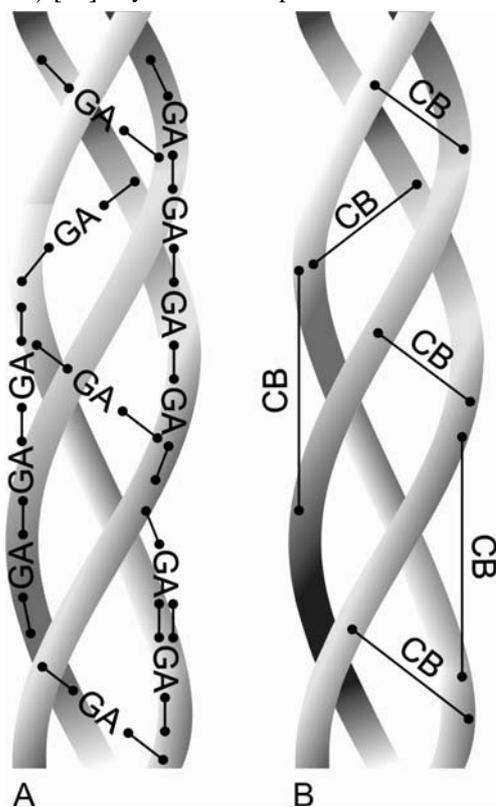
be an especially interesting method stabilizing the tissue structure. The most important advantage of this method is the formation of covalent bonds between amino acids without introducing "cytotoxic linkers". Covalent bonds (CB) are formed directly between amino acid residues (Fig. 1b). Moreover, this stabilization process does not contribute to excessive stiffness of the tissue [32, 40] and its premature calcification [2, 43].

The aim of the present study was to investigate (by means of the radioisotopic method) changes in tissue density of native BP, GA-crosslinked and photooxidized in the presence of methylene blue (MB). Crosslinking density in the tissue was evaluated by determination of the accumulation effect of radioactive cobalt ions ($^{60}Co^{2+}$) and tissue permeability to these ions.

Experimental

Materials

BP came from hearts of 5–6 month old domestic cattle (*Bos taurus*) which were obtained from the local abattoir. The tissues were collected according to the protocol of multiple organ procurement adopted from the human model [36]. Next, the tissues were transported to the laboratory in the sterile and cooled (4°C) buffered physiological saline solution (PBS; pH 6.5; Sigma). The procedures of pericardium selection for bioprosthetic heart valves described by Simionescu and co-workers were used [37]. Moreover, fibrous pericardium was mechanically separated from other parts of the pericardial sac. This intervention did not cause any damage of the tissue structure. Before stabilization, tissue fat, heavy vasculatures and ligaments were removed.

Pericardial tissues have a varying thickness throughout their area. This depends on the number of collagen layers, the density of slits in tissues, and water content. Thus, the differences in thickness and weights of the prepared samples in our experiments were unavoidable. Taking into account these differences and the fact that no measurement method is perfect for determining the pericardium tissue thickness [26], we used a special procedure for the selection of samples for testing as described below. We assumed that the samples of BP which had the same weight and permeability to the PBS solution were the tissues having the same thickness and density of structure, while the samples of BP which had different weights and simultaneously did not differ with respect to permeability of the PBS solution represented tissues of the same structure type but of different thickness (the higher the weight the higher the thickness) [9].

Methods

Experimental procedures

The experiments were carried out in three main stages: (i) selection of BP samples; from among the tissue pieces (20 mm in diameter) of equal weights, we selected those demonstrating identical permeability to the PBS solution during 24 h; as a result, we obtained three groups

Fig. 1. A schematic diagram of crosslinks that may occur in collagenous tissues: after stabilization using various forms of glutaraldehyde (GA), i.e. a monomer (-GA-), a dimer (-GA--GA-), a trimer (-GA-GA-GA-), a product of aldol condensation (-GA=GA-) and a polymer (-GA-GA-GA-GA-) (A); after photooxidation leading to the formation of covalent bonds (CB) between the amino acid residues (B).

of samples with the weight of 0.14 ± 0.01 g ($n = 8$), 0.21 ± 0.02 g ($n = 8$) and 0.29 ± 0.03 g ($n = 7$) designed for photooxidation studies as well as four groups of samples with the weight of 0.12 ± 0.01 g ($n = 8$), 0.14 ± 0.03 g ($n = 8$), 0.15 ± 0.01 g ($n = 8$) and 0.16 ± 0.02 g ($n = 7$) designed for GA treatment; (ii) stabilization (using MB-mediated photooxidation or GA crosslinking) of tissue samples demonstrating equal weight and permeability to the PBS solution; (iii) radioisotopic assays for testing the binding capacity of the pericardium samples (native, photooxidized and GA-treated) and their permeability to $^{60}Co^{2+}$ [9].

Permeability of tissues to PBS

Tissue permeability to PBS was evaluated based on the rate of penetration of this solution through them [9]. Tissue samples (20 mm in diameter) were attached via a ring on the bottom end of the flow column which was filled with 25 cm^3 of PBS. The column was placed upright in a glass beaker with 100 cm^3 of PBS. The PBS solution flows by gravity through the tissue to the beaker. Some similarly functioning research systems have been used recently for testing the permeability of biomaterial membranes [13].

MB-mediated photooxidation

The photooxidation process was carried out during 5, 15, 30, 45, 60, 90 and 120 min, using visible light (12 W light bulb) and a 0.05% solution of MB (Sigma) in PBS (pH 6.5), at a temperature of 23°C. Before photooxidation, samples of BP were soaked in a 50% solution of saccharose (Sigma) in PBS for 1 h. Then, the tissues were pre-incubated in the presence of MB and cleaned air. Finally, the samples were irradiated in the presence of the dye and cleaned air. The distance between the light source and the surface of the dye solution was 15 cm.

GA crosslinking

The crosslinking with GA was carried out for 30 min using a 0.2% solution of GA (Sigma) in PBS (pH 6.5), at a temperature of 23°C, without lighting.

Investigation of crosslinking density by the radioisotopic method

Measurements of the $^{60}CoCl_2$ penetrability through tissue samples were made immediately after their preparation and selection. The PBS solution of the volume equalling 16 cm^3 supplemented with $^{60}CoCl_2$ (2.59 MBq/dm^3) and cooled to a temperature of 4°C penetrated the tissue sample (placed on the bottom of the column) during 24 h.

The permeability of fibrous pericardium to cobalt ions was tested every time on the same side of the tissue, i.e. the side of serous pericardium. Samples of 1 cm^3 filtrates were measured at the beginning of the $^{60}Co^{2+}$ permeability test (0 h), using the tissue samples of 0.14 g weight and after 24 h of $^{60}Co^{2+}$ penetration through tissue samples of all investigated weights (24 h). Radioactivity of the tissue samples (24 h) and the filtrates penetrating those samples (0 h and/or 24 h) was examined using a γ-spectrophotometer (Packard) and reported as counts per minute (cpm). Both the $^{60}Co^{2+}$-accumulation effect in the tissue samples as well as their permeability to cobalt ions were examined. The same tissue samples were tested before and after (MB)-mediated photooxidation or GA crosslinking in this test. Non-treated tissues (described as native) were used as a control.

Results

Changes in crosslinking density between native, photooxidized and GA-crosslinked tissues were revealed. The efficiency of stabilization processes was evaluated on the basis of $^{60}Co^{2+}$ accumulation in the tissue samples (TS) and of their permeability to cobalt ions. Significant decreases in radioactivity (mean values reported as counts per minute; cpm; the coefficient of variation did not exceed 3%) were detected in the thinnest tissue samples (weight: 0.14 ± 0.01 g) after their photooxidation as well as in filtrates penetrating the same samples (filtrate samples – FS; Table 1). These effects indicate that the structure density increases in stabilized tissues. Data in the "0 min" column represent results obtained for non-photooxidized control samples after the 24 h permeation of $^{60}Co^{2+}$. On the other hand, data described as "FS; 0 h; 0.14 g" represent results obtained for filtrates after the penetration through non-photooxidized and

Table 1. The influence of the photooxidation time (5, 15, 30, 45, 60, 90 and 120 min) on the pericardium density evaluated based on radioactivity of the tissue samples (TS) of various weights (0.14, 0.21, 0.29 g) and 1 cm^3 samples of filtrates (FS) penetrating these TS

Samples; time of $^{60}Co^{2+}$ penetration	TS weight (g)	The tissue photooxidation time (min)							
		0	5	15	30	45	60	90	120
		Sample radioactivity (cpm)							
TS; 24 h	0.14	19 391	16 300	15 600	15 601	14 100	12 500	10 000	10 000
	0.21	4 783	4 956	4 800	4 800	4 607	4 430	4 478	4 113
	0.29	2 541	3 863	3 794	2 757	2 615	2 462	2 545	2 316
FS; 24 h	0.14	21 267	11 426	8 567	8 433	8 270	8 262	7 040	6 922
	0.21	2 944	1 683	1 394	1 527	1 330	1 141	1 268	1 100
	0.29	1 524	1 220	681	523	302	212	225	315
FS; 0 h	0.14	1 766	1 758	1 774	1 774	1 733	1 720	1 701	1 657

Table 2. Specific radioactivity of BP and filtrates penetrating these samples

Tissue samples	Sample weight (g)	Specific radioactivity (cpm/mg)	
		Tissue samples	Filtrates
Native	0.14	139	152
	0.21	23	14
	0.29	9	5
Photooxidized	0.14	72	49
	0.21	20	5
	0.29	8	1
GA-treated	0.12	57	36
	0.14	49	24
	0.16	42	17

Fig. 3. Dependence between the $^{60}Co^{2+}$-specific radioactivities in crosslinked pericardium samples (bound ions) and the filtrates penetrating those tissue samples (free ions).

photooxidized tissue samples (weight: 0.14 ± 0.01 g) at the beginning of the $^{60}Co^{2+}$ permeability test. Their radioactivity remained at almost the same level during 120 min (the arithmetic mean and standard deviation: 1735 ± 41; coefficient of variation CV = 2.4%). This confirms both the structural uniformity of the examined tissues as well as lack of tissue changes during that time. The photooxidized pericardium samples of a larger thickness (weights of 0.21 ± 0.02 and 0.29 ± 0.03 g) did not reveal any significant changes in the accumulation of cobalt ions or permeability after photooxidation at different times.

Tissue permeability to $^{60}Co^{2+}$ and the accumulation of these ions in photooxidized samples increased with the decrease of their weight (corresponding to the thickness, Table 1). Moreover, it was visible for the sample group of the weight of 0.14 ± 0.01 g that the permeability of tissues and the accumulation of $^{60}Co^{2+}$ in them were dependent on the photooxidation duration. An effect of lowering radioactivity in the stabilized tissue samples as well as in filtrates penetrating the same samples suggests an increase in the density of their structure and may confirm the tissue crosslinking effect.

A similar weight-dependence for $^{60}Co^{2+}$ permeability was observed in the case of filtrates penetrating tissues crosslinked by GA (Fig. 2), whereas accumulation of $^{60}Co^{2+}$ in the GA-treated tissue samples remained at the same level (the arithmetic mean and standard deviation: 6819 ± 328; coefficient of variation CV = 4.8%).

Data concerning the permeability of tissue samples to $^{60}Co^{2+}$ and the binding of those ions in the tissues
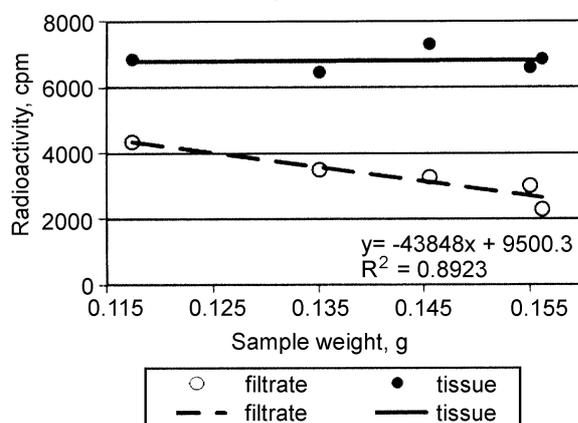
Fig. 2. Radioactivity of the GA-treated pericardium samples and filtrates penetrating these samples.
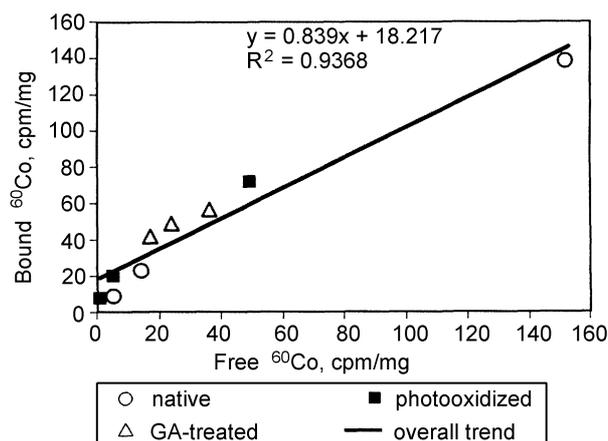
were recalculated with reference to the weight of the samples. As a result, the values of specific radioactivity of the investigated samples were obtained (Table 2). These data showed that the crosslinking of components in the extracellular matrix may influence a decrease in the specific radioactivity calculated for the tissue samples and filtrates.

Thus, we searched for a relationship between the ^{60}Co-specific radioactivities of crosslinked BP samples (indicative of cobalt ions bound by the unit weight of the tissue) and filtrates penetrating these tissues (indicative of free cobalt ions passed with the filtrate by the unit weight of the tissue). As a result, we obtained an almost directly proportional dependence which is presented in Fig. 3. These results suggest that the tissues – native and stabilized by various methods – which are able to accumulate fewer cobalt ions in the unit of mass and which may simultaneously transport/pass up fewer of these ions (an effect evaluated based on the ^{60}Co-specific radioactivity present in a filtrate after penetration of the tissue mass unit) are the tissues of higher density, i.e. heavily crosslinked tissues.

Discussion

Collagen, elastin and other proteins (to a lesser extent) belong to the structural elements of various tissues that decide on their structural integrity [15]. The collagenous tissues are often treated using various methods to obtain biomaterials.

Pericardium is commonly utilized for the production of biomaterials especially useful in the manufacturing of biological heart valves due to a more homogenous structure as compared with other collagenous tissues. Collagen type I is the basic component of pericardium (71.8 ± 2.1% of dry weight) [34]. This protein plays an important role in the structure organization of tissues [15, 24]. Fibrous pericardium is composed of two main layers formed of collagen type I fibers. The arrangement of the collagen fibers in each layer is parallel, but the fibers in both these layers are perpendicular towards each other. The structure of pericardium components creates molecular sieves which are permeable (to a different extent) for ions, low-molecular proteins and

polysaccharides – depending on their dimension and form [3, 25]. Undoubtedly, such molecular sieves obtain other properties after tissue stabilization because of introducing additional crosslinking bonds into the structure. The crosslinking density and character of the crosslinks depend on the mechanism of tissue stabilization. Photostabilization and GA treatment influence the formation of covalent bonds in the tissue substance in different ways. Photostabilization is based on oxidative processes. One of the hypotheses which concern crosslinking through photooxidation postulates alteration in the imidazole ring of histidine, leading to the formation of side chains containing aldehyde groups [42] or imidazole peroxide [1]. On the other hand, GA crosslinking is a result of interactions occurring between various GA forms and the tissue proteins. In this case, chemical bonds of various lengths are created (Fig. 1). Regardless of the mechanisms of stabilization processes, the crosslinking density increase in tissues influences the decrease in their susceptibility to degradation [10, 11, 20].

In our experiments, significant changes in density between non-stabilized and stabilized tissues have been revealed. These changes were dependent on the way of tissue treatment.

The reduced ability of $^{60}Co^{2+}$ binding in the photooxidized tissues (Table 1) may be an evidence for the decrease in the number of free bonding sites due to an effective formation of intra- and intermolecular crosslinks in and between protein molecules present in the tissue structure. On the other hand, the lowering radioactivity in filtrates penetrating the same samples may point to an increase in the tissue structure compactness due to the crosslinking processes (Table 1). These phenomena were demonstrated in our previous studies using various investigation methods [10, 11, 20–22, 41]. We showed that structural proteins in tissues which were stabilized both by photooxidation as well as by the use of various chemical reagents (i.e. tannic acid, dimethyl suberimidate and GA) were less susceptible to enzymatic degradation and/or extraction [10, 11, 20, 41]. It was also confirmed that stabilized extracellular matrix was more resistant to protein extraction because of higher structure density [10, 11, 20, 32]. The more of crosslinked structures the smaller the amounts of free space for enzyme action, as well as of free binding sites enabling ion trapping.

Moreover, data presented in this study as well as in some previous works show that the results of photooxidation processes are time-dependent [32, 39, 40]. This is a typical effect for various stabilization processes which proceed with crosslinking reactions [11, 25].

Other experimental data confirming the increase of the tissue crosslinking density were also discovered in this study. The decrease in the permeability of photooxidized tissue samples to $^{60}Co^{2+}$ (Table 1) may point to obtaining a modified tissue acting as a "molecular sieve" of higher density, in comparison with the native tissue. It was demonstrated that the structure of the thinnest tissues which were represented by samples of the lowest weight was the most susceptible to crosslinking during the photooxidation processes. The differences between groups of samples representing tissue pieces of various weight/thickness (each group was composed of samples of the same weight) in terms of their permeability to $^{60}Co^{2+}$ as well as accumulation of these ions in the tissues may result from the diversity of their structures. The samples of a greater weight may have a denser structure and/or more complex layers of collagen fibers, which may influence the penetration of the photoactive dye into the tissue structure and consequently affect the efficiency of photostabilization. Prolongation of the photooxidation process caused a significant decrease in radioactivity of the tissue samples and filtrates penetrating these samples only in the case of the thinnest samples, with the weight of 0.14 ± 0.01 g (Table 1). The reduced ability to bind $^{60}Co^{2+}$ in the tissues and simultaneously their lower permeability to these ions may be attributed to both their higher compactness and thickness.

Our data revealed differences between the tissues which were stabilized through the dye-mediated photooxidation and GA crosslinking, and the changes that occurred in the tissues during these processes. The results of $^{60}Co^{2+}$ radioactivity measurements in filtrates after penetration through GA-treated tissues demonstrated weight-dependence, whereas radioactivity of the cobalt ions accumulated in these tissues remained at the same level (Fig. 2). This phenomenon reflects differences in the kind of crosslinking processes that took place during pericardium photooxidation and GA treatment. GA creates a variety of crosslinks of various lengths (Fig. 1). Besides, the spatial structure of the crosslinked structure is not uniform: the tissues are the most compact in external layers, which prevent continued crosslinking of inner layers. On the other hand, the lower binding capacity in the GA-treated tissue samples as compared with the photooxidized samples of the equal weights (thickness) may point to a lower crosslinking efficiency during the photooxidation processes.

The dependence on weight in the case of both the increase in compactness of the crosslinked tissues as well as the simultaneous decrease in their binding abilities may be much better reflected by the changes in the specific radioactivities of samples (Table 2). The relationship between values of specific radioactivity, which were calculated using data obtained for tissue-bound, and free $^{60}Co^{2+}$ seems to be unequivocal, i.e. the dependence between these values is almost directly proportional regardless of type of the crosslinking process or its lack (Fig. 3). Besides, taking into account the results presented in Table 2 and Fig. 3, it may be stated that thinner tissues (represented by samples of lower weights) are more weakly crosslinked as compared with the thicker ones. It seems especially important to note that the thinner native tissues are those of lower density (Table 2) and that the compactness of those tissues may be substantially increased by their crosslinking using various methods, such as dye-mediated photooxidation or GA treatment. It may also be stated that the stabilization effect in photomodified pericardium depends on the tissue thickness and the time of its exposition to the light and dye. The exposition time is of special importance in the case of thin tissue photooxidation.

Over the recent years, many different opinions, concerning the efficiency of photooxidation of collagenous tissues useful for biomaterial production, have been formulated, and both advantages and disadvantages

have been discussed [4, 39, 43]. The most significant beneficial feature of the photostabilized biomaterial seems to be the reduction of calcification [43]. On the other hand, the abrasion of some tissue parts in bioprosthetic heart valves was observed [4]. However, photostabilized pericardium tissues are available all over the world in commercial products.

Conclusions

It has been demonstrated that MB-mediated photo-oxidation may lead to obtaining the biomaterials with advantageous properties. This process influences the increase of tissue density. The decrease in the ion--binding capacity seems to be one of the essential results of tissue crosslinking which may be of importance for mitigation of calcification and for the durability of biomaterials. The important feature of photooxidation is the lack of surface effect associated with aggregation and polymerization of the chemical reagents. It has been demonstrated that the key factor determining the result of crosslinking during photooxidation is the compactness and the thickness of the tissue. The period of tissue exposition to the light and dye is equally important. Photooxidation belongs to promising methods of tissue stabilization, but it should be emphasized that the crosslinking efficiency is lower in comparison with GA treatment. On the other hand, stiffness of such biomaterial is not as significant as in the GA-treated tissues. Therefore, such biomaterials are easier to use in implantology.

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