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OPHTHALMOLOGY

Comparison of the biomechanics and fixation index of crosslinking between lysyl oxidase and genipin on guinea pig sclera, an animal model of defocus-induced high myopia

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Abstract. The aim of this study was to investigate the crosslinking effect of lysyl oxidase (LOX) alone and in combination with genipin (GNP) on the sclera of myopic eyes.

A total of 116 guinea pigs were used in this study. Among them, 16 guinea pigs were used as normal controls. The others were established as a lens-induced myopia model. The study included 80 guinea pigs with myopia higher than –6.0D. There were 12 groups of guinea pigs (A–L). Groups A and B included guinea pigs with normal eyes treated with saline and 0.1% LOX, respectively. Groups C, D, E, F, G, H, I, J, K, and L had myopic eyes. Groups C, D, E, and F were treated with saline, 0.1% LOX, 0.5% LOX, and 1% LOX, respectively. Groups G, H, and I were treated with 0.1% LOX + 1% GNP, 0.5% LOX + 1% GNP, and 1% LOX + 1% GNP, respectively, and allowed to react for 4 h. Groups J and K were treated with 1% LOX + 1% GNP for 8 h and 12 h, respectively. Group L was treated with 1% GNP for 4 h. The biomechanical features and fixation index of crosslinking between the groups and among 16 eyes in each group were compared. The isolated sclerae were treated with various agents in vitro. The elastic modulus and tensile strength of the sclerae were measured in 10 eyes from each group using an electronic microtensile machine. Samples from the other six eyes of each group were used to determine the fixation index via a ninhydrin assay.

Significant differences were observed in the elastic modulus and tensile strength measurements between groups A (saline) and B (0.1% LOX) with normal eyes ($P < 0.05$), between groups C (saline) and D (0.1% LOX) with myopic eyes ($P < 0.05$), and between group C (saline) and groups D (0.1% LOX), E (0.5% LOX), and F (1% LOX) ($P < 0.01$). However, there were no differences among groups D, E, and F ($P > 0.05$). Moreover, there were significant differences among groups I (1% LOX + 1% GNP, 4 h), J (1% LOX + 1% GNP, 8 h), and K (1% LOX + 1% GNP, 12 h) ($P < 0.05$). There was a significant difference in the mean elastic modulus between group L (1% GNP) and groups F (1% LOX) and I (1% LOX + 1% GNP) ($P < 0.01$), but there was no difference between group F and group I ($P = 0.412$).

LOX had a crosslinking effect on the sclera of normal as well as on myopic eyes. The effect of crosslinking of LOX was weaker than that of GNP, and no catalytic crosslinking effect of LOX was shown with GNP in the present study.

Key words: lysyl oxidase, genipin, scleral crosslinking, high myopia, biomechanics.

INTRODUCTION

While the etiology of myopia is currently under intense investigation, it is clear that the optical reason of myopia is an elongated eye. It has been demonstrated that changes in scleral collagen are the underlying cause of axial growth [1–3]. The decomposition and biomechanical changes of the posterior scleral collagen are considered to be the factors that induce myopia or the pathological progression of myopia in juveniles [4,5]. In the prevention

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and control of myopia, scleral collagen crosslinking has been shown to increase the biomechanical strength; thus, controlling the axial length growth could be a treatment strategy [3,6,7]. Scleral crosslinking includes physical crosslinking and chemical crosslinking. The former combines ultraviolet A rays with riboflavin. However, its application is limited due to damage to tissues and the need for special lighting equipment [8]; it is more suitable for the ocular surface and the cornea. Chemical crosslinking has attracted more attention because it does not require special equipment and is not limited by the anatomical location. The traditional crosslinking agents used for animal experiments at present, such as glutaraldehyde and glyceraldehyde, have obvious toxic and side effects [9–11].

The natural compound genipin (GNP) has a low cytotoxicity and a good crosslinking effect. It is a gardenia glycoside hydrolysate that is derived from gardenia fruit; its molecular formula is $C_{11}H_{14}O_5$. It reacts with amino acids to form blue pigment. The higher its concentration, the darker the blue staining [12,13]; therefore, the use of GNP is limited to the white sclera [14]. It is important to find a crosslinking agent that can reduce or avoid GNPs blue staining.

Lysyl oxidase (LOX) is a 32-kDa extracellular copper ion-dependent amino acyl oxidase. Research has shown that LOX, with the help of copper ions, can form special covalent bonds in the extracellular matrix to oxidize certain amino acid residues on collagen and elastin [15]. Thus, LOX can catalyze collagen formation and elastin maturation and is the key enzyme responsible for the stability of collagen [16–18]. LOX has been studied in many fields of medicine. For example, the decrease of LOX expression can affect the elastic fibers, which leads to a decreased stability of the pelvic support structure [19]. In addition, LOX is upregulated in many tumors and is closely related to the invasion and metastasis of tumors [20,21]. Can LOX act directly on the sclera of myopic eyes and become an effective crosslinking agent? Or, can LOX be used in conjunction with GNP to enhance the crosslinking effect and reduce the adverse reactions of GNP? The present study was performed to answer these questions, and hopefully will lay the groundwork for further treatment options for myopia.

MATERIALS AND METHODS

Reagents and equipment

Custom-made polymethyl methacrylate contact lenses for guinea pig (Beijing Jingde Jia-Run Trade Co., Ltd.) were used in this study. The parameters of the lenses were as follows: –8.00D, peripheral diameter of 3 mm, total diameter of 18 mm, base arc of 8.00 mm, and optical zone of 12 mm. The reagents and equipment used for the biomechanical test and for the fixation index of crosslinking are shown in Tables 1 and 2, respectively.

Table 1. Reagents and equipment used for the biomechanical test

	Manufacturer	Model
Lysyl oxidase recombinant protein	ImmunoClone, Huntington Station, NY, USA	_
Genipin	Linchuanzhixin Biological Technology Co., Ltd., Fuzhou, Jiangxi, China	_
Digital micrometer calipers	Shanghai Measuring Tool & Cutting Tool Co., Ltd.	0–100 mm
Computer-controlled electronic biomechanical tensile testing machine	Shanghai Heng Yi Precision Instrument Co., Ltd.	M221C

Table 2. Reagents and equipment used to determine the fixation index of crosslinking

	Manufacturer	Model
Anhydrous ethanol	Sinopharm Chemical Reagent Co., Ltd., China	10009218
Acetic acid	Qiangsheng Chemical Co., Ltd., Jiangsu, China	64-19-7
Anhydrous sodium acetate	Sinopharm Chemical Reagent Co., Ltd., China	10018818
Glycine	Bioengineering Co., Ltd., Shanghai, China	A610235
Ninhydrin	Bioengineering Co., Ltd., Shanghai, China	A610378
Centrifuge	Thermo Scientific™, USA	MicroCL 17
Electric heated water bath	Beijing Changfeng Instrument Co., Ltd., China	HW-SY11-K P2
Aquapro super pure water meter	Aquapro Corporation, USA	AJY-0501
PH meter	Metter-Toledo GmbH, Germany	LP115
Electronic balance	Beijing Sartorius Instrument Systems Co., Ltd., China	CPA
Microplate reader	BioTek, Winooski, VT, USA	EPOCH2
Centrifuge	Thermo Scientific™, USA	Legend Micro 21R

Induction of myopia in guinea pigs by lenses

The animal experiment was reviewed and approved by the Ethics Committee of Shanghai Experimental Animal Society. A total of 116 3-week-old normal tricolor guinea pigs from England (provided by the Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), weighing 180–200 g, were used in this study. Two guinea pigs were raised in each cage at a constant temperature (23–25 °C). The intensity of the indoor fluorescent lighting was about 500 Lx, with a 12-h light/dark cycle. The defocused myopia induction process was as follows: First, a Velcro ring washer was fixed on the head of a guinea pig with skin glue. Then a concave lens was pasted on the Velcro ring washer. The lens vertex distance was about 5 mm from the cornea (Fig. 1). During the experiment, the lenses were cleaned twice daily, and the guinea pigs wore glasses continuously except during the cleaning of the lens [22]. After 21 days of lens treatment, the refractive state was determined by two optometrists using a streak retinoscope, and the eyes of the guinea pigs with a mean value more than –6.0D were included in this study.

Sampling and grouping

The guinea pigs were sacrificed by using overdose anesthesia (0.2 g/2 mL, xylazine hydrochloride, Huamu Animal Health Products Co., Ltd., Jilin, China). The eyeballs were removed, and the scleral tissues were isolated from each eye within 4 h after sacrifice under constant temperature and humidity conditions (25 °C, 40% humidity). A total of 32 normal eyes and 32 myopic eyes of guinea pigs were randomly divided into four groups (16 eyes per group). Scleral strips of about 4 mm × 10 mm were cut from 10 eyes in each group. The scleral tissues were placed into the following groups: group A (normal eyes, balanced salt solution), group B (normal eyes, 0.1% LOX solution), group C (myopic eyes, balanced salt solution), and group D (myopic eyes, 0.1% LOX solution), and allowed to react for 4 h (Table 3). After the crosslinking reaction was completed, the scleral surface was washed with a balanced salt solution, and then the strips were subjected to a tensile test to measure the elastic modulus and tensile strength. The remaining six samples in each group were tested for the degree of crosslinking. The differences in biomechanical and crosslinking degrees between groups A and B and between groups C and D after the reaction with the different crosslinking solutions were compared.

Eighty myopic eyes of guinea pigs were collected as described above, randomly divided into five groups (16 eyes each): group E (0.5% LOX), group F (1% LOX), group G (0.1% LOX + 1% GNP), group H (0.5% LOX + 1% GNP), and group I (1% LOX + 1% GNP), and allowed to react for 4 h. The same tests as described above were used to compare the differences among groups D, E, and F, as well as among groups G, H, and I.

Next, 32 myopic eyes, collected as described above, were randomly divided into two groups (16 eyes) and put into the same solution (1% LOX + 1% GNP), wherein group J was crosslinked for 8 h, and group K was crosslinked for 12 h. The same tests as described above were used to compare the differences among groups I, J, and K.

Subsequently, for group L, 16 myopic eyes, collected as described above, were put into 1% GNP for 4 h. The same tests as described above were used to compare the differences among groups L, F, and I.

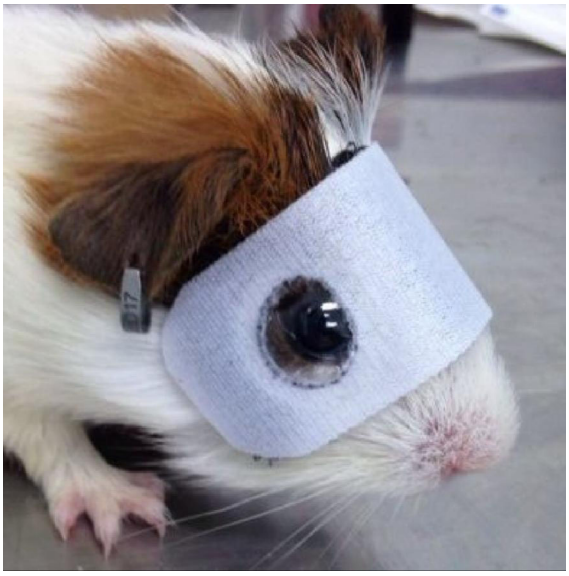

Fig. 1. Defocused myopic guinea pig model wearing contact lenses.

Table 3. Scleral tissue groups

Group	Eyes	Agent	Reaction time
A	16 normal eyes	Saline	4 h
B	16 normal eyes	0.1% LOX	4 h
C	16 myopic eyes	Saline	4 h
D	16 myopic eyes	0.1% LOX	4 h
E	16 myopic eyes	0.5% LOX	4 h
F	16 myopic eyes	1% LOX	4 h
G	16 myopic eyes	0.1% LOX + 1% GNP	4 h
H	16 myopic eyes	0.5% LOX + 1% GNP	4 h
I	16 myopic eyes	1% LOX + 1% GNP	4 h
J	16 myopic eyes	1% LOX + 1% GNP	8 h
K	16 myopic eyes	1% LOX + 1% GNP	12 h
L	16 myopic eyes	1% GNP	4 h

Biomechanical test

The biomechanical test was performed at room temperature (25 °C) and 40% humidity. The system configuration of the computer-controlled electronic biomechanical tensile testing machine was set as follows (Fig. 2): force sensor of ± 10 N, sampling speed of 25 times per second, automatic fracture determination, automatic return after fracture, preload of 0.005 N, and loading speed of 5 mm/min. The both ends of the scleral strip specimen were fixed on the clips and clamped. The distance between the upper and lower clamps was adjusted through the computer software so that the scleral strips were not folded, packed, or over-stretched. The thickness and width of each sample were measured by using electronic digital micrometer calipers and computer software for data analysis. The curve abscissa represented the stress, the ordinate represented the strain, and then they were entered in the computer. The machine was programmed to stretch the scleral strips at a constant speed. The computer automatically collected the sample stress–strain values and automatically generated the stress–strain curve and the maximum tensile strength.

Test for the fixation index of crosslinking

The ninhydrin assay [23] was performed, and the standard curve was established. First, 0, 40, 80, 120, 160, or 200 µL of glycine solution (200 µg/mL) was added to a 1.5-mL Eppendorf tube, and distilled water was added to a volume of 200 µL. Then to each tube 200 µL 2 M acetate buffer of pH 5.4 and 200 µL of 0.1% ninhydrin color developing solution were added. After thorough mixing, the tube was capped, heated in a 100 °C water bath for 15 min, and then cooled to room temperature. Finally, 0.6 mL of 60% ethanol was added to each tube, and the optical density (OD) value of each tube was measured using a microplate reader at a wavelength of 570 nm. The standard curve was drawn with OD 570 nm as the vertical axis, and the amino acid content as the abscissa. For sample processing, the scleral samples were equilibrated for 24 h at constant temperature and constant humidity, and 5 mg of each sample was accurately weighed. Next, 300 µL of deionized water and 450 µL of 0.1% ninhydrin solution were added, and the mixture was heated at 100 °C for 20 min and then cooled to room temperature. Subsequently, distilled water was added to a volume of 2 mL, the solution was set aside for 10 min at room temperature, and finally the OD value was measured with a microplate reader at a wavelength of 570 nm. For the calculation of the degree of crosslinking, the measurement was repeated three times for each sample, and the number of the free amino groups in the sample was calculated based on the standard curve. The formula is as follows: Fixation index % = (Free amino content before crosslinking – Free amino content after crosslinking) / Free amino content before crosslinking × 100% [24].

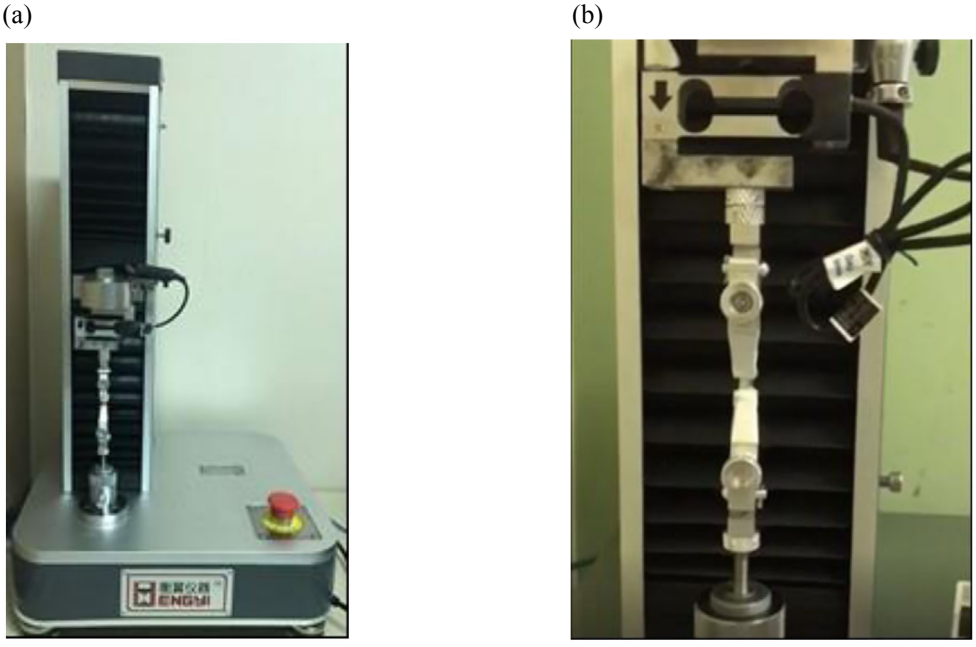

Fig. 2. (a) Electronic biomechanical tensile testing machine. The lower clip was fixed, and the upper clip could move up and down for the stretch test. (b) Enlarged picture of the clips. The white tissue shown between the two clips is a scleral strip for testing. The clip has a customized 5-mm-wide fixture tip.

RESULTS

The results ($\bar{x} \pm s$) for the elastic modulus and tensile strength showed that there was a significant difference in the overall mean values between groups A and B ($t = –5.157, –4.562$, $P < 0.05$) as well as between groups C and D ($t = –7.340, –7.360$, $P < 0.05$). Groups A and C were not treated with the crosslinking solution; therefore, there were no data for the fixation index (Table 4). The measurements of amino acids in the sclerae of groups A and C were only used as a baseline for comparison with the crosslinking groups.

There were statistically significant differences in the elastic modulus and tensile strength between group C and groups D, E, and F ($P < 0.01$). However, there was no statistically significant difference in the fixation index among groups C, D, E, and F ($P > 0.05$, Table 5). Moreover, there were no significant differences in the biomechanics and fixation index among groups G, H, and I ($P > 0.05$, Table 6).

There were statistically significant differences in the biomechanics and fixation index between group I and groups J and K ($P < 0.05$). With the extension of the crosslinking time, not only the biomechanical strength and the fixation index were obviously higher, but the degree of blue staining of the sclera was obviously increased as well (Table 7, Fig. 3).

There were statistically significant differences in the mean elastic modulus, tensile strength, and fixation index between group L and groups F and I ($P < 0.01$). However, there was no significant difference in the mean elastic modulus, tensile strength, or fixation index between group I and group L ($P > 0.05$, Table 8).

Table 4. Effects of LOX on elastic modulus, tensile strength, and fixation index in normal and myopic eyes

Group	Treatment	Time	Elastic modulus (Mpa)	Tensile strength (Mpa)	Fixation index
A (normal eyes)	Saline	4 h	190.501 ± 9.457	2.986 ± 0.176	
B (normal eyes)	0.1% LOX	4 h	231.517 ± 14.501	3.657 ± 0.308	10.4 ± 1.6%
C (myopic eyes)	Saline	4 h	171.833 ± 18.225	2.312 ± 0.328	
D (myopic eyes)	0.1% LOX	4 h	227.667 ± 25.751	3.131 ± 0.358	10.2 ± 1.1%
Group A vs Group B	*t*		–5.157	–4.562	No data
	p		0.04	0.006	
Group C vs Group D	*t*		–7.340	7.360	No data
	p		0.001	0.001	

Table 5. Effects of LOX concentrations on elastic modulus, tensile strength, and fixation index in myopic eyes

Group	Treatment	Time	Elastic modulus	Tensile strength (Mpa)	Fixation index (Mpa)
C	Saline	4 h	171.833 ± 18.225	2.312 ± 0.328	
D	0.1% LOX	4 h	227.667 ± 25.751	3.131 ± 0.358	10.2 ± 1.1%
E	0.5% LOX	4 h	239.143 ± 19.726	3.465 ± 0.513	11.7 ± 1.5%
F	1% LOX	4 h	246.276 ± 20.890	4.060 ± 0.417	12.9 ± 1.6%
	F		153.768	72.540	1.046
	P		0.000	0.000	0.375

Table 6. Effects of combined applications of LOX and GNP in myopic eyes

Group	Treatment	Time	Elastic modulus	Tensile strength (Mpa)	Fixation index (Mpa)
G	0.1% LOX + 1% GNP	4 h	315.708 ± 17.262	4.965 ± 0.274	21.1 ± 1.7%
H	0.5% LOX + 1% GNP	4 h	319.013 ± 11.095	5.302 ± 0.447	21.8 ± 2.1%
I	1% LOX + 1% GNP	4 h	322.622 ± 15.145	5.493 ± 0.776	22.3 ± 2.6%
	F		0.872	2.444	1.046
	P		0.438	0.121	0.375

Table 7. Effects of different exposure times in 1% LOX + 1% GNP treatment

Group	Treatment	Time	Elastic modulus	Tensile strength (Mpa)	Fixation index (Mpa)
I	1% LOX + 1% GNP	4 h	322.622 ± 15.145	5.493 ± 0.776	22.3 ± 2.6%
J	1% LOX + 1% GNP	8 h	340.167 ± 16.804	6.701 ± 0.431	26.1 ± 1.2%
K	1% LOX + 1% GNP	12 h	353.833 ± 13.867	8.560 ± 0.422	32.9 ± 1.3%
	F		17.737	53.581	81.426
	P		0.000	0.000	0.000

Fig. 3. The appearance of guinea pig sclerae in 1% LOX + 1% GNP solution after crosslinking for 4 h, 8 h, and 12 h.

Table 8. Effects on scleral biomechanics and fixation index in diferent treatments

Group	Treatment	Time	Elastic modulus	Tensile strength (Mpa)	Fixation index (Mpa)
F	1% LOX	4 h	246.276 ± 20.890	4.060 ± 0.417	12.9 ± 1.6%
I	1% LOX + 1% GNP	4 h	322.622 ± 15.145	5.493 ± 0.776	22.3 ± 2.6%
L	1% GNP	4 h	336.097 ± 19.204	5.971 ± 0.467	23.8 ± 2.3%
	F		22.156	21.841	36.377
	P		0.000	0.000	0.000

DISCUSSION

The present study was designed to answer the following questions: Does LOX have a direct crosslinking effect on the sclera? Are the crosslinking effect of the LOX concentration and the exposure time related? What is the effect when LOX is combined with GNP?

To the best of the authors' knowledge, there are no reports on the use of LOX in scleral crosslinking in the literature at present. Therefore, the present study was a preliminary study to determine whether LOX has a crosslinking effect in testing the biomechanical features and the fixation index.

The elastic modulus is used to evaluate the mechanical properties of materials; it is the ratio of material stress to the related strain, which represents the ability of materials to resist elastic deformation. When the strength of the material increases, also the elastic modulus as well as the tensile stress will increase under the same strain of the pull. The ninhydrin method was used to test the fixation index. The fixation index reflects the degree of crosslinking in the collagen sample. Ninhydrin reacts with the free α-amino acids to form a blueish-purple pigment, and the amount of the pigment is proportional to the amount of free α-amino acids [23,24]. The fixation index reflects the percentage of the amino acids that react with the crosslinking agent: the higher the value, the more complete the reaction (*see* Materials and Methods for the formula to calculate its value).

In the present study, the sclerae were immersed in the LOX solution, and the effect of LOX was direct and thorough. Therefore, we selected 0.1% as the starting concentration of LOX and increased the concentration to 0.5% and 1% to compare the crosslinking effect of various concentrations. There are many reports on the crosslinking effect of various concentrations of GNP in isolated sclerae. The most commonly used concentrations of GNP are 0.01%, 0.1%, and 1%, and the immersion times in the GNP solution vary from 1.5 h to 24 h [25,26]. We applied 1% GNP and an immersion time of 4 h in the present study.

The guinea pigs we used had too small eyeballs for an in vivo injection procedure. If we perform the experiment in vivo, the crosslinking agents need to be injected subconjunctivally and may not be distributed evenly on the surface of the sclera. The crosslinking effect could also be affected by other factors, such as catabolism in vivo, which will affect the final results of the study. Therefore, we designed our study as an in vitro experiment in order to allow the agents to be in direct contact with both sides of the sclera strips and to ensure their maximum effect. We will perform further studies in vivo only when the experiments show effective crosslinking in vitro.

The results of the study showed the following:

- 0.1% LOX had a crosslinking effect on the sclera of normal and myopic eyes.
- Although the mean value of the fixation index increased as the LOX concentration increased, the crosslinking effect did not show a statistical difference under the experimental conditions of the present study. This finding may be related to the low concentration of LOX used. Furthermore, these results may also be related to the nonactivation of LOX because no copper ions were added [15–17]; a further study is warranted.
- We did not find any difference in the fixation index between the groups by adding 1% GNP to different concentrations of LOX. The present study indicated that the major effect was from GNP when it was used in combination with LOX.
- According to our study, the best combination was 1% LOX + 1% GNP, and the fixation index was time-dependent. The longer the crosslinking time, the higher the fixation index.

These results are consistent with a previous report [12]. However, in our experiments, the role of LOX in combination with GNP was not determined. Therefore, we performed one more experiment to compare the crosslinking effects of 1% GNP, 1% LOX, and 1% GNP + 1% LOX. The results demonstrated that 1% LOX had the weakest effect. There was no significant difference in the crosslinking effect between the 1% GNP group and the 1% GNP + 1% LOX group. The combination of LOX with GNP did not enhance the crosslinking effect when compared with GNP alone. However, the combination significantly reduced the blue staining of the sclera (Fig. 4), which indicated that LOX and GNP may compete for the same crosslinking binding site. For further research, we will explore the experimental conditions, concentration ratio, and crosslinking mechanism of LOX and GNP.

Although the effectiveness of LOX on scleral crosslinking was confirmed in the present study, some questions still remain to be explored. For example: What is the optimal and safe concentration of LOX on the sclera? Is LOX superior to other chemical crosslinkers in terms of effectiveness and safety? What are the reaction intensity and toxicity when copper ions are added? What are the mechanisms of the crosslinking reaction of LOX and GNP and their possible reaction site in the sclera? What are the effects of LOX when it reacts with an extracellular matrix, e.g. matrix metalloproteinases, collagen, and other factors in a myopic sclera? These questions will be explored in our future studies.

Fig. 4. Group A: control; group F: 1% LOX; group I: 1% LOX + 1% GNP; group L: 1% GNP. The crosslinking time for all groups was 4 h.

CONCLUSIONS

In the present study, LOX showed a crosslinking effect on the sclera of normal as well as myopic eyes from guinea pigs. However, no correlation between the effect and the LOX concentration was found in the present study. Better experimental conditions, e.g. temperature or addition of copper ions, may reveal a better crosslinking effect of LOX. Comparison of treatment with

1% LOX, 1% LOX + 1% GNP, and 1% GNP showed that the group treated with 1% LOX only had the weakest crosslinking effect. No significant difference in the cross-linking effect was found between the 1% LOX + 1% GNP and 1% GNP groups. LOX did not show a catalytic crosslinking effect with GNP in the present study.

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