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Evaluation of the Degree of Cross-linking in UV Irradiated Porcine Valves

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Abstract

A porcine heart valve was irradiated by Ultraviolet (UV) rays (10 W, 254 nm) for 2, 4, 8 and 24 hours at 4°C to cross-link the structural collagen matrix. The degree of cross-linking was evaluated by assaying the released amount of hydroxyproline (Hyp) from the matrix, and comparing it with the positive controls of valves treated by glutaraldehyde (GA) solution (0.625 wt%) and the negative controls of non-treated fresh valves. The undigested weight ratio of the specimens increased by increasing the UV irradiation time. The undigested weight of the leaflets, tunica interna and tunica externa of the fresh, GA-treated and UV-irradiated specimens after collagenase digestion was compared. As UV irradiation increased, the amount of released hydroxyproline was gradually decreased until 8 hours of irradiation, after which the released hydroxyproline-reduction occurred slightly until 24 hours of irradiation time in this system. A total 47.68% of the hydroxyproline in the valve was cross-linked by UV irradiation after 24 hours, while 73.74% of the hydroxyproline in the positive control was crossed-linked. Light microscopic observation revealed that the typical crimp pattern of collagen fibers decreased and was rearranged into a dense flattened pattern as the UV irradiation induced interfibrilar cross-linking. GA-treated valves demonstrated a denser matrix pattern than the UV-irradiated specimens. Cross-linked collagenous tissue prepared by UV irradiation would be useful for improving durability and reducing the disadvantages related to using a chemical cross-linking agent.

Key Words: Porcine valve, UV irradiation, cross-linking, hydroxyproline assay

INTRODUCTION

Collagen is a principal structural protein in the vertebrate extracellular matrix which integrates cells to form tissues, organs, and finally a body. The characteristics of collagen have been extensively studied for possible use as a biomedical material with various modifications.[1-5]

A heart valve is a typical collagenous tissue that consists of type I collagen, and an impaired or damaged valve is replaced by xenografts such as a porcine valve.[6] Since collagenous tissues begin to be denatured immediately after excision, deterioration of tissue must be arrested and deferred to exploit the tissues as clinical materials, preferably beyond the recipient's natural life.[7,8]

Preparatory methods of collagen are mainly concerned with the enhancement of mechanical properties by increasing intramolecular or intermolecular cross-linkings in the collagenous materials.[9] This is to prolong the materials' original structural and mechanical integrity. Removing and/or at least neutralizing the antigenic properties attributed to collagenous materials is also an advantage of cross-linking.[7,10-14] The amino acids in the collagen-peptide chain contain pendant groups such as amines (NH_2), acids (COOH) and hydroxyls (OH). Together with the amide bond of the polymers, these are points for possible chemical reactions on the collagen cross-link.[15-17]

Chemical methods typically utilize bifunctional chemicals that interact with collagen at two different sites. The functional groups of the chemical agent react with those on the amino acid residues of collagen, such as the ε-amino function on lysine and hydroxylysine or the carboxyl function on aspartic and glutarmic acid, giving rise to the cross-link between the collagen molecules.[18,19] Glutaraldehyde (GA) is a remarkably effective tanning agent; however, the GA cross-linking may lead to the presence of residual non-reacted functional groups (aldehyde) in the colla-

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gen matrix, which can result in a cytotoxic reaction during degradation of the collagen.[20-23]

On the other hand, calcification is a multi-effect event which is caused by a number of factors such as the presence of phospholipids in tissue that can attract calcium ions, voids and cavities in the tissue created by the removal of proteoglycans during processing or cellular degradation. This predisposes, in the tissue cross-linked by glutaraldehyde, potential points that can trap foreign particles which may lead to nucleation centers for calcium. Increased calcium uptake leads to a build-up of calcium phosphate, which in time mineralizes into calcium phosphates.[24-26]

Physical cross-linking methods include drying, heating, or UV irradiation or gamma irradiation.[27-29] Unlike chemical cross-linking methods, these methods do not introduce toxic chemicals into the tissues. UV irradiation is a faster and more effective cross-linking method than any other physical method, though the cross-linking rate depends on the width of the collagenous tissue.[30]

In this study, to evaluate the degree of cross-linking, porcine heart valves were cross-linked by UV irradiation and the cross-linking degree was evaluated by hydroxyproline assay. A control group was treated with low concentration GA solution (0.625 wt.%) for more than 24 hours to ensure optimum cross-linking and also used a fresh valve as another control group.[31,32]

MATERIALS AND METHODS

Specimen preparation

Porcine aortic conduit including valves were procured under non sterile conditions at a local slaughterhouse and stored in Hanks' solution (GIBCO, Gaithersburg, MD, U.S.A.) containing antibiotics at 4°C immediately after excision. Within 24 hours after procurement, all extraneous muscle, fatty and fibrous tissues were dissected from the procured specimen. The valve root was dissected into three parts; valve leaflet, tunica interna, and tunica externa (Fig. 1).

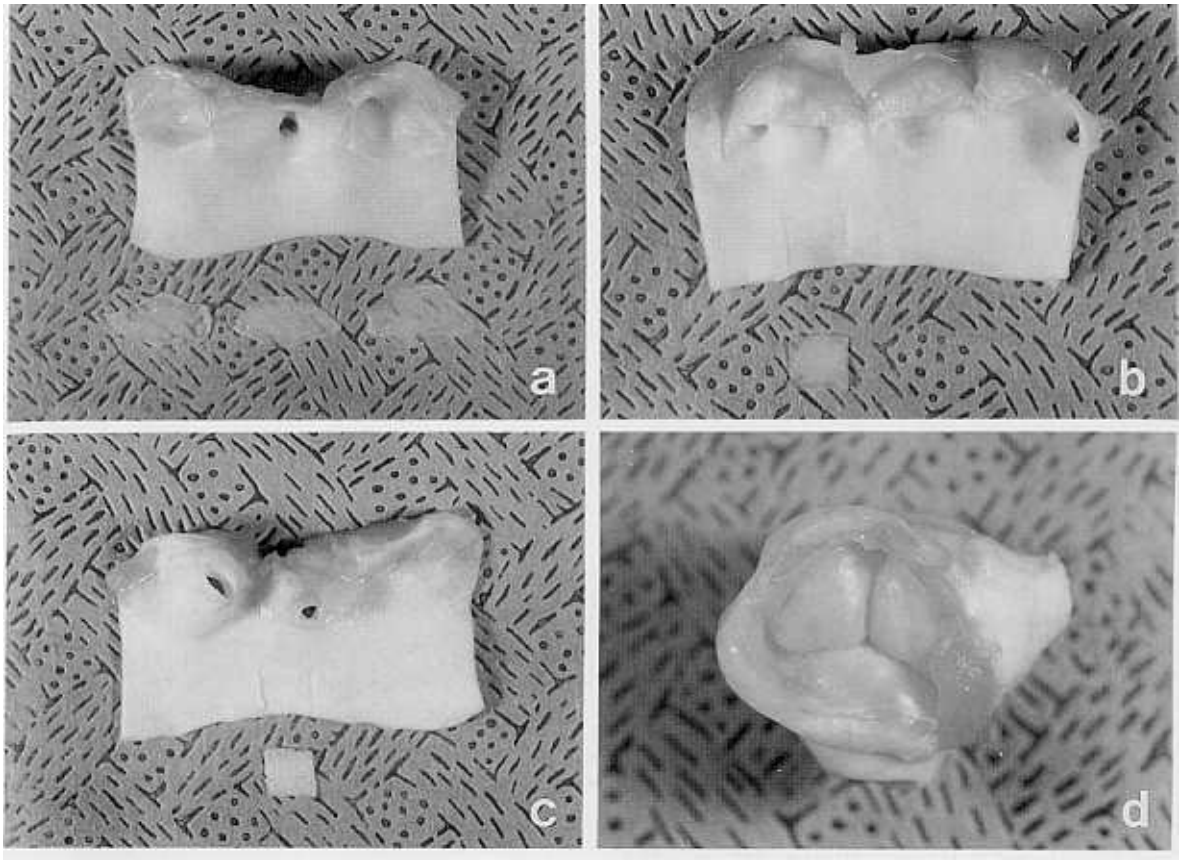

Fig. 1. UV-treated porcine valves; (a) leaflet, (b) tunica interna, (c) tunica externa and (d) GA-treated porcine valve root.

Glutaraldehyde treatment

GA solution was prepared by placing glutaraldehyde (grade I, glutaraldehyde, Aldrich, Milwaukee, U.S.A.) into phosphate buffered saline and thoroughly stirred at pH 7.4. The concentration of GA was controlled to 0.625% (w/w), and the procured porcine heart valve roots were placed in the solution for 24 hours at 4°C. The treated valves were thoroughly washed by distilled water and used as positive controls.

UV irradiation

UV irradiation of porcine valves was achieved by placing valve roots in a poly methylmethacrylate (PMMA) case in a custom-made UV cross-linking chamber (Fig. 2). Valves were exposed to six 10W UV lamps, which were allocated at upper / lower / front / back / right and left side walls of the UV irradiation chamber, for 2, 4, 8 and 24 hours at 4°C. The distance between each light source and the valves was 13 inches.

Collagenase digestion

The UV-irradiated and GA-treated specimens were lyophilized at 40°C in a freeze dryer (LABCONCO 4.5). Each 20 mg of leaflets, tunica interna and tunica externa were weighed and placed in polypropylene centrifuge tubes, and 4 ml of collagenase solution (43 units/ml, Type IA, Sigma, St. Louis, MO, U.S.A.) was added to each tube. The tubes were capped and placed in a water bath to incubate at 37°C for 24 hours. The supernatant was extracted from the solution and used as a collagenase digested solution specimen for hydroxyproline assay.

Hydroxyproline assay

Leaflet, tunica interna and tunica externa of the fresh, GA-treated and UV-irradiated valves were separated, and the amount of hydroxyproline from the tissues was measured by Neuman and Logans assay method.[31]

To obtain standard hydroxyproline amount calibration, dry 18×150 mm glass-made test tubes were employed, and each 1 ml of distilled water for the blank setting, 5 mg, 10 mg, 15 mg, 20 mg, 25 mg and 50 mg of hydroxyproline was delivered into the tubes.

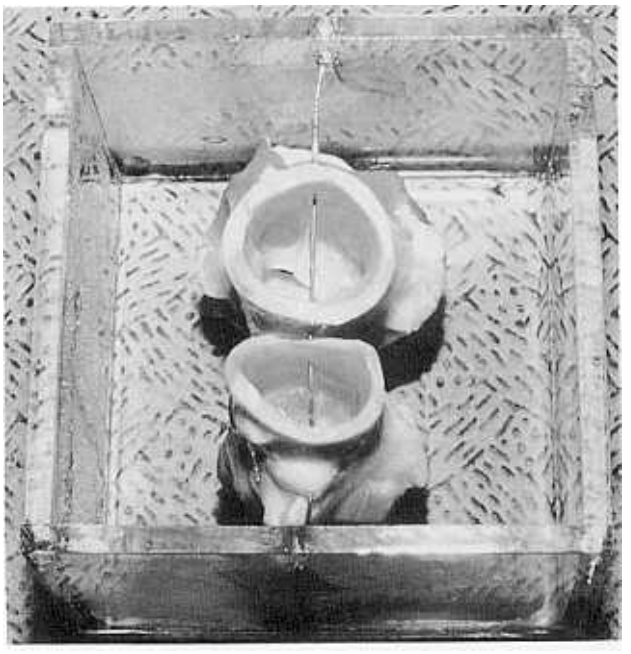

Fig. 2. UV irradiation in the self-made PMMA case.

One ml each of 0.01 M copper sulfate solution, 2.5 N sodium hydroxide, and 6% hydrogen peroxide were added into each test tube in succession. The solutions were mixed and shaken occasionally for 5 minutes, and placed in a water bath at 80°C for 5 minutes with continuous shaking to destroy the excess peroxide. The remaining peroxide decreased in color formation and produced an orange-red hue. The tubes were immediately chilled in an ice-water bath, and 4 ml of 3.0 N sulfuric acid and 2 ml of ρ-dimethylaminobenzealdehyde solution were added with agitation. The tubes were placed in a water bath at 70°C for 16 minutes, and then cooled in tap water. The contents were transferred to selected UV absorption tubes and light absorbance was read at a wave length of 580 nm, and the standard calibration curve was obtained. Copper sulfate, sodium hydroxide, hydrogen peroxide, sulfuric acid, and ρ-dimethylaminobenzealdehyde were purchased from the Aldrich chemical company.

The amount of hydroxyproline in each 1 ml of supernatant obtained from the GA-treated and UV-irradiated specimens after collagenase digestion was investigated. Each experimental group consisted of 8 solution specimens, and the measured values were statistically analyzed using a paired Student's t-test. Statistical difference was regarded as $p<0.05$.

Table 1. Released Hydroxyproline after Collagenase Digestion (μg/20 mg specimen)

	Fresh	GA	UV2	UV4	UV8	UV24
Leaflet	2871.16±230.31	733.56±56.70	2259.60±511.21	1828.80±419.61	1682.80±456.43	1644.99±635.58
T. Interna	2568.16±178.06	681.50±64.13	2023.88±463.72	1754.24±366.22	1569.60±623.11	1273.71±410.75
T. Externa	2555.53±112.96	682.84±54.64	1565.59±386.56	1071.37±232.39	1084.04±209.37	924.54±72.10

Fresh, non-treated group, negative control; GA, 0.625% glutaraldehyde in PBS saline solution for 24 hrs, positive control; UV2, UV (254 nm) irradiated group, 2 hrs; UV4, UV (254 nm) irradiated group, 4 hrs; UV8, UV (254 nm) irradiated group, 8 hrs; UV24, UV (254 nm) irradiated group, 24 hrs; Mean±S.D., n=8.

Histology

For histological observation, specimens were washed in phosphate buffered saline (pH 7.4) and distilled water, then fixed by 10% buffered formalin as a routine preparatory process. Paraffin-embedded histological sections were cut in 15 μm thicknesses and stained with haematoxylin and eosin (H & E).

RESULTS AND DISCUSSION

Each released amount of hydroxyproline from the leaflets, tunica interna and tunica externa of fresh valves was investigated (Table 1), and the amount from the negative control was considered to be 100% (Fig. 3).

In general, the heart valve mainly consists of Type I collagen, of which each molecule contains three polypeptide chains, termed αchains; two are of one kind, termed α1(I), and the third is of another, termed α2(II). They are similar and homologous in peptide arrangement. Both consist of repeating triplets of sequence glycine-X-Y where X and Y are any amino acid; proline is frequently in the X position, and hydroxyproline in the Y position. Hydroxyproline is derived from proline and thus their positions are the result of enzymatic specificity.[33-35]

Formation of cross-links during UV irradiation (254 nm) is thought to be initiated by free radical formation on aromatic amino acid residues such as tyrosine and phenylalanine. Bonding between this radical then forms cross-links.[36]

In this study, comparing with negative controls, the amounts of released hydroxyproline from the GA-treated positive controls were 25.55% from leaflets, 26.54% from tunica interna, 26.72% from tunica externa, and 26.27% as a total gross average. This showed that about 73.73% of the original amount of hydroxyproline in a valve remained in the valve by cross-linking through GA treatment.

The released hydroxyproline from the leaflets treated by UV irradiation for 2, 4, 8 and 24 hours were 78.70%, 63.70%, 58.61%, and 57.29% respectively in comparison with the negative control. This showed that about 42.71% of the hydroxyproline in the leaflets was cross-linked by 24 hours of UV irradiation, and that the cross-linking amount was 31.74% less than the positive control against the negative control in leaflets.

In tunica interna, the amounts of released hydroxyproline after UV irradiation for 2, 4, 8, and 24 hours, in comparison with the negative control, were 78.81%, 68.31%, 61.12% and 49.60%, respectively. It is assumed that 50.40% of the hydroxyprolines in the specimen were cross-linked by UV treatment for

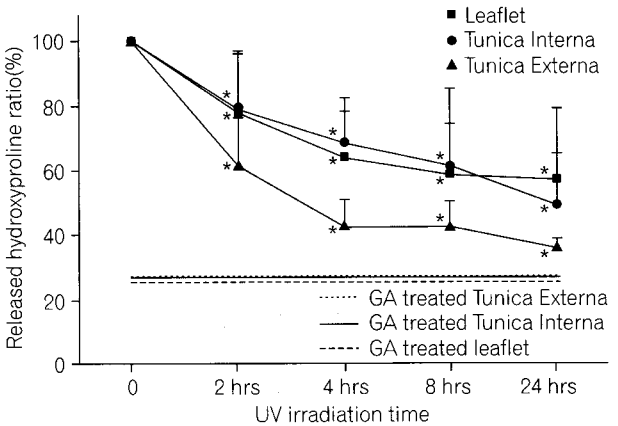

*Fig. 3. Released hydroxyproline ratio after collagenase digestion, released amount of hydroxyproline from the glutaraldehyde and UV-treated specimens were compared with fresh specimens, respectively (*significantly different compared to the fresh group ($p<0.05$, $n=8$), 0: non-treated group, negative control, GA: 0.625% glutaraldehyde in PBS saline solution for 24 hrs, positive control, 2 hrs: UV (254 nm) irradiated group, 2 hrs, 4 hrs: UV (254 nm) irradiated group, 4 hrs, 8 hrs: UV (254 nm) irradiated group, 8 hrs, 24 hrs: UV (254 nm) irradiated group, 24 hrs, 100%: released hydroxyproline amount of fresh specimen.*

24 hours, and were less cross-linked than the positive control by as much as 23.05% against the negative control.

In comparison with the negative control, the amounts of hydroxyproline released from UV-irradiated tunica externa were 61.26%, 41.92%, 42.42%, and 36.18%, and it showed that 63.82% of the hydroxyproline in the specimens was cross-linked, and 9.49% were less cross-linked than the positive control against the negative control. Though the amount of hydroxyproline released from the specimen irradiated by UV for 4 hours was higher than that released from the specimen irradiated for 8 hours, there was no statistical significance.

In general, the released amount of hydroxyproline from tunica externa was the lowest in comparison with the others, in which leaflets released a higher amount of hydroxyproline than tunica interna.

In leaflets, the amounts of released hydroxyproline were gradually decreased until 8 hours of UV irradiation, and this could be related to the fact that cross-linking was partially induced at the initial stage. However there was no statistical difference between 8 hours and 24 hours of irradiation time, therefore, maximum total cross-linking had probably occurred after 8 hours of irradiation. But in tunica interna, the released hydroxyprolines were continuously decreased until 24 hours, because the UV rays would reach the area which is histologically looser than the leaflet. Though the released hydroxyprolines also decreased gradually in tunica externa until 24 hours of UV irradiation, the statistical differences between 4, 8 and 24 hours of irradiation were not significant. This phenomenon could be related to the fact that the area is directly exposed to UV rays, which induced earlier cross-linking than the others.

The primary reason for limitations to the rate and extent of cross-linking may be the number of aromatic amino acid residues and the attenuation of UV rays by the collagenous tissues. We could assume that the cross-linking degree for each part of the valves did not increase any further after UV irradiation for 8 hours, relatively. This result suggests that after 8 hours of UV irradiation, almost all of the residues capable of being affected had taken part in cross-linking. In total average, the amount of hydroxyproline dissociated from the valves treated for 24 hours has decreased as much as 52.32% in comparison with the fresh negative controls, and 199.23% of that from the GA-treated positive controls, relatively.

As a result, it can be assumed that about 47.68% of the hydroxyproline in the valve is cross-linked by UV irradiation for 24 hours, while 73.74% of it is crossed-linked by GA treatment, in total average. This means that the magnitude of the cross-linking rate after 24-hours of UV irradiation is 64.66% less

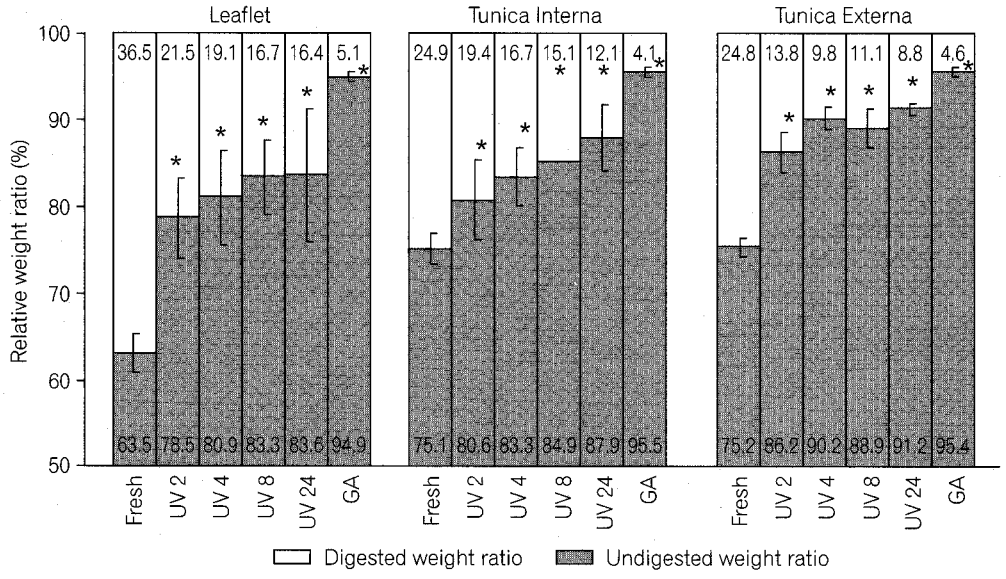

*Fig. 4. Relative weight ratio of specimens after collagenase digestion (*significantly different compared to the fresh group ($p < 0.05$, $n=8$), Fresh: non-treated group, negative control, GA: 0.625% glutaraldehyde in PBS saline solution for 24 hrs, positive control, UV2: UV (254 nm) irradiated group, 2 hrs, UV4: UV (254 nm) irradiated group, 4 hrs, UV8: UV (254 nm) irradiated group, 8 hrs, UV24: UV (254 nm) irradiated group, 24 hrs, 100%: initial weight of specimens used to collagenase digestion, 20 mg.*

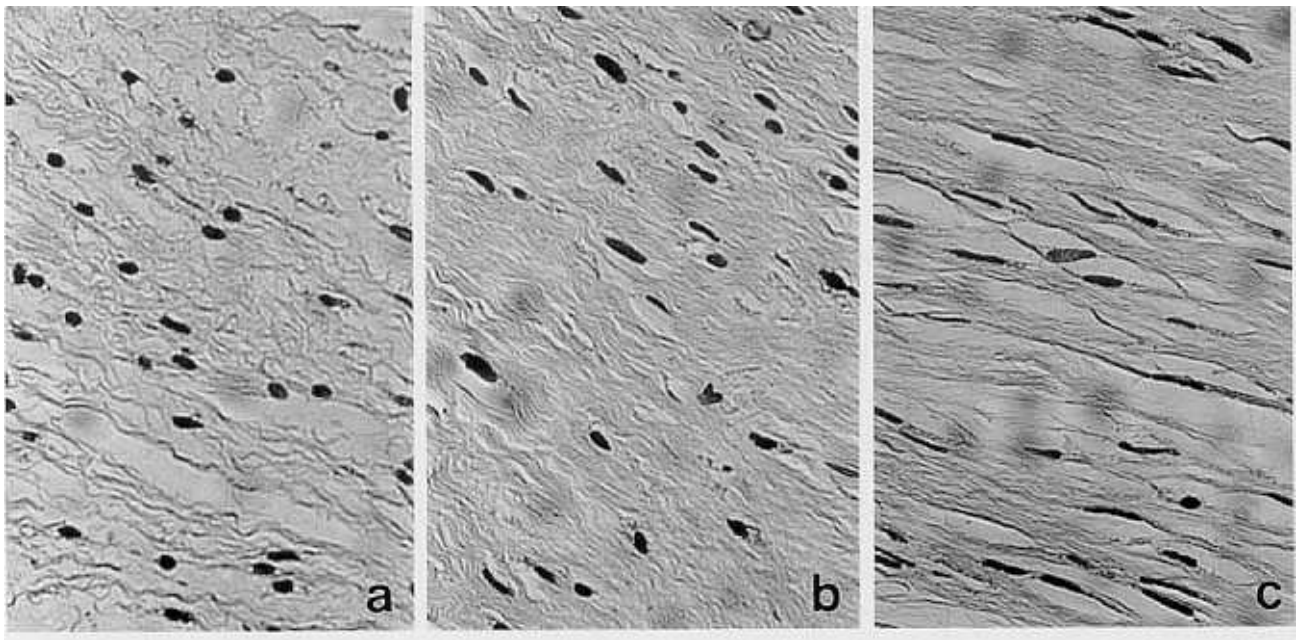

Fig. 5. Light photomicrographs of porcine valve leaflets with H & E stain: (a) fresh tissue, (b) UV-irradiated tissue, and (c) GA-treated tissue.

than the positive control.

The undigested weight of leaflets, tunica interna and tunica externa of the fresh, GA-treated and UV-irradiated specimens after collagenase digestion was compared with the digested weight of specimens (Fig. 4), and the initial weight of specimens was regarded as 100% (20 mg).

The amounts of released hydroxyproline gradually decreased by increasing the UV irradiation time, and this might be explained by the increasing degree of cross-linking. Therefore, the increase in the undigested weight ratio of the specimens depending on the increased UV irradiation time is a natural consequence and this result correlates well with Table 1.

However, the digested total weight is not entirely the weight of hydroxyproline, for collagen is cleaved not in the hydroxyproline form but in the leucine form when collagen is digested by collagenase.

In light microscopic observation, the natural porcine valve leaflet displayed a typical collagenous fibrous layer, arranged in a wavy pattern attributed to the collagen crimp (Fig. 5a). As shown in figures 5b and 5c, UV-irradiated and GA-treated valves each revealed a roughly similar morphology. However, the collagen crimp pattern of GA-treated sample became denser than the UV-irradiated one by the difference in the degree of cross-linking. Though the UV-irradiated valves were somewhat less stable for collagenase digestion than those of GA-treated valves, the valves demonstrated relatively higher resistance against enzyme digestion in comparison with fresh valves.

In this study, we evaluated the cross-linking effects of porcine heart valves for use as replacement devices. Collagenous tissue prepared by UV irradiation may be useful for improving durability and reducing the antigenic properties. Further experiments are required to determine the mechanical properties, *in-vitro/in-vivo* calcification test, and cross-linking efficiency of UV irradiation according to the valve depth.

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