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Evaluation and ultrastructural changes of amniotic membrane fragility after UVA/riboflavin cross-linking and its effects on biodegradation

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Abstract

This study aims to evaluate the changes of fragility and ultrastructure of amniotic membrane after cross-linking by UVA/riboflavin.

Forty-nine fresh amniotic membranes were randomly divided into 3 groups. Eighteen were in group A (CX group) and immersed in 0.1% riboflavin solution for 10 min for UVA/riboflavin cross-linking. Sixteen were in group B (B2 group), soaked for 10 min with 0.1% riboflavin. After soaking, membranes in group A and B were transferred into corneal preservation solution. Fifteen pieces were in group C, directly into corneal preservation solution. The biomechanical and ultrastructural changes of the amniotic tissue before and after cross-linking were examined (CX group = 13, B2 group = 11, C group = 15). The amniotic membrane tissue of group A (n = 5) and B (n = 5) was transplanted into 16 eyes of the rabbits, respectively, and the dissolution time of the amniotic membrane tissue was investigated.

After cross-linking, compared with the control group, the elastic modulus of the low-stress area of the amniotic membrane (E_{low}) was higher, while the elastic modulus of the high-stress area of the amniotic membrane (E_{high}) was lower, with no significant difference in the tensile strength. Also, the collagen fibers showed coarse and bamboo-like changes. In group A, amniotic membranes began to dissolve 4 weeks after conjunctiva transplantation, and all amniotic membranes were dissolved and absorbed 6 weeks after conjunctiva transplantation. In group B, some amniotic membrane tissues were still visible 6 weeks after conjunctiva transplantation.

This study suggested that after amniotic membrane cross-linking, the brittleness was increased, the hardness was enhanced, and the morphology of the collagen fiber was changed. The cross-linked amniotic membrane showed resistance to tissue dissolution.

Abbreviations: CXL = cross-linking, GA = glutaraldehyde.

Keywords: amniotic membrane, biomechanics, cross-linking, electron microscopy, tissue dissolution, UVA

1. Introduction

Amniotic membrane exhibits anti-inflammatory, anti-bacterial, anti-fibrosis, anti-scarring, analgesic effects, and promotes epithelialization. It has been used as a wound dressing for many years for burns, diabetic foot ulcers, fistulas, and venous leg ulcers.[1,2] Amniotic membrane is also applied to ocular surface tissue repair in ophthalmology. It is used in glaucoma surgery to inhibit tissue scar formation and provide a mechanical barrier.[3] Amniotic membrane cannot only be used as a substitute for oral mucosa,[4] but also a good carrier for in vitro cell culture.[5] However, the application of amniotic membrane is limited as it requires suturing during surgery. It is fragile and firmly adheres to the wound. It may cause bleeding and pain when changing the bandage.[1] At the same time, the amniotic membrane will be gradually dissolved in human tissue. For example, in glaucoma surgery, the amniotic membrane will be dissolved in the sclera about 2 weeks after surgery,[6] and the amniotic tissue will be maintained in the ocular surface covering operation for about 2 weeks and then dissolved.[7,8] Therefore, Wollensak et al believe that biomechanical improvement can improve the clinical application and expand the application range of the amniotic membrane.[9]

Wollensak et al first used UVA/riboflavin cross-linking to treat keratoconus in 2003.[10] Cross-linking refers to the establishment of chemical bonds between molecules through chemical reactions. Cross-linking can change the connection between

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collagen fibers.[11] In the UVA/riboflavin corneal cross-linking process, riboflavin releases oxygen monomer, which induces cross-linking between corneal collagen fibers. The collagen cross-linking is associated with the vulnerability of collagen-containing tissues,[12,13] and the amnion contains laminin, proteoglycan, fibronectin, and collagen of type I, III, IV, V, and VII.[14] Therefore, we tried UVA/riboflavin cross-linking (CXL) on the amniotic membrane, observed the changes in biomechanics before and after cross-linking, and examined the ultrastructural changes of the amniotic membrane after cross-linking by transmission electron microscopy.

2. Materials and methods

2.1. Preparation of amniotic membrane tissue

Placenta was obtained from a cesarean section of healthy pregnant women with negative HbsAg, HIV, HBV, BCV, and syphilis tests. The material was taken under aseptic conditions, and the removed amniotic membrane was rinsed with physiological saline and immersed in saline containing gentamicin (40 μ/mL) and amphotericin (2.5 mg/L) for 15 min. The amniotic membrane was cut into 1 cm × 1 cm pieces in size, placed in corneal preservation solution and stored in a 4°C refrigerator. Written informed consent was obtained from all participants, and the study followed the Tenets of the Declaration of Helsinki.

2.2. Grouping

The fresh amniotic membrane samples in this experiment were divided into three groups: cross-linking group (CX Group), riboflavin group (B2 Group), and the control group (C Group). The samples were processed as follows:

Group	CX group	B2 group	C group
..n	18	16	15
Procedure	16 h after cross-linking and was placed in the corneal preservation solution	Amniotic membrane was placed in 0.1% riboflavin 10 min, and then in the corneal preservation solution for 16 h	Amniotic membrane was placed in the corneal preservation solution for 16 h

2.3. Collagen cross-linking of amniotic tissue

The amniotic membrane tissue was completely and adequately soaked for 10 min in 0.1% riboflavin (0.1%; Sigma–Aldrich Inc, St. Louis, MO). A cross-linking system for UV-A illumination (ALL-1; Sunvision Meditech Co. Ltd, China, wavelength 365 nm, irradiance 9 mW/cm^2, total dose 5.4 J/cm^2) was used for continuous illumination of 10 min.[15] 0.1% riboflavin solution was applied to keep the amniotic membrane moist during the illumination. The amnion tissue after cross-linking was placed in the corneal preservation solution and stored in a refrigerator at 4°C.

2.4. Mechanical experiment

Thirteen and 11 amniotic membrane tissues were taken from CX group and B2 group, respectively, together with 15 amniotic membrane tissues from C group (the remaining amniotic membrane tissues were used for other studies). Each sample was subjected to a uniaxial tensile test, preloaded (6 cycles) before the experiment, and then subjected to a tensile test until the elongation was 0.01 mm/s. A stress–strain curve was derived and divided into three regions, namely: low stress linear region (AB), nonlinear region (BM), high stress linear region (MN) (Fig. 1), from which three mechanical parameters, low-stress elastic modulus (E_{low}), high-stress region linear modulus (E_{high}), and tensile strength, were determined.

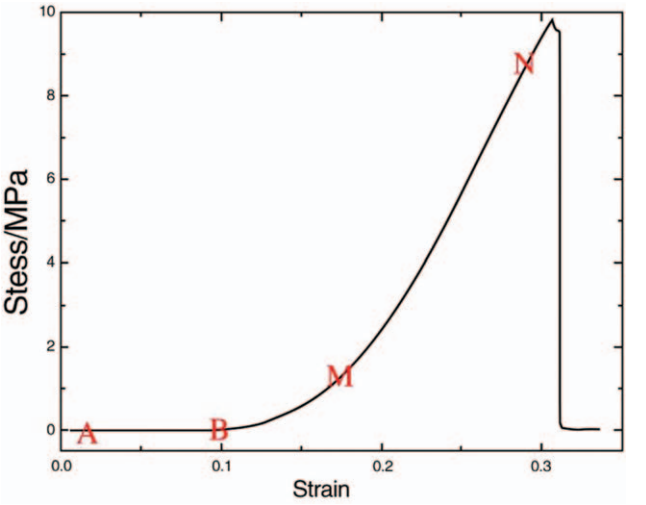

Figure 1. Illustration of the stress–strain curve.

2.5. Transmission electron microscopy of amniotic membrane

Five amniotic membrane tissues were randomly selected from CX Group and B2 Group for electron microscopic imaging and transplantation, respectively.

2.6. Transplantation of amniotic membrane tissue into rabbit's conjunctiva and quantification of dissolution and absorption time

Eight New Zealand white rabbits (weighing 1.5–2 kg) in group A and B were randomly selected. In group A, the corneal limbus was used as the base of both eyes, and the bulbar conjunctiva was cut 6 mm behind the corneal limbus. The cross-linked amniotic membrane (size 5 mm × 5 mm) was laid flat and implanted under the bulbar conjunctiva, and 10/0 nylon thread was used to suture the bulbar conjunctiva. In group B, the amniotic membrane was immersed in riboflavin solution. Two rabbits in group A and B were sacrificed at 1, 2, 4, and 6 weeks after operation. Eyeballs were taken and fixed with formalin and embedded. Longitudinal sections perpendicular to corneal limbus were made at the center of amniotic membrane transplantation. The dissolution and absorption of amniotic membrane tissue were examined by HE staining.

2.7. Statistics

All measurement data were expressed as mean ± standard deviation, processed by SPSS l3.0 statistical software package,

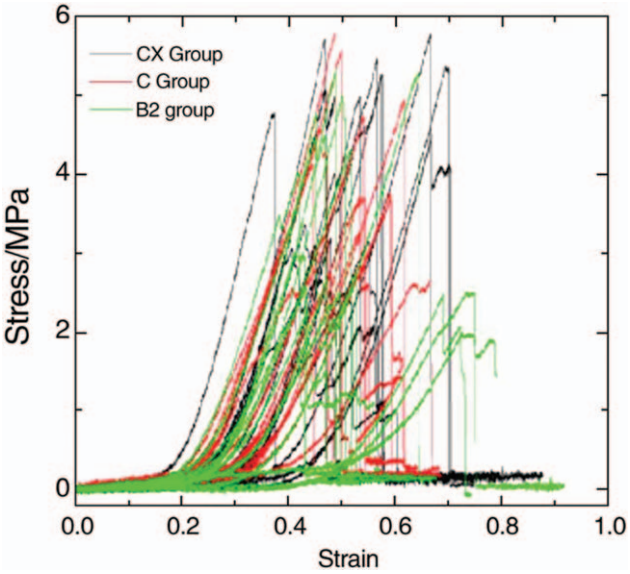

Figure 2. The stress–strain curves of the three groups.

using independent sample mean t test. $P < .05$ was considered statistically significant.

3. Results

1. The stress–strain curves of the three groups are shown in Figure 2. The mechanical parameters are summarized in Table 1 and Figure 3. The E_{low} of the CX Group was 0.6225 ± 0.1724 MPa, which was 1.3 and 1.5 times larger than that of the B2 Group and the C Group, respectively. The E_{high} of the CX Group was 16.9081 ± 2.9775 MPa, which was smaller than B2 Group and C Group (0.93 times that of the B2 Group, and 0.79 times that of the C Group). No statistical significance was detected in the tensile strength of the three groups.
2. After amniotic membrane cross-linking, the collagen fibers showed coarse and bamboo-like changes (Fig. 4).
3. The dissolution of subconjunctival amniotic membrane is shown in Figure 5. In Group A, some amniotic membrane tissues were still present under the conjunctiva at 4 weeks, and more inflammatory cells were observed. By the end of 6 weeks, no amniotic membrane tissues and fewer inflammatory cells were observed. In Group B, no obvious amniotic membrane tissue was observed under the conjunctiva at 4 weeks, and fewer inflammatory cells were present.

Table 1

The length, width, and mechanical parameters of the CX group, the B2 group, and the C group.

No.	Width (mm)	Length (mm)	Aspect ratio	E_{low} (MPa)	E_{high} (MPa)	Tensile strength (MPa)
CX-2	2.22	5.52	2.49	0.5192	15.3448	2.7411
CX-3	2.58	5.60	2.17	0.7061	13.8016	3.2706
CX-4	2.46	5.07	2.06	0.5810	19.6350	5.5914
CX-5	2.67	5.90	2.21	0.6283	16.9695	4.1290
CX-6	2.31	5.55	2.40	0.7995	16.4872	3.1694
CX-7	2.18	6.20	2.84	0.9485	13.4422	2.8466
CX-8	2.03	6.47	3.18	0.6451	22.5900	5.7915
CX-9	2.42	5.84	2.41	0.5508	11.4954	2.6714
CX-10	2.52	5.28	2.09	0.6223	18.9089	4.5722
CX-11	2.11	5.27	2.50	0.6495	17.7346	4.9640
CX-12	1.73	6.05	3.50	0.7227	18.5993	4.7496
CX-13	2.47	5.92	2.40	0.5094	15.8082	3.7246
CX-14	2.76	5.98	2.17	0.2097	18.9883	3.7623
Mean	2.34	5.74	2.49	0.6225	16.9081	3.9987
SD	0.28	0.40	0.43	0.1724	2.9775	1.0651
B2–1	2.50	5.48	2.20	0.2024	17.9336	5.3196
B2–2	2.44	6.20	2.54	0.3927	12.9172	2.5188
B2–3	2.35	5.55	2.36	0.4575	17.5674	2.7179
B2–4	2.00	6.50	3.24	0.6672	20.3026	4.4972
B2–5	2.43	5.39	2.21	0.5502	14.2595	2.2687
B2–6	2.33	4.70	2.01	0.5832	19.7854	4.0232
B2–7	2.59	5.47	2.12	0.4522	12.6998	2.5749
B2–9	2.39	5.64	2.36	0.4076	22.0082	4.9983
B2–12	2.15	5.14	2.38	0.3314	19.4967	3.0101
B2–13	2.43	6.68	2.75	0.4674	24.6636	5.3468
B2–15	2.69	5.32	1.97	0.3735	17.5309	4.3137
Mean	2.39	5.64	2.38	0.4441	18.1059	3.7808
SD	0.19	0.59	0.37	0.1270	3.7337	1.1923
C-1	2.40	5.74	2.39	0.1016	21.8400	5.3785
C-3	2.17	7.50	3.46	0.6669	23.8071	3.0733
C-4	2.19	5.40	2.47	0.3836	20.2791	5.7823
C-5	2.25	5.65	2.52	0.4652	25.5247	5.7182
C-6	2.59	5.54	2.14	0.3783	23.2085	5.2338
C-7	2.54	5.21	2.05	0.4949	19.8375	3.3647
C-8	2.47	4.69	1.90	0.3938	23.9736	5.4763
C-9	1.87	6.87	3.68	0.2641	19.7876	4.5254
C-10	2.19	5.70	2.60	0.2524	19.4828	3.1865
C-11	1.99	7.93	3.99	0.7059	27.6664	4.7947
C-12	2.27	6.29	2.77	0.2810	24.0030	5.0840
C-13	2.69	5.89	2.19	0.3850	21.0230	5.2714
C-14	2.76	6.14	2.23	0.3690	16.4324	3.9756
C-15	2.68	6.40	2.39	0.5210	12.8193	3.1558
C-16	2.45	6.51	2.66	0.4829	21.0991	4.9922
Mean	2.37	6.10	2.63	0.4097	21.3856	4.6008
SD	0.26	0.86	0.61	0.1566	3.6591	0.9869

4. Discussion

Amniotic membrane has been applied in the treatment of wounds such as burns, diabetic foot ulcers, fistulas, ocular defects, and venous leg ulcers. As a stent, amniotic membrane tissue can delay dissolution and maintain hardness.[2] For example, the amniotic membrane was used under the scleral flap for glaucoma small beam resection to delay the closure of the filtration channel. If the amniotic membrane is of a certain strength and the dissolution rate is reduced, the filtration channel will remain for a longer time.[6] Using the glutaraldehyde (GA) to chemically crosslink the amniotic membrane, Spoerl et al found that the force of the amnion cross-linked with GA at 2.5 mm elongation was significantly increased by 175% versus fresh amnion and 76.8% versus the cryopreserved amnion.[16] Through this cross-linked amniotic tissue, collagenase digestion was almost completely resisted in ocular surface tissue repair. It remained insoluble for up to 90 days and displayed good clinical effects. However, compared with fresh or frozen preservation, the amniotic tissue was dissolved on the 7th day. At present, cross-linking technology is well studied and mainly applied to the cross-linking of the cornea in ophthalmology and used in corneal diseases.[17] Some of the tests have shown that the corneal strength after cross-linking treatment by riboflavin combined with UVA irradiation can be increased by 300%, and the collagen

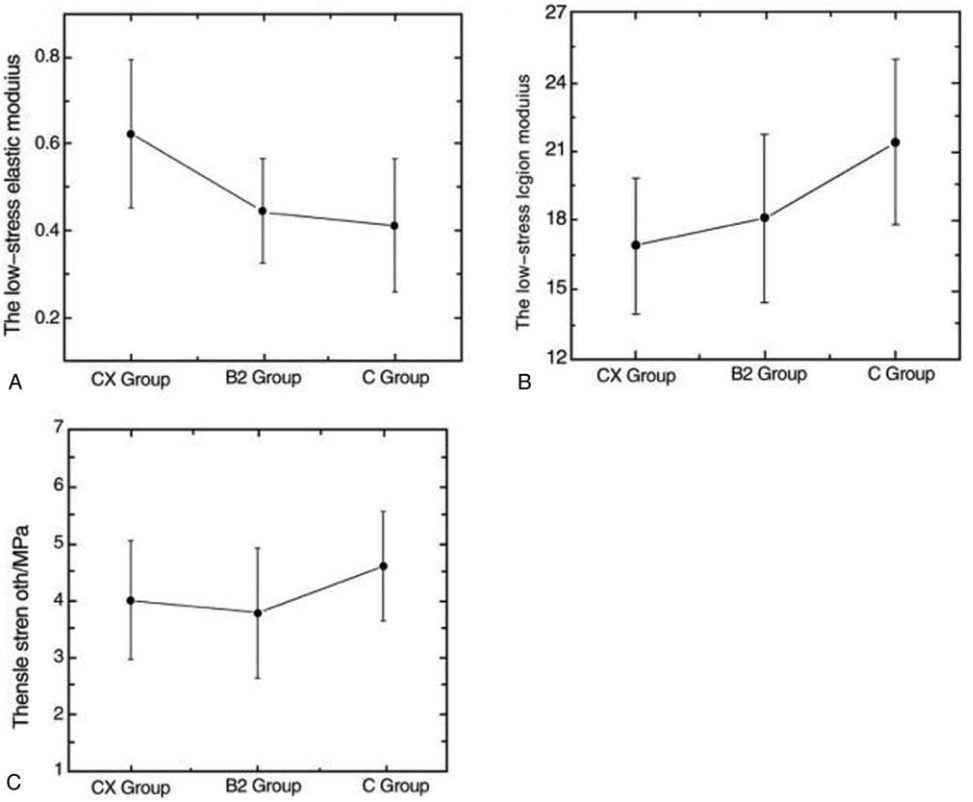

Figure 3. (A) The low-stress elastic modulus (E_{low}) of the low-stress linear region of the three groups. (B) The high-stress region linear modulus (E_{high}) of the high-stress linear region of the three groups. (C) The tensile strength of the three groups.

fibers in the anterior corneal matrix are changed to a more compact state, with the tissue hardening effect similar to formaldehyde.[18] CXL can significantly prolong the dissolution time of corneal collagen tissue.[19] The human amniotic membrane was cross-linked with $Al_2(SO_4)_3$ by Sekar, about 125% increase in the tensile strength was observed in the cross-linked human amniotic membrane compared to human amniotic membrane.[20] Fujisato et al[21] crosslinked amniotic membrane by radiation and chemical methods. Radiation cross-linking with gamma-ray and electron beam and chemical cross-linking with GA were used to investigate the effect of cross-linking on its physicochemical and biodegradation properties. They found that cross-linking took place in the interior of the fiber assembly without impairing the mesh structure, and the cross-linking amniotic membrane was implanted subcutaneously in mice. Also, the dissolution time of amniotic membrane was delayed. Therefore, cross-linking was considered to be an effective approach to reduce the biodegradation rate of amniotic membrane. Lai et al[22] found that when amniotic membrane was treated with glycine, lysine, or glutamic acid, such chemical cross-linking changed the tensile strength and provided a good medium for cell culture. No blood vessels, nerves, or lymphatic vessels are present in the amniotic membrane. It is a flexible tissue with a thickness of about 0.02 to 0.05 mm, divided into an epithelial layer, a basement membrane, a dense layer, a fibroblast layer, and a sponge layer. Like the cornea, the amniotic membrane also contains a large number of collagen fibers. The amniotic basement membrane and the stromal layer contain different collagens, mainly type I, III, IV, V, and VII collagen and fibronectin, laminin, and other components. Amniotic membrane can act as a "transplanted basement membrane" and work as a new healthy, and suitable matrix to promote tissue epithelialization.[14] As amniotic and corneal tissue share certain collagen similarity, the cross-linking technology will also affect the biomechanics and ultrastructure of amnion tissue. Indeed, our research confirmed that the collagen fibers of the amniotic membrane after cross-linking treatment showed a coarse and bamboo-like change. Also, we found that the dissolution time of cross-linked amniotic membrane tissue was prolonged by transplanting the cross-linked amniotic membrane tissue under conjunctiva. The amniotic membrane treated with GA for a longer duration exhibited a greater extent of molecular aggregation, thereby leading to a considerable increase in nanofiber diameter and resistance against collagenase degradation.[23] In this study, we found that after cross-linking treatment, the amniotic membrane underwent biomechanical and ultrastructural changes (Figs. 2 and 3); the collagen fibers of amniotic membrane underwent bamboo-like changes (Fig. 4); and the biodegradation rate was prolonged (Fig. 5). The relationship between the resistance to tissue dissolution and the changes in physical and chemical properties of amniotic membrane after cross-linking needs further study. In addition, we did not study the effect of ultraviolet intensity and irradiation time on the amniotic membrane. In one study, Lai et al used the Blak-Ray high intensity UV lamp (365 nm) to cross-link the riboflavin-treated amniotic membrane, by exposing for different periods of time (i.e., 0–150 min). Results showed that the number of cross-links of amniotic membrane significantly increased with

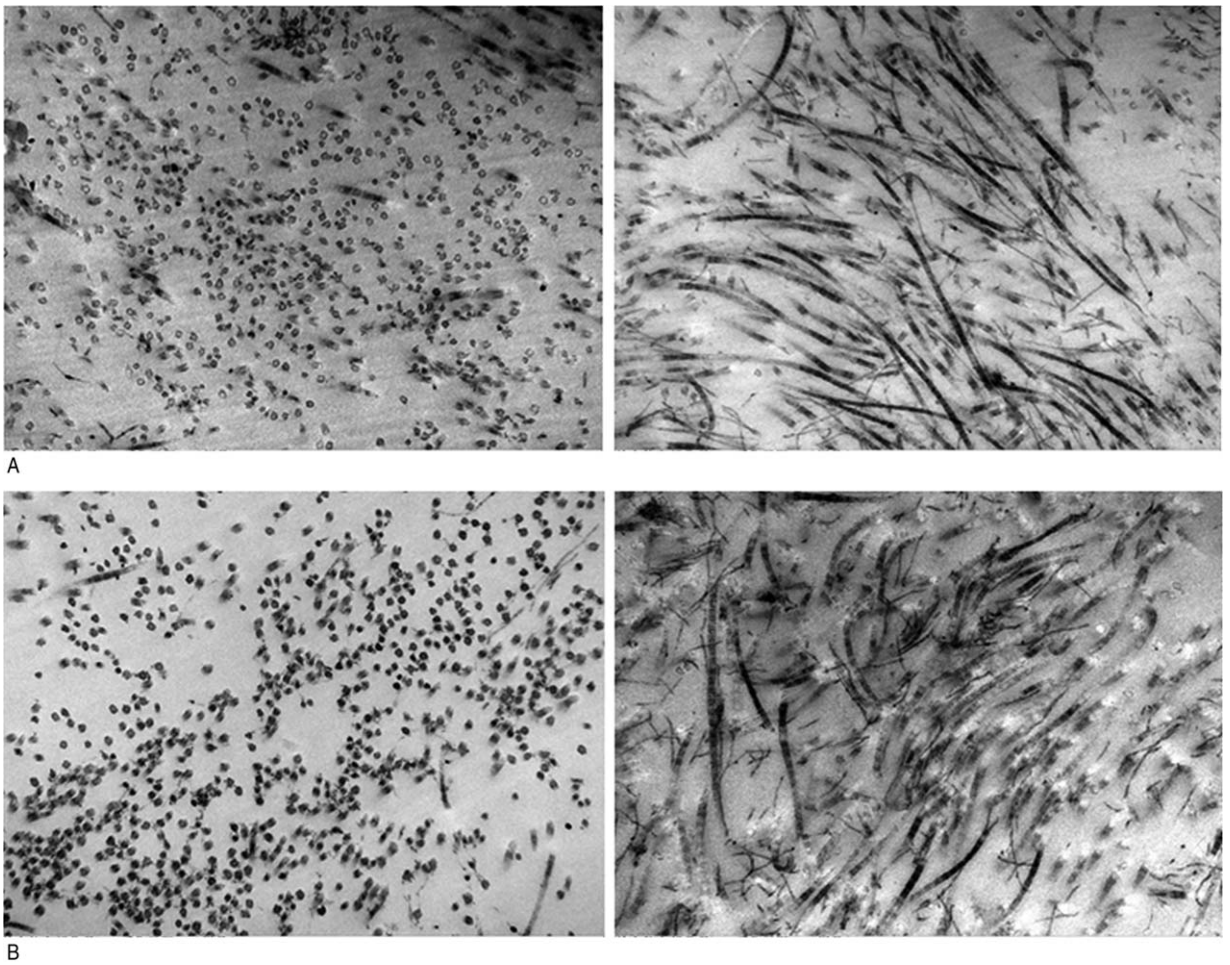

Figure 4. Electron microscopy of the uncross-linked amniotic membrane (A) and the cross-linked amniotic membrane (B). (A) The collagen fibers were smooth and arranged neatly. Magnification: 60,000×. (B) The collagen fibers showed a bamboo-like change, which was coarse and disorderly arranged. Magnification: 60,000×.

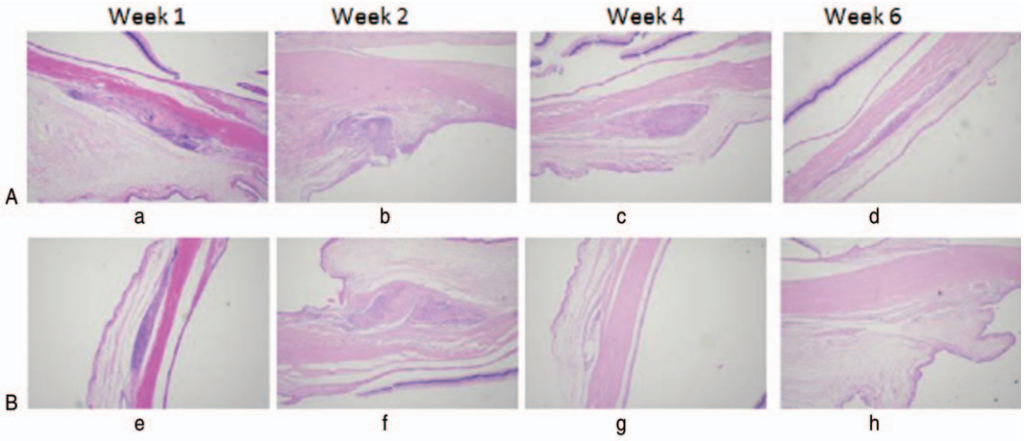

Figure 5. (A) a: visible amniotic membrane, many inflammatory cells; b: visible amniotic membrane, fewer inflammatory cells; c: visible amniotic membrane, fewer inflammatory cells; d: visible amniotic membrane, few inflammatory cells; (B) e: visible amniotic membrane, many inflammatory cells; visible amniotic membrane, fewer inflammatory cells; f: invisible amniotic membrane, few inflammatory cells; g: invisible amniotic membrane, no inflammatory cells.

increasing illumination time from 5 to 50 min. However, cross-linking was inhibited by longer irradiation time (i.e., 150 min). Thus, the UV irradiation time may have a profound influence on the fabrication of cross-linked amniotic membrane.[24] Finally, further analysis of ultrastructure could also be one direction of future research. In summary, this study provides experimental data for the biomechanical improvement of amniotic membrane by ultraviolet cross-linking. These findings may improve the clinical efficacy of amniotic membrane tissue application.

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Author contributions

Chenming Zhang and Tingting Du and Qiaoling Wang participated in the design of this study and performed the statistical analysis. Chenming Zhang and Tingting Du carried out the study and collected important background information. Qiaoling Wang drafted the manuscript. Guoying Mu, Jia Wang and Xin Gao carried out the concepts, design, definition of intellectual content, literature search, data acquisition, data analysis and manuscript preparation. Guoying Mu, Jia Wang and Fumin Long provided assistance for data acquisition, data analysis and statistical analysis. Xin Gao, Jia Wang and Fumin Long carried out literature search, data acquisition and manuscript editing. Guoying Mu performed manuscript review. All authors have read and approved the content of the manuscript.

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