·Basic Research·

External sclerostomy with the femtosecond laser versus a surgical knife in rabbits

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Abstract

• AIM: To experimentally compare the external sclerostomy produced using a femtosecond laser with that made by a surgical knife and to evaluate the healing patterns, efficacy and technical advantages of femtosecond laser sclerostomy.

• METHODS: In a prospective randomized, controlled, masked-observer study, 10 pigmented rabbits underwent external sclerostomy with a femtosecond laser in the right eye; 10 additional rabbits underwent sclerostomy with a surgical superblade in the right eye. Clinical characteristics, which included bleb morphology and intraocular pressure, were recorded for 1 month after surgery. Six additional rabbits underwent external femtosecond laser sclerostomy in the right eye and mechanical sclerostomy in the left eye and were killed at day 14 after surgery. Histologic staining, immunohistochemistry and scanning electron microscopy were subsequently performed to assess the morphology of the filtering fistula. The titanium-sapphire femtosecond laboratory laser was operating at a repetition rate of 1 kHz, 0.4 mJ pulse energy, a central wavelength of 800nm and a pulse duration of 50 femtoseconds. Mann-Whitney and Kaplan-Meier tests were used for statistical analysis.

• RESULTS: Successful complete sclerostomy was achieved in each laser-treated eye which was hit only once by the laser. The laser treated time was approximately 15s-16s. In the laser-treated group (n=16), 2 eyes (12%) developed mild hyphema at the site of entry and 8 eyes (50%) showed transient edema in the corneal periphery adjacent to the laser impact zone. The differences between the groups in duration of function blebs and pressure reduction were statistically significant (P=0.025 and 0.016, respectively). The success rate of the laser-treated group was significantly higher than the knife group (P=0.005). Histologically, the subconjunctival connective tissue was loosely arranged with partially patent sclerostomy in the laser-treated eyes at postoperative day 14. This contrasted with the completely scarred sclerostomy tract in the knife group. The mean numbers of fibroblasts and new vessels as well as the amount of new collagen deposition at bleb site were significantly decreased in the laser group (P= 0.045, 0.013 and 0.036, respectively).

• CONCLUSION: The current study demonstrates that external femtosecond laser sclerostomy may offer a safe and effective alternative for the minimally invasive surgical management of glaucoma.

• KEYWORDS: femtosecond laser; sclerostomy; fibrosis; glaucoma

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INTRODUCTION

Conventional filtration surgery is the most frequent technique applied to reduce the intraocular pressure for patients with medically uncontrolled glaucoma. Unfortunately, the long-term success is often impaired by subconjunctival fibroblastic proliferation and subsequent scar formation in response to the mechanical manipulation and surgically-induced tissue trauma [1-3].

In 1990s, laser sclerostomy has gained great interest as an alterative approach that can minimize tissue dissection during the surgical procedures [4]. The usefulness of nanosecond or picosecond pulses of Nd:YAG [5], Er:YAG [6]

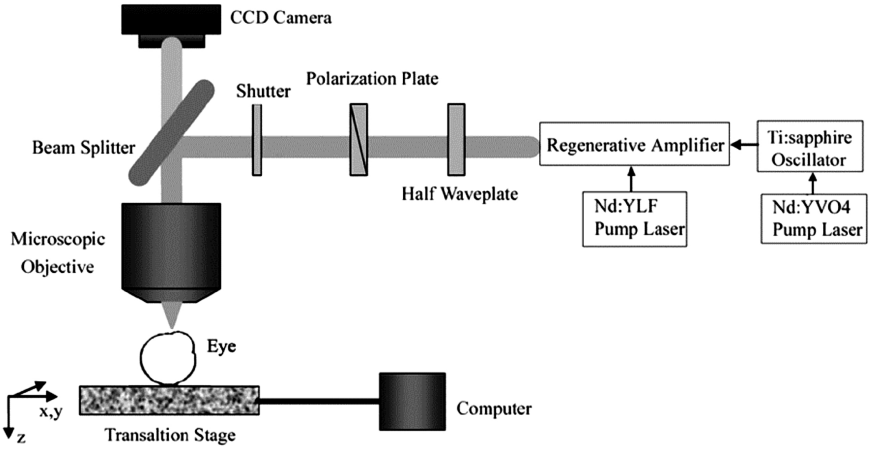

Figure 1 The experimental setup for laser sclerostomy.

and Ho:YAG [7] lasers has been previously investigated in laser sclerostomy. However, these treatments were ineffective or not widely accepted, because of excessive laser-induced thermal damage and mechanical relaxation occurring during the pulse [8]. The severe collateral damage stimulates fibrosis and scarring, which can subsequently result in the obstruction of filtering fistula, eventually leading to the surgical failure.

The generation of femtosecond pulsed laser offers a completely new possibility for minimizing the collateral damage during the laser surgery process [9-12]. Owing to its ultrashort pulse duration, extremely high intensity, and high precision ablation effect, it is distinctly advantageous for minimally invasive surgery in various medical fields, such as ophthalmology to create corneal flaps for refractive surgery, otolaryngology to treat ossicular bones, and cardiovascular surgery to treat arteriosclerosis [12].

Previously, our laboratory demonstrated the feasibility of minimally invasive laser sclerostomy by a femtosecond laser at an 800nm wavelength in hydrated rabbit sclera *in vitro*, and identified the appropriate patterns of laser ablation and relevant parameters [13]. The current study was undertaken to extend our previous study to a rabbit model *in vivo*. The purpose of this study was to compare the effect of an external sclerostomy performing using a femtosecond laser with one using a surgical superblade, and to evaluate the healing patterns, efficacy and advantages of femtosecond laser sclerostomy.

MATERIALS AND METHODS

Materials Twenty-six adult pigmented female rabbits weighing 1.5-2.2kg, 10-12 weeks of age were used in this prospective randomized, controlled, masked-observer study. Animals were obtained from the Animal Center of Tongji Medical College (Wuhan, China) and acclimatized for 1 week before the experiments started. All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Methods

Groups of treatment The experiment was performed in two phases. In the first phase, 20 rabbits were randomly divided into two groups: 10 underwent external sclerostomy performed with a femtosecond laser in the right eye and 10 underwent external sclerostomy with a surgical superblade in the right eye. The nonsurgical left eye served as a control. Clinical characteristics of the operated eyes, which included bleb morphology and intraocular pressure, were recorded after surgery. The second phase of our experiment involved an additional 6 rabbits, with the right eye treated with femtosecond laser and the left eye treated with mechanical sclerostomy on the same day. These 6 rabbits were killed for histology analysis at day 14 after surgery. In the present study, all clinical and histology assessments were made by an observer who was masked to the treatment received by each rabbit.

Laser system A schematic of the experimental setup is shown in Figure 1. Briefly, a mode-locked Ti:sapphire oscillator (Tsunami, Spectra Physics) pumped by a neodymium yttrium vanadate ($Nd:YVO_4$) laser (Millennia Vs, Spectra Physics) generates 80 MHz, 50 fs pulses. The oscillator output is amplified by a regenerative amplifier (Spitfire, Spectra Physics) to produce 800 nm, 50 fs pulses with pulse energy of 2 mJ at a 1 kHz repetition rate. The pulse energy of the amplified laser could be attenuated to a desirable level by rotating a half wave plate followed by a linear polarizer. A fast acting shutter was used to precisely control the laser exposure time or the pulse number delivered to the sample. A He-Ne laser (632nm) was used as a guiding beam because the surgical femtosecond laser beam is barely visible at 800nm. Both beams were focused onto the outer surface of sclera by a 4×/numerical aperture (NA) 0.1 microscope objective, which was mounted in an optical microscope (Eclipse 50i, Nikon) with a charge coupled device (CCD) camera so that the scleral surface and the process of laser ablation could be observed concurrently, using a beam splitter. The laser spot size at the surface of

sclera was 10μm in diameter, which was measured with the help of CCD image.

External laser sclerostomy General anesthesia was induced by ear-vein injection of 20% ethyl carbamate (Urethane, Shangpu Chemical Corp, Shanghai, China) 1g/kg. With rabbits under local anesthesia with 0.4% oxybuprocaine (Benoxil, Santen, Osaka, Japan), a lid speculum was inserted to expose the globe. A fornix-based conjunctival flap was raised, after which a blunt dissection of the subconjunctival space was performed of approximately 5mm along the limbus and 8mm posteriorly. The rabbit head was then mounted on a motorized computer-controlled three-dimensional translation stage driven by stepper motor controllers (SC102, Beijing Optical Instrument Corp, China). The stage was precisely positioned to bring the focus of the laser onto the region of corneo-scleral junction. The laser was then fired at a pulse energy of 0.4mJ with the beam path parallel to the iris. Meanwhile, the translation stage was triggered to move horizontally, which allowed the femtosecond pulses to scan over the limbus from the 12- to 1- o' clock positions in a 2 mm long straight line pattern. A full-thickness sclerostomy was indicated by observing the escape of aqueous humor from the surgical site and the He-Ne aiming beam into the anterior chamber. The translation speed was set to be 0.13mm/s. The pulse energy and the translation speed of the stage used in the surgery were derived from our previous *in vitro* study [13]. All treated eyes was hit only once by the laser. After laser ablation process, the conjunctiva was repositioned and the wound was closed with 8-0 interrupted Vicryl sutures (Ethicon, Piscataway, New Jersey, USA). The operative and fellow control eyes received topical 0.025% dexamethasone (Wujing Medicine Corp, Wuhan, China) and 0.25% chloramphenicol drops (Qianjiang Pharmaceutical Corp, Hubei, China) 4 times daily starting the day of surgery and continuing for 1 week.

External mechanical sclerostomy The rabbit was anesthetized, and the conjunctiva was raised and dissected as described above. A disposable surgical superblade (Jinhuan Medical Products Corp, Shanghai, China) was used to create a 2mm long limbal incision from the 12- to 1- o' clock positions, with patency being ensured by observing the tip of the blade in the anterior chamber. The blade was then removed and the conjunctival wound was closed with 8-0 interrupted Vicryl sutures. The operative and fellow control eyes received topical 0.025% dexamethasone and 0.25% chloramphenicol drops 4 times daily starting the day of surgery and continuing for 1 week.

Clinical evaluation Slit-lamp observations were performed at different time intervals after surgery to assess the filtering bleb status and the overall inflammatory state of the eye. The intraocular pressure (IOP) was measured with a handheld applanation tonometer (Tono-Pen XL, Medtronic Solan, Jacksonville, Florida, USA) with animals under topical anesthesia (0.4% oxybuprocaine, 1 drop per eye). The difference in IOP between the operated right eye and the left control eye was monitored before surgery and after surgery on the designated days until the filter was considered failed. The measurements were performed in triplicate and averaged. As the difference between both eyes of each animal was 2 mmHg or less before intervention, clinical success was defined by ⩾3mmHg difference in IOP.

Pathology At day 14 after surgery, 6 rabbits in the second phase were killed with a lethal intravenous injection of ethyl carbamate. All operated eyes were enucleated and bisected vertically in an anterior posterior plane at the sclerostomy site. One half was fixed in 10% formaldehyde and embedded in paraffin for light microscopy. The second half of the globes was placed in 2.5% glutaraldehyde for scanning electron microscopy.

For light microscopic examination, ten serial sections, each 5μm thick, were prepared from each specimen. The sections were stained with hematoxylin and eosin (H&E) for general histologic observation and inflammation cells, Masson trichrome stain to assess the level of collagen deposition, vimentin immunohistochemistry (Dako, Denmark) to identify the distribution of fibroblasts, and factor VIII immunohistochemistry (Dako, Denmark) to check the density of new blood vessels.

Light microscopic analysis was performed using a ×40 objective of a standard light microscope (BX-50 Olympus Photomicroscope). The numbers of inflammatory cells, fibroblasts as well as blood vessels per square centimeter were counted in each section by the help of an eyepiece inserted in the light microscope. The values obtained from the cell counts of 10 serial sections were presented as arithmetic means ±standard deviation. The number of inflammatory cells was divided into 4 grades: 0, less than 10 cells; 1, 10-50 cells; 2, 50-100 cells; 3, more than 100 cells. The level of collagen deposition was examined with an appropriate software (HMISA-2000 medical image analysis system, Champion Corp, Wuhan, China) which was connected to the light microscope, and also divided into 4 grades: 0, less than 25% of the subconjunctival space was filled by new collagen deposition; 1, 25%-50%; 2, 50%-75%; 3, more than 75%.

Statistical Analysis The Mann-Whitney test in SPSS for Windows (Version 12.0) was performed to compare IOP, time to bleb failure and the values obtained from the histological evaluations between the two treatment groups. Survival analyses were carried out for surgical success using the Kaplan-Meier log rank test. A P value<0.05 was considered significant.

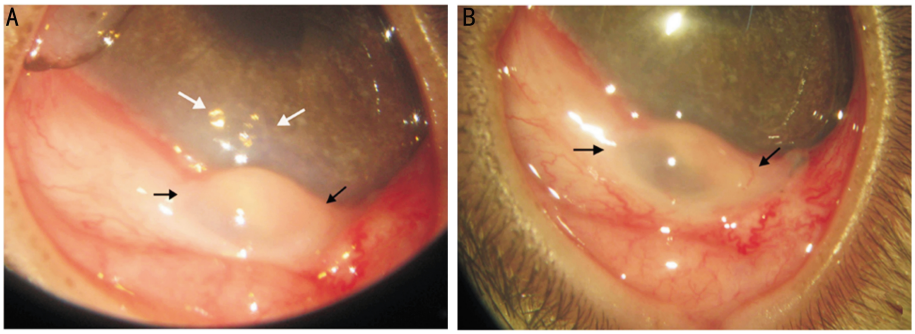

Figure 2 Functioning bleb after laser surgery A: Four days after laser surgery, an elevated, functioning bleb is visible along with focal perilimbal corneal edema; B: At day 10, the bleb remains functioning with resolution of corneal edema. Black arrows: edges of the bleb; white arrows: focal corneal edema.

RESULTS

Clinical Appearance There was little difficulty in technique with the femtosecond laser sclerostomy. A full-thickness sclerostomy was easily created with the laser in all 16 right eyes. The ablation time was 15-16 seconds, which was significantly shorter than that with the knife (4-5 minutes).

Localized corneal edema adjacent to the sclerostomy site was detected in 8 eyes (50%) in the laser group and was present for an average of 8.5 days (Figure 2), as opposed to 3 eyes (19%) and an average of 4.6 days in the knife group. Two eyes (12%) in the laser group developed mild hyphema at the site of entry which was induced by laser injury to iris root, but resolved spontaneously within 3 days. Conjunctiva hyperemia lasted an average of 7.8 days in the eyes that had undergone laser sclerostomy versus 5.6 days in the eyes that had undergone sclerostomy with a superblade. Trace flare was present in all treated eyes in both groups and resolved by 7 days postoperatively. No intraocular infection was seen in any rabbit at any time in the study.

The mean change in IOP difference in the laser group and knife group was exhibited in Figure 3. All of the rabbits in the knife group met the criterion for failure by 14 days postoperatively. In comparison, surgeries in 50% (5 of 10) of the laser-treated eyes at day 21 were successful. Two of the animals in this group were successful up to 30 days.

Blebs were noted to persist in the eyes that had undergone laser sclerostomy for an average of 9.9 days; blebs persisted in the eyes that had undergone sclerostomy with a blade for an average of 4.8 days. Three of ten of the eyes that had undergone sclerostomy with a blade never showed a clinically detectable bleb at all after surgery. Table 1 summarizes the numbers of eyes with functional blebs and IOP reduction over time. The differences in duration of functional bleb and IOP reduction were statistically significant (P=0.025 and P=0.016, respectively). Pressure reduction out-lasted the duration of the functional bleb in all cases. The cumulative probability of success was greater in the laser-treated group than the mechanical surgical group (Figure 4). The difference between the groups was statistically significant (P=0.005).

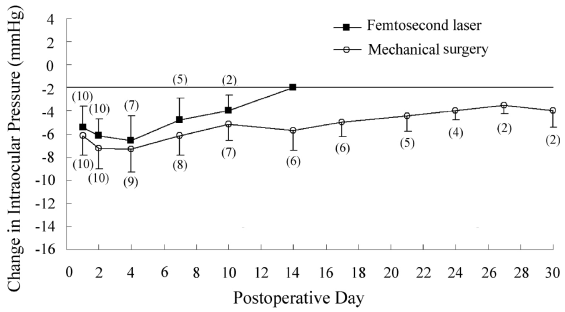

Figure 3 Mean changes in pressure in the laser group and the knife group Values in parentheses indicate number of rabbits measured at each time interval.

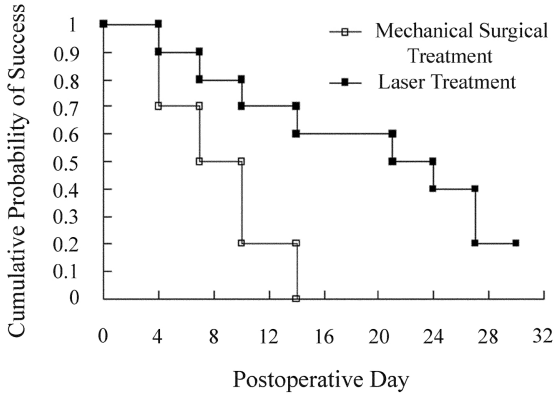

Figure 4 Survival curves of the laser group and the knife group (P=0.005).

Histologic Appearance As shown in Figure 5A, histologic examination confirmed a narrow but patent full-thickness sclerostomy in the laser group at day 14 postoperatively. The collagen tissue adjacent to the site of laser ablation maintained normal architectural features, with minimal collateral damage and absence of thermal coagulation. Tissue repair of the fistula was slight, with mild deposition of amorphic eosinophilic collagenous extracellular matrix at the internal ostium. Hyperemia of the iris root was obvious. In comparison, in the knife group at day 14, the sclerostomy became completely occluded by highly cellular fibrous tissue, with evidence of new collagen deposition in the subconjunctival space (Figure 5B).

Table 1 Duration of functional blebs and IOP reduction[1] in laser versus knife groups *n*(%)

Group	Duration(days)			P^2
	⩽7	8-14	>14	
	No. of eyes with functional blebs			0.025
Knife(*n*=10)	8(80)	2(20)	0(0)	
Laser(*n*=10)	3(30)	6(60)	1(10)	
	No. of eyes with IOP reduction			0.016
Knife(*n*=10)	5(50)	5(50)	0(0)	
Laser(*n*=10)	2(20)	2(20)	6(60)	

IOP=intraocular pressure.
[1]The decrease in IOP greater than 2 mmHg compared with the contralateral untreated eye, which is defined as the clinical success
[2] Mann-Whitney *U* test

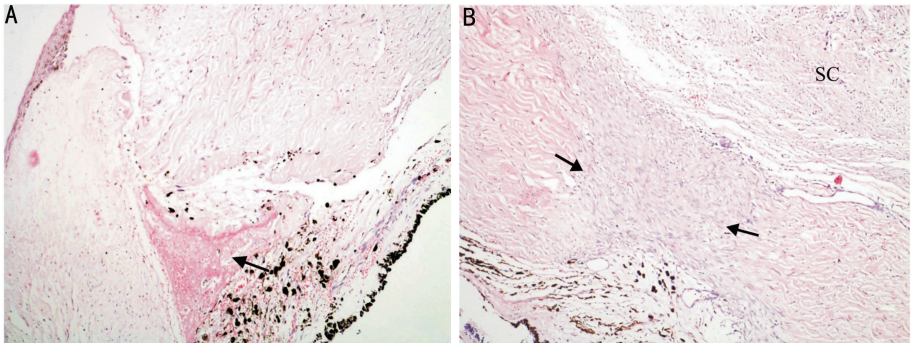

Figure 5 Light micrograph of sclerostomy at day 14 A: Laser group. Note the narrow but patent fistula, absence of a collateral coagulative effect, slight wound-healing reaction characterized by mild deposition of amorphic eosinophilic collagenous extracellular matrix (arrow) at the internal ostium; B: Knife group. The fistula has become obstructed by highly cellular fibrous tissue (arrow), with evidence of new collagen deposition in the subconjunctival space. SC: subconjunctival collagen fibers. (Hematoxylin and eosin stain,×200).

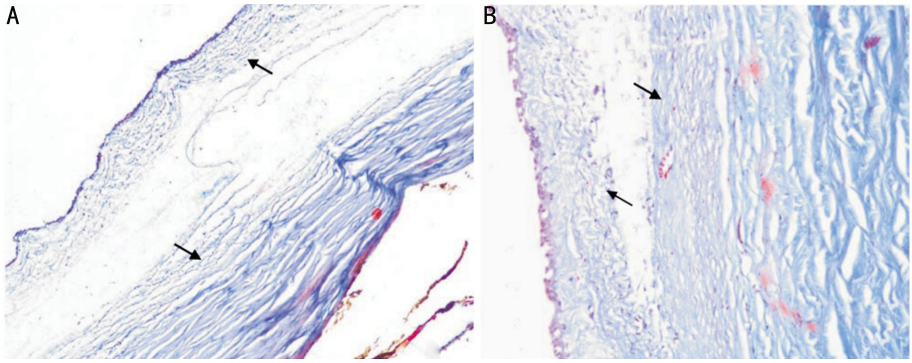

Figure 6 Masson trichrome stain to evaluate the level of collagen deposition at day 14 A:Laser group; B: Knife group Arrow: Level of linear collagen deposition (×200).

Chronic inflammation was observed by light microscopy in the filtering zone of both laser- and superblade-treated eyes, and there was no significant difference in the quantity of inflammatory cells (P=0.652). However, the amount of collagen deposition (Figure 6), as well as the mean numbers of fibroblasts and vessels (Figures 7 and 8, Table 2) in the filtration site were significantly less in the laser group than in the knife group (P=0.036, 0.045, 0.013, respectively).

We compared scanning electron microscopic (SEM) characteristics of the filtering fistula in both laser and knife groups at day 14 after surgery. Morphologically, SEM demonstrated a partially patent sclerostomy tract and loose subconjunctival bleb architecture in the laser group (Figure 9A). In contrast, in the knife group the fistula tract and subconjunctival space had scarred closed due to bulk filling by thick, dense collagenous connective tissue (Figure 9B).

Table 2 The mean number ± SD of fibroblasts and blood vessels at the bleb site in the study groups

Group	*n*	Fibroblasts	Blood vessels
Knife	6	54.67±12.96	18.67±4.89
Laser	6	[1]39.17±11.27	[2]10.17±4.17

Numbers were determined in a ×400 field
SD = standard deviation
[1]Mann-Whitney *U* test, P=0.045
[2]Mann-Whitney *U* test, P=0.013.

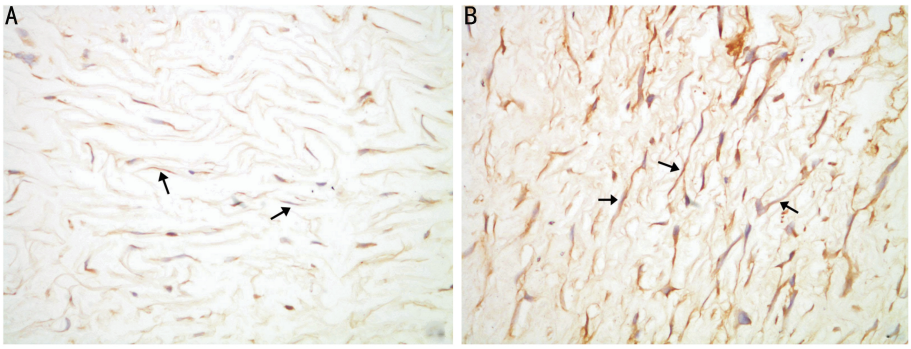

Figure 7 Vimentin immunohistochemical stain of the bleb site shows a reduction in the number of fibroblasts (arrow) at day 14 A: Laser-treated eyes; B: Superblade-treated eyes (×400).

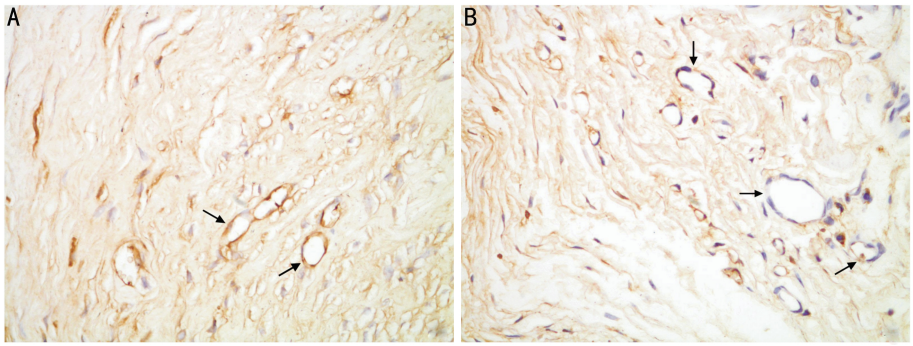

Figure 8 Factor VIII immunohistochemical stain of the bleb site shows the blood vessels (arrow) at day 14 A: Laser-treated eyes; B: Superblade-treated eyes (×400).

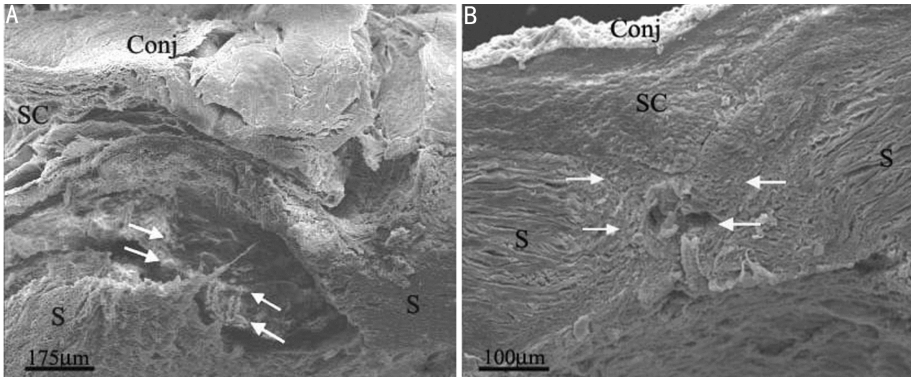

Figure 9 Scanning electron microscopic view of the filtering fistula at day 14 postoperatively A: The sclerostomy site created with the laser remained patent (arrow) and was associated with looser subconjunctival bleb architecture. Bar, 175μm. B: In the knife group the fistula tract and subconjunctival space completely closed (arrow) due to bulk filling by thick, dense collagenous connective tissue in the knife group. Bar, 100μm. Conj: conjunctiva; S: sclera; SC: subconjunctival collagen fibers.

DISCUSSION

Properties and advantages of femtosecond laser ablation This study demonstrated the feasibility of the femtosecond laser in external sclerostomy surgery in *in vivo* rabbit eyes. It is well established that the femtosecond laser is a minimally invasive tool for highly precise ablation of biological tissue [12]. The fundamental advantages in using femtosecond laser for ablation, as compared with nanosecond or picosecond pulsed lasers, have been outlined previously [14-16]. With ultrashort pulse duration, the femtosecond laser is able to produce extremely high power intensity capable of creating plasma formation and photodisruption, at quite a low energy-per-pulse level and decreased fluence threshold. Consequently, the overall thermal and mechanical load on the tissue is significantly reduced. In addition, the pulse duration of femtosecond laser is sufficiently short to minimize thermal diffusion and shock wave propagation from the volume of energy deposition into the unablated tissue. All these properties of femtosecond laser contribute to thermal and stress confinement, thereby reducing collateral tissue damage during the ablation process [17,18].

At present, corneal flap creation in laser in situ keratomileusis (LASIK) surgery is the most common application of the femtosecond laser in clinic [9]. Besides, due to its high precision and very low thermal and

mechanical side effects, femtosecond laser may also potentially be well suited for minimally invasive glaucoma laser therapy [11,14-16,19,20]. Up to date, however, only a few research groups have investigated the potential applications of femtosecond pulses in laser sclerostomy through *in vitro* experimental studies [11,19,20]. In the previous study, our laboratory demonstrated predictable sizes and shapes of photodisruption in hydrated rabbit sclera *in vitro* using an 800nm femtosecond Ti:sapphire laser and obtained the appropriate relevant parameters for laser sclerostomy [13]. The current study was undertaken to extend our previous efforts to *in vivo* eyes and compare femtosecond laser sclerostomy technique with conventional mechanical full-thickness procedure.

Comparison of the two sclerostomy techniques In the present study, a limbus full-thickness incision 2mm long was successfully created within 16 seconds in all treated eyes which were hit only once by the laser. In comparison with the mechanical surgery, sclerostomy was achieved faster and more easily with the laser due to the high power intensity produced by the femtosecond laser focusing.

The complications that were apparent with the laser group included localized damage of the adjacent cornea and iris, which was mainly due to misalignment of the laser beam and subsequent imprecision of the localization of the laser incision. Following improvement in laser delivery techniques such as the introduction of an optical fiber [21,22], the direction of laser beam should be controlled more precisely, and, thus, such complications may be minimized. In addition, the rabbit has a very shallow anterior chamber angle and an anterior ciliary body [23,24]. The anatomic characteristics may predispose inadvertent damage to the iris after penetrating the limbus.

In the current study, we defined clinical success as dependent on changes in IOP. In our experience, IOP measurements are less subjective and better quantifiable than bleb appearance and survival. Furthermore, throughout the course of the experiment, we monitored the difference in IOP between the operated right eye and the left control as opposed to measuring the absolute value of the operated eye in order to exclude interindividual, cyclic, and anesthesia-related variations in IOP [25]. It is worth to mention that IOP reduction lasted longer than the conjunctival bleb in both groups in the present study. Similar findings were described also in previous studies of performing sclerostomy using Nd:YAG [5,26], Er:YAG [27] and pulsed dye laser [28]. Possibly, several other mechanisms may be involved, such as a complete but narrow sclerostomy allowing filtration of aqueous but no bleb formation, a cyclodialysis effect, or reduced aqueous production from either direct trauma or subsequent uveitis.

On histologic examination, we verified that using the femtosecond laser to perform filtering procedures gave a more patent and durable fistula, with no evidence of collateral damage to the surrounding tissue. In addition, the laser-treated eyes showed a significant reduction in the amount of fibroblasts, blood vessels and collagen deposition at postoperative day 14 in comparison to the superblade-treated animals. Within the histologic parameters of our experimental model, we demonstrated that external sclerostomy with a femtosecond laser could effectively decrease fibrosis and scar formation after filtration surgery. The mechanism may be that the femtosecond laser produced highly efficient ablation of sclera with little collateral damage, thereby minimizing the stimulus for fibrosis.

To our knowledge, this is the first study to assess the safety and efficacy of performing filtration surgery with a femtosecond laser in an *in vivo* model. Compared with conventional external filtration surgery, femtosecond laser sclerostomy has been shown to remain functional for a longer period of time and have less fibrosis and scarring. Therefore, our results indicate a viable alternative for minimally invasive glaucoma surgical therapy.

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