The change of collagen in rabbit conjunctiva after conjunctiva cross-linking

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Abstract

Background We aimed to determine the ultrastructural changes of collagen fibrils in the rabbit conjunctiva after conjunctiva cross-linking using riboflavin and UVA light of 45mW / cm² irradiation intensity. Conjunctiva cross-linking may increase conjunctiva stiffness.

Methods The super-temporal quadrant of the right eyes of twenty-four adult rabbits were treated with topical riboflavin solution (0.25%) followed by irradiation with UVA light (45mW/cm²) for 4 min. After 3 weeks, the collagen fibrils in fibril bundles were examined with electron microscopy. Immunohistochemical staining was applied to detect the expression of collagen I and III in the rabbits' conjunctiva.

Results The diameter of collagen fibrils in the fibril bundles varied slightly and ranged from 30 to 60 nm in control group conjunctival stroma. While in the treatment group, the diameter of collagen fibrils ranged from 60 to 90 nm. Thickest collagen fibrils were observed in the treatment group (fibril diameters up to 90 nm), whereas thickest collagen fibrils in control group conjunctival stroma are considerable smaller (up to 60 nm in diameter). However, both of the thickness of collagen fibrils displayed a unimodal distribution. Collagen I and collagen III were increased after treatment with riboflavin and UVA light of 45 mW/cm².

Conclusions The data indicate that in rabbits, conjunctiva cross-linking with riboflavin and UVA light of 45 mW/cm² for 4 min is relatively safe and does not induce ultrastructural alterations of conjunctiva cells. The conjunctiva cross-linking riboflavin and UVA light of 45 mW/cm² can increase the diameter of collagen fibrils, but the average density of collagen I and collagen III have no statistical significance.

Background

Conjunctivochalasis(CCh) is a common age-related eye disease and characterized by progressive thinning and accumulation in the lower eyelid causing foreign body sensation, tears and other symptoms^[1]. Conjunctivochalasis was associated with the biomechanical properties of conjunctiva^[1,2]. As we know, the reduction in the size of a single collagen fiber and the reduction in the number of conjunctival collagen fibers are the main pathogenic factors of progressive CCh. During this process, the conjunctiva is remodeled, resulting in a thinning and weakening of the conjunctival tissue^[2]. Despite extensive research, there is still no effective method to prevent the progression of CCh. In recent years, riboflavin/UVA-induced collagen cross-linking was successfully used to prevent the progression of keratoconus and other corneal expansion^[3,4]. CCh and keratoconus have similar pathogenesis, which are typical changes in collagen tissue, so we hypothesized that conjunctival collagen cross-linking can enhance the conjunctiva to prevent CCh progression.

On this basis, we performed an electron microscopy and immunohistochemical staining study aimed to provide a comprehensive structural and ultrastructural description and a morphometric analysis of the conjunctival structure to explore collagen cross-linking technology used in conjunctivochalasis.

Methods

Animals and anesthesia

All animals were bred, handled, and finally euthanized in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-four adult New Zealand White rabbits (age 3–4 months) were obtained from the Shanghai Laboratory Animal Centre (Shanghai, China). Animals were anesthetized with 1% pentobarbital sodium (30 mg/kg) by intravenous injection. Conjunctival cross-linking was performed on the right eye of the rabbit, the left eye was used as a control group.

Conjunctiva cross-linking

Animals were given general anesthesia and exposed the super-temporal quadrant of the right eyes. Then, apply adequate ParaCel (the formula contains 0.25% riboflavin, HPMC (Hydroxy propyl methyl cellulose), EDTA (Ethylene Diamine Tetraacetic Acid), Trimethylmethylamine, Acetic acid n-butyl ester) to completely cover the super-temporal conjunctiva and repeat this process every 90s for 4 min. Thoroughly flush the conjunctiva surface with the VibeX Xtra (formulation contains 0.25% riboflavin, hypotonic Saline). Apply sufficient VibeX Xtra to completely cover the super-temporal conjunctiva surface and repeat this process every 90s for 6 min. Rinse the conjunctiva completely with BSS. Ultraviolet irradiation apparatus was used for rapid trans-epithelial collagen cross-linking treatment. The treatment plan was to use 45mW / cm² irradiation intensity, irradiation spot diameter 9mm, pulse irradiation mode (pulse irradiation interval [1s, 1s]), a total of 320s irradiation, to obtain a total irradiation energy of 7.2J. Rinse the conjunctiva completely with BSS. After the irradiation procedure, the 0.05% levofloxacin eye drops were given.

Electron microscopy

Three weeks after conjunctiva cross-linking, all the rabbits were sacrificed by intravenous injection of pentobarbital sodium. Each rabbit's eyes were picked and used four stitches to mark the treatment site. After removing the eyeball, the marked tissue pieces (10 x 10 mm), including the conjunctiva and sclera, were cut from the limbus. Tissues were immediately fixed in phosphate buffer (Biochrom, Germany) containing 2.5% glutaraldehyde (Sigma, Germany) overnight. Then, tissue pieces were rinsed three times with 0.1 M phosphate buffer for 15 minutes each time, and fixed with 1% citric acid for 2 hours. Thereafter, the tissue preparations were subjected to gradient dehydration using acetone (30, 50, 70, 90, 100%; 15–20 minutes each time). Then, the tissue pieces were embedded in acetone-epoxy resin (2:1) at room temperature for 4 hours and acetone-epoxy resin (1:2) at room temperature overnight and pure epoxy resin at 37 degrees for 3 hours. After that, semi-thin (500 nm) and ultra-thin (70nm) sections were cut from the tissue blocks with a microtome. The semi-thin sections were stained with toluidine blue (0.1 %) at 70 °C and embedded in balsam. For electron microscopy from the tissue observed in light microscope, ultra-thin sections were transferred onto resin-laminated slot grids, which were firstly stained

with lead citrate for 10 minutes, washed twice with double distilled water, stained with uranyl acetate for 30 minutes, and washed twice with double distilled water. After ultra-thin sections drying, the sections were examined with an electron microscope (Zeiss, Germany) at 4000-fold magnification with a slow-scan CCD Camera (proScan, Germany).

Immunohistochemistry (IHC)

Each rabbit's eyes were picked and used four stitches to mark the treatment site. After removing the eyeball, each rabbit's eyes were soaked for 24h in 4% paraformaldehyde (phosphate buffer saline (PBS) buffered). After dehydration using a sucrose gradient method, the eyeball was cut along the limbus under a microscope to remove the anterior segment. Serial sections treated with the microtome were frozen to the treatment conjunctiva (thickness 10 μ m) using an optical cutting temperature compound (Tissue Tek, Sakura, Japan). After drying at room temperature for 24 hours, the samples were stored in a refrigerator at 4 °C.

Immunohistochemical staining procedure

The sample sections were taken out of the freezer and placed at room temperature for 30min, soaked in acetone at 4 °C for about 10min, and washed with PBS for 3 times for 5min each time. Samples were incubated in 3% hydrogen peroxide for 10min to eliminate enzymatic activity and washed twice with PBS for 5min. Then, the samples were sealed with 5% goat serum (PBS dilution) and incubated for 10min at room temperature. The serum was removed (no wash) and they were dropped into the primary antibody (1:125 dilution) overnight at 4 °C. On the next day, the samples were washed with PBS for 5min, three times, dropped into a biotin-labeled secondary antibody (1:125 1% bovine serum albumin dilution in phosphate buffered saline (BSA-PBS)), and incubated for 20min at 37°C. The sample was washed with PBS for 5min, three times, and then dropped into streptavidin labeled with horseradish peroxidase (diluted with PBS) and incubated at 37 °C for 20min. Finally, the sample was washed with PBS for 5min, three times, placed into a color developing agent 3,3 N-Diaminobenzidine (DAB) Tetrahydrochloride Horseradish Peroxidase Color Development Kit or 3-amino-9-ethylcarbazole (AEC)), washed with running water and dyed again; the film was sealed and then the images were taken. *Histology*

After sacrificing, the treated area was positioned with 10-0 silk thread and the removed eyeballs were immersed in neutral buffered formalin for 2d. Subsequently, 2 mm x 4 mm strips, including the conjunctiva and sclera, were cut for histological analysis and all the sections were embedded in paraffin. To compare the histological change, 4 μ m thick paraffin sections of the conjunctiva and sclera were prepared and stained with hematoxylin and eosin (HE). Terminal deoxynucleotidyl transferase (TdT) mediated biotin dUTP nick-end labeling (TUNEL) assay was performed using the TUNEL assay kit (Promega, USA), and according to the manufacturer's instructions. The expression of collagen I and collagen III is a reliable marker of fibroblast proliferation. To evaluate this, mouse monoclonal anti-collage

antibody (diluted 1:1 in PBS, Merck KGaA, Darmstadt, Germany) and biotinylated goat anti-mouse IgG (code B – 6398; Sigma-Aldrich) was used together as a secondary antibody, according to the manufacturer's instructions. To evaluate this, mouse monoclonal anti-collagen antibody (1:100 dilution in PBS, Merck KGaA, Germany) and biotinylated goat anti-mouse IgG (code B – 6398; Sigma-Aldrich) were used together as the secondary antibody, according to the manufacturer's instructions. All sections were examined by light microscopy (Axioplan 2 imaging; Zeiss, Germany). Five fields were randomly selected from each section at a magnification of 400× for histological evaluation.

Data analysis

As previously mentioned, the longitudinal (filamentous), frontal (circular) and oblique (elliptical) profiles of collagen fiber bundles were found based on the orientation in the images^[5]. The diameter of 100 adjacent fibers in one fiber bundle was measured with the analysis software (Image-Pro Plus 6.0), and fiber distribution maps were generated. The counting function of the software can be automatically and continuously numbered, and the measured distances would be automatically generated to the corresponding excel data sheets. The measured collagen fiber diameter values were fitted to the normal distribution using IBM SPSS Statistics 19. The data were expressed as mean ± SD, and bar charts were generated from the fibril data. Significance was determined with T-test and was accepted at $P < 0.05$.

The density (the number of collagen fibrils per μm^2) was analyzed with Analysis and Prism software. The collagen fraction (the relative area filled with dark collagen fibrils within the bright interfibrillar matrix) was evaluated with a custom-made software that recognized brightness thresholds. All data was analyzed from the frontal profiles of collagen fibers.

Results

Ultrastructure of the rabbit conjunctiva

In the conjunctival, there were collagen fibril bundles, which were long and seem to be intertwined (Fig.1). After conjunctiva cross-linking, the ultrastructure of the conjunctival stroma and the morphology of the cells were not different between treated and control groups (not shown). We observed that fibroblasts had cellular activation and degeneration, mainly characterized by cell process thickening, endoplasmic reticulum expansion and cytoplasmic vacuoles (not shown).

Thickest of collagen fibril

We measured the diameter of individual collagen fibrils in the conjunctival stroma and explored whether conjunctival cross-linking caused a change in conjunctival collagen fibril thickness. The average diameter of the collagen fibers was calculated by measuring the diameters of 100 adjacent fibrils, and the density

of the fibrils was expressed by the number of corresponding fibrils per μm^2 area. While, the collagen fraction showed filling in the matrix between the fibrils, there was a relative area of collagen fibrils. In the control rabbit conjunctival stroma, the diameter of collagen fibrils in the fibril bundles varied slightly, ranging from 30 to 60 nm (Fig. 2a). While in the treatment group, the diameter of collagen fibrils ranged from 60 to 90 nm (Fig. 2a). The thickest collagen fibers were observed to be 90 nm in the treatment group, whereas the thickest collagen fibers in the control rabbit conjunctival matrix were 60 nm. However, both of the thicknesses of collagen fibers showed a unimodal distribution.

The density of collagen fibrils was 117.33 ± 5.2 fibrils per μm^2 and 79.57 ± 5.3 fibrils per μm^2 in the controls and treatment group (Fig.2b). The collagen fraction was 0.33 ± 0.01 and 0.39 ± 0.02 (Fig.2c). As shown that, compared with the control, the diameter of the collagen fibrils in the conjunctival matrix was significantly increased with riboflavin and UVA light of $45 \text{ mW} / \text{cm}^2$ treatment ($P < 0.01$) (Fig. 2d), and the density of collagen fibers in the collagen fiber bundle was significantly decreased (Figure 2b). In addition, after treatment with riboflavin and UVA light of $45 \text{ mW} / \text{cm}^2$, the collagen fraction was not significantly ($P > 0.05$) increased.

Expression of collagen fibrils in the rabbit conjunctiva by HE and Masson

To detect the expression of collagen fibrils in the rabbit conjunctiva, we used HE and Masson in our study. From Figure 3 and 4, we could see that the expression of collagen fibril increased in the treatment group compared with the control group, and the collagen fibers in the treatment group were arranged more tightly and the fibers were thicker.

Expression of collagen I and collagen III in the rabbit conjunctiva by IHC

In our study, the frozen sections were evaluated using immunohistochemical staining. Collagen I and collagen III were observed in both the control group and the treatment group. The positive staining of collagen I and collagen III was located outside the cell and presented a brown color. Semi-quantitative analysis of immunohistochemical staining results was conducted using image-pro-plus 6.0.

The average optical density of collagen I were 0.18 ± 0.02 and 0.19 ± 0.04 in control group and treatment group (Fig.5). The average density of collagen III were 0.17 ± 0.01 and 0.18 ± 0.01 in control group and treatment group (Fig.6). In our study, both of the average optical density of collagen I and collagen III have no significantly ($P > 0.05$) increased after treatment with riboflavin and UVA light of $45 \text{ mW} / \text{cm}^2$ (Fig.7).

Discussion

Collagen cross-linking (CXL) was used to increase the tension and stability of collagen fibers, which causes covalent bonding between intra- and inter-molecular. The principle is to use a wavelength of 370 nm UVA to irradiate the tissue infiltrated by the photosensitizer riboflavin, and the riboflavin molecule is excited to a triplet state, resulting in an active oxygen species dominated by singlet oxygen^[6, 7]. Reactive oxygen species can react with various molecules and induce chemical cross-linking reactions between the amino groups of collagen fibers, thereby increasing the mechanical strength of collagen fibers and their ability to resist protease digestion.

Currently, it was used to treat keratoconus. The pathogenesis of conjunctivochalasis is similar to keratoconus^[8]. The type, number, and spatial structure of collagen are changed and its mechanical tension is reduced. Therefore, in this study, we found that the ultrastructure of collagen fibers after conjunctival cross-linking has changed, affecting the amount and diameter of collagen fibers in bundles. In addition, Fibroblasts had obvious signs of cell ular activation.

Our results confirm that conjunctival collagen cross-linking is safe and does not cause inflammatory reactions and degeneration in the conjunctival tissues. Some studies have suggested that the diameter, distribution and orientation of collagen fibers determine the biomechanical properties of the conjunctiva^[9,10]. The results of this study found that the diameter of collagen fibers changed slightly, and ranged from 30 to 60 nm in control group. While in the treatment group, the diameter of collagen fibrils ranged from 60 to 90 nm. This is similar to the previous study^[5]. However, our study showed that the diameter of the collagen fibrils exhibits a unimodal distribution.

In our study, we detected the expression of collagen fibrils in the rabbit conjunctiva by HE and Masson. From Figure 6 and 7, we could see that the expression of collagen fibril increased in the treatment group, compared with the control group, the collagen fibers in the treatment group were arranged more tightly and the fibers were thicker.

Our experiment also applied frozen section immunohistochemical staining to detect the collagen I and collagen III expression in the rabbit conjunctiva, changing the conjunctiva morphology. We observed the positive staining of collagen I and collagen III collagen was located outside the cell and presented a brown color. Both of the average density of collagen I and collagen III weren't increased after treatment with riboflavin and UVA light of 45mW/cm².

Conjunctivochalasis is associated with a thinning of conjunctival collagen fibrils, which may be one reason for the decreased conjunctival stiffness and thinning^[11, 12, 13]. We found that treatment with riboflavin and UVA light of the 45 mW/cm² intensity induced a significant increase in the diameter of collagen fibrils in the fibril bundles. In the conjunctiva, activated fibroblasts and macrophages may produce exogenous enzymes that degrade collagen.

Current results may indicate that cross-linking results in remodeling of the conjunctival extracellular matrix, including degradation of collagen fibers and/or de novo synthesis of collagen fibers ^[14, 15]. However, conjunctival cells are temporarily damaged after conjunctival cross-linking. The rearrangement of conjunctival cells in treated tissues leads to the regeneration process and may result in prolonged biomechanical enhancement ^[5]. Conjunctival remodeling may also contribute to conjunctival scarring and stiffening. However, extensive conjunctiva light irradiation should be avoided at the time; it bears the risk of tissue damage in choroid and retina. Conjunctival remodeling can also cause scarring and hardening of the conjunctiva. However, we should to avoid extensive conjunctival irradiation because it has the risk of damaging the choroid and retinal.

Conclusions

We will use different energies and irradiation times in order to explore the safety and effectiveness of the conjunctival cross-linking method, without compromising the cross-linking efficiency while preventing the harmful effects of high-intensity phototherapy.

Declarations

- Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Putuo District Central Hospital in Shanghai.

- Consent for publication

Not applicable

- Availability of data and materials

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

- Competing interests

The authors declare that they have no competing interests

- Funding

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- Authors' contributions

QSL and LJM conceived and designed the study. LJM, HMW, LH and YXG performed the experiments. LJM and QSL wrote the paper. LJM, QSL, HMW, LH and YXG reviewed and edited the manuscript. All authors read and approved the manuscript.

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Not applicable

Abbreviations

UVA: ultraviolet A

CCh: Conjunctivochalasis

PRK: photorefractive keratectomy

LASIK: laser in situ keratomileusis

HPMC: Hydroxy propyl methyl cellulose

EDTA: Ethylene Diamine Tetraacetic Acid

IHC: Immunohistochemistry

PBS: phosphate buffer saline

BSA: bovine serum albumin

DAB: 3,3 N-Diaminobenzidine

AEC: 3-amino–9-ethylcarbazole

HE: hematoxylin and eosin

TdT: Terminal deoxynucleotidyl transferase

TUNEL: dUTP nick-end labeling

CXL: Collagen cross-linking

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Figures

Figure 1

The electron microscopical image of the conjunctiva showing fibroblasts (fb) embedded in the interfibrillar matrix (gs, ground substance) collagen fibril bundles (cf). Fibroblasts show an elliptical nucleus (n) and thin cytoplasmic processes (p) which are primarily parallel to the surface of the eyeball. Fibroblasts in the conjunctiva contain large amounts of rough endoplasmic reticulum (*).

Fig.2a

Fig.2b

Fig.2c

Fig.2d

Figure 2

a Examples of collagen fibril profiles in electron microscopical images of the conjunctiva of control eyes and of eyes treated with UV light of an intensity of 45 mW/cm². b Number of collagen fibrils per μm^2 in fibril bundles of the conjunctiva of control eyes and of eyes treated with UV light of an intensity of 45 mW/cm². c Collagen fraction (the relative area filled with collagen fibrils within the interfibrillar matrix of the conjunctiva) of control eyes and of eyes treated with UV light of an intensity of 45 mW/cm². d The diameter of single collagen fibrils in the control eyes and treated with UV light of an intensity of 45 mW/cm². (*: $P < 0.05$)

Figure 3

The expression of collagen fibrils in the rabbit conjunctiva by HE (×400, A: control group, B: treatment group)

Figure 4

The expression of collagen fibrils in the rabbit conjunctiva by Masson (×400, A: control group, B: treatment group)

Figure 5

Immunohistochemical staining of frozen sections. Collagen I was expressed in the rabbit conjunctiva. When compared with the control group, there was higher expression in the treatment group. (A: control group, B: treatment group)

Figure 6

Immunohistochemical staining of frozen sections. Collagen III was expressed in the rabbit conjunctiva. When compared with the control group, there was higher expression in the treatment group. (A: control

group, B: treatment)

Figure 7

Comparing the control group, the average optical density of collagen I and collagen III have no significantly increased after treatment with riboflavin and UVA light of 45 mW/cm² ($P > 0.05$).

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