·Basic Research·

The influence of He-Ne laser on scar formation after trabeculectomy in rabbits

Jun Hu[1], Gui-Gang Li[1], Hong Zhang[1], Nan Xiang[1], Zhi-Qi Chen[1], Rui-Lin Wang[1], Yan Xiang[1], Bin Li[1], Zheng-Jia Li[2], Yan-Yan He[2], Li-Fang Wang[2]

Foundation item: Supported by the Natural Science Foundation of Hubei Province, China (No. 2007ABA108)

[1]Department of Ophthalmology, Tongji Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

[2]Wuhan National Laboratory for Optoelectronics, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Correspondence to: Hong Zhang. Department of Ophthalmology, Tongji Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology, Wuhan430030, Hubei Province, China. zhanghongtjh@163.com

Received:2010-05-10 Accepted:2010-06-01

Abstract

• AIM: To investigate the influence of He-Ne lasers on scar formation in the filtration canal after trabeculectomy in a rabbit model, as well as to explore the mechanisms for preventing scar formation when using He-Ne lasers *in viva*

• METHODS: Experiment 1: Four groups were established (four eyes in each group). In 12 eyes, the upper nasal limbus area next to the upper rectus muscle received 10 minutes of He-Ne laser irradiation (100, 150, 200mW/cm^2; 60, 90, 120J/cm^2) every day for three days. Four eyes served as controls. Twenty-four hours after the final irradiation, the rabbits were sacrificed and the irradiated tissue was excised, fixed with paraformaldehyde and tested for proliferating cell nuclear antigen (PCNA), connective tissue growth factor (CTGF) and apoptosis (TUNEL). Experiment 2: Forty-two rabbits were randomly divided into two groups and standard trabeculectomy was performed in the right eyes either after 200mW/cm^2 He-Ne laser irradiation or not in the filtration area. The expression of PCNA and CTGF, apoptosis and collagen density in the filtration area were tested on the 7th, 14th and 28th day after surgery.

• RESULTS: Experiment 1: There were no more PCNA and CTGF positive cells in the He-Ne irradiation group than in the control group. No apoptotic cells were found in either group. Experiment 2: The expression of PCNA and CTGF was lower in the He-Ne irradiation group than in the control group on the 7th and 14th day after trabeculectomy surgery ($P< 0.05$); no apoptotic cells were detected in either group. Collagen density was significantly lower in the He-Ne irradiation group than in the control group on the 14th and 28th day after surgery ($P< 0.05$).

• CONCLUSION:Pretreating the filtration area with 200mW/cm^2 (120J/cm^2) of He-Ne laser irradiation may be helpful in preventing scar formation after trabeculectomy, possibly due to the downregulation of the expression of PCNA, CTGF and collagen synthesis in fibroblasts.

• KEYWORDS: He-Ne laser; trabeculectomy; scar formation

DOI:10.3980/j.issn.2222-3959.2010.02.09

Hu J, Li GG, Zhang H, Xiang N, Chen ZQ, Wang RL, Xiang Y, Li B, Li ZJ, He YY, Wang LF. The influence of He-Ne laser on scar formation after trabeculectomy in rabbits. *Int J Ophthalmol* 2010;3 (2):132-136

INTRODUCTION

Scar formation on the filtration bulb is the most common cause of trabeculectomy surgery failure [1]. The human body's healing process after trabeculectomy surgery comprises complicated biological processes involving an extensive network of cytokine, cells and extracellular matrix (ECM). Fibroblasts from Tenon's cyst are the most important type of cell involved in the healing and scarring process following surgery. The surgery trauma actives the fibroblast, and the fibroblast cell migrates, proliferates and synthesizes ECM such as collagen protein, stress protein and proteoglycan, which then leads to scar formation and failure of the filtration bulb [2-6]. Therefore the key to improving the outcome for filtration surgery-and an important area of research in this field-is to prevent scar formation of the fibroblast [7-9]. Scientists have found that He-Ne lasers have the ability to suppress the proliferation and collagen synthesis of skin fibroblast, and induce cell apoptosis *in vitro* [10-18]. The potential of He-Ne lasers to prevent scar formation after trabeculectomy therefore has been established. What we need to determine is how best to use He-Ne laser-in other words, what is the optimal amount of irradiation to be both effective and safe. Studies have shown that the influence of He-Ne laser on cells is bidirectional, depending on the energy and power density. Hawkins *et al* [10]. reported that He-Ne laser irradiation of 10J/cm^2 energy density could

damage normal and injured skin fibroblasts *in vitro*. Gross *et al* [11] reported that kidney epithelial proliferation was suppressed by He-Ne laser irradiation of 40mW/cm^2 power density (energy density 11.9-142J/cm^2) when administered every other day, but promoted by 142J/cm^2 administered a single time. AlWatban *et al* [12] reported a 39.1% increase in cell proliferation with irradiation of 1.25mW/cm^2 power density (energy density 180J/cm^2) over the course of three days. In Shu *et al* [13-15], He-Ne laser irradiation of 30J/cm^2 promoted the proliferation of scar fibroblasts *in vitro* while irradiation of 60J/cm^2 and higher reduced it. Thirty minutes of He-Ne laser irradiation of 100 or 150mW/cm^2 every day for three consecutive days reduced the amount of collagen synthesis and induced cell apoptosis [6]. Yasukawa *et al* [16] reported that fifteen seconds of He-Ne laser irradiation of 17mW/cm^2 every other day accelerated the healing of operative wounds in rat skin. Hawkins *et al* [17] found that 5J/cm^2 He-Ne laser irradiation for two continuous days could promote the migration and proliferation of skin fibroblasts *in vitro*, while 10 or 16J/cm^2 suppressed it. In Houreld *et al* [18], He-Ne laser irradiation of 5J/cm^2 every other day promoted migration while 16J/cm^2 suppressed it, with damage to the DNA.

In this study, we observed the influence of He-Ne laser irradiation at 200mW/cm^2 power density on scar formation after trabeculectomy *in vivo* in a rabbit model. We also examined changes in proliferating cell nuclear antigen (PCNA) and connective tissue growth factor (CTGF) and collagen synthesis to achieve a better understanding of the mechanism by which this occurs.

MATERIALS AND METHODS

Materials Fifty healthy sanitary New-Zealand white rabbits were used in this study, under the supervision of the Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (HUST). The animals, including both sexes and weighing approximately 2kg on average, were provided by the animal center of Tongji Medical College. The He-Ne laser used in this study (power density 100-300mW/cm^2; light spot diameter 4mm) was manufactured by Wuhan National Laboratory for Optoelectronics. Goat-anti-rabbit PCNA monoclonal antibody, goat-anti-rabbit CTGF monoclonal antibody, apoptosis kit (TUNEL) and a collagen trichrome stain kit were purchased from Wuhan Boster Biotechnology Company, China.

Methods The irradiation area was the naso-upper quadrant limbus next to the upper rectus muscle, with a diameter of 4mm. Irradiation was performed for ten minutes a day for three consecutive days, ending 24 hours before examination or trabeculectomy. Rabbits were anesthetized through intravenous injection of 30g/L pentobarbital sodium 1mL/kg.

Table 1 Expression of PCNA and CTGF in rabbit fibroblast cells (mean±SD, %, n=4)

He-Ne laser	PCNA	CTGF
Control	1.8±0.6	3.9±0.80
100mW/cm^2	2.0±0.9	4.0±1.9
150mW/cm^2	1.6±0.6	3.4±0.7
200mW/cm^2	1.7±0.3	4.1±1.8
F value	0.321	0.166
P value	0.810	0.917

Standard trabeculectomy was performed on the He-Ne laser irradiation site, a zone with the fornix-based conjunctival flap, a 3mm×3mm two-thirds full-thickness scleral flap, a 1.5mm×1mm trabeculum flap, a 1.5mm×1.5mm basal iridectomy, a scleral flap fixed with two interrupted sutures using 10-0 nylon and a conjunctival flap with a continuous suture.

Experiment one: sixteen eyes of eight rabbits were divided into four groups at random: a control group and three He-Ne laser groups, each with four eyes. The He-Ne laser groups received ten minutes of He-Ne laser irradiation (100, 150, 200mW/cm^2; 60, 90, 120J/cm^2) every day for three consecutive days, ending 24 hours before the animals were sacrificed (under the supervision of the Animal Ethics Committee). The irradiated tissue was then excised, fixed with 40g/L paraformaldehyde, tested for PCNA and CTGF expression (immunohistochemistry) and apoptosis (TUNEL) of the fibroblasts. The expression of PCNA and CTGF was semi-quantified using image analysis. Experiment two: forty-two rabbits were randomly divided into two groups: a control group and a He-Ne laser group. The rabbits in the He-Ne laser group received ten minutes of He-Ne laser irradiation (200mW/cm^2, 120J/cm^2) every day for three consecutive days, ending 24 hours before trabeculectomy in the left eye. Seven rabbits were sacrificed (under the supervision of the Animal Ethics Committee) and the filtration area was excised, fixed with 40g/L paraformaldehyde, and on the 7th, 14th and 28th day post-surgery, tested for PCNA, CTGF and collagen expression and apoptosis of fibroblasts.

Statistical Analysis SPSS version 13.0 software was used for data analysis, to explore the difference among or between groups with Chi-square test or t test. P<0.05 was considered statistically significant.

RESULTS

Experiment One The difference between the ratio of PCNA- to CTGF-positive fibroblasts in the control and He-Ne laser irradiation groups was not significant statistically (P>0.05). No apoptotic cells were found in either the control or He-Ne laser irradiation group (Table 1).

Experiment Two The expression of PCNA in fibroblast cells peaked on the 7th day postoperatively, decreasing dramatically on the 14th and 28th days (Figure 1). The ratio of

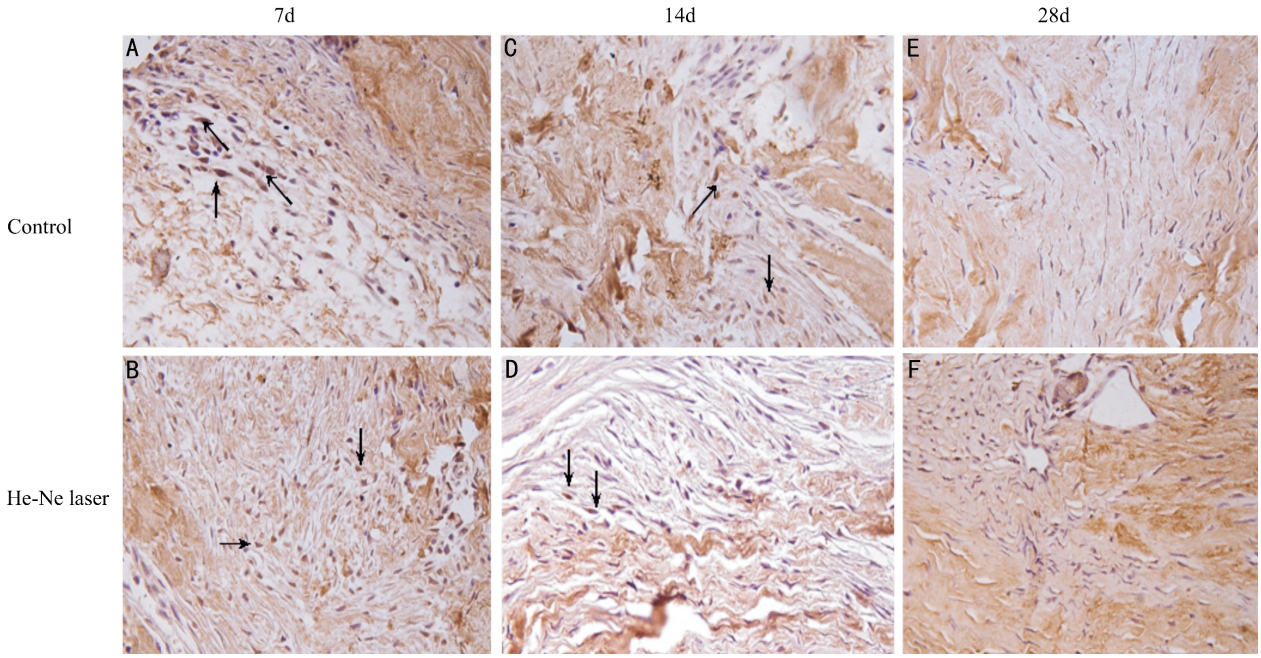

Figure 1 Expression of PCNA in fibroblasts after trabeculectomy in rabbit (SABC×400) PCNA positive fibroblasts were stained brown in the nuclei (arrowheads)

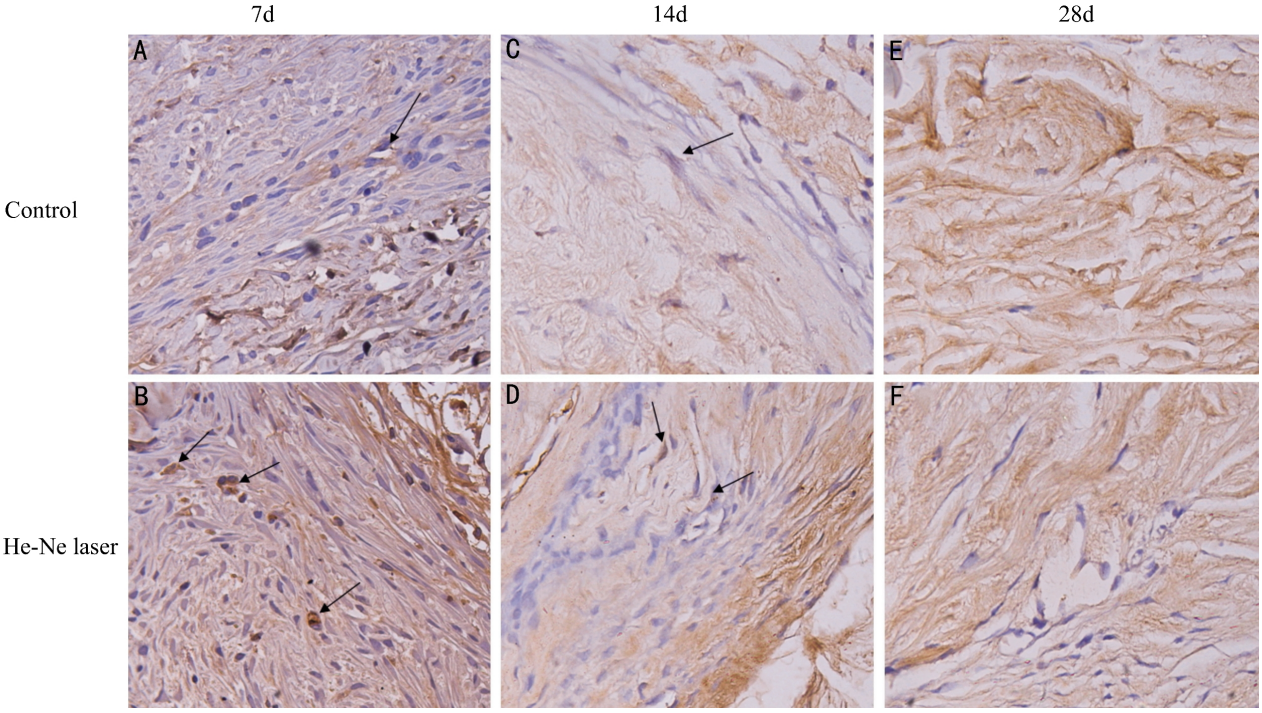

Figure 2 Expression of CTGF in fibroblast cells after trabeculectomy in rabbit (SABC×400) CTGF positive fibroblasts were stained brown in the cytoplasm (arrowheads)

PCNA-positive fibroblasts was lower in the He-Ne laser irradiation group than in the control group on the 7th and 14th day (t=5.968; 6.605, P=0.004; 0.003), but there was no statistically significant difference on the 28th day (t=0.256, P= 0.810). Expression of CTGF in fibroblast cells peaked on the 14th day postoperatively, decreasing dramatically on the 28th day (Figure 2). The ratio of CTGF-positive fibroblasts was lower in the He-Ne laser irradiation group than in the control group on the 7th and 28th day (t=4.606; 6.299, P= 0.043; 0.003), while there was no statistically significant difference on the 14th day (t =2.196, P =0.093). The collagen density in the filtration site was lower in the He-Ne laser irradiation group than in the control group on the 14th and 28th day (t=2.914; 3.032, P=0.013, 0.010)(Figure 3).

No apoptotic cells were found in either the control or the He-Ne laser irradiation group on the 7 th, 14th or 28th day postoperatively (Table 2).

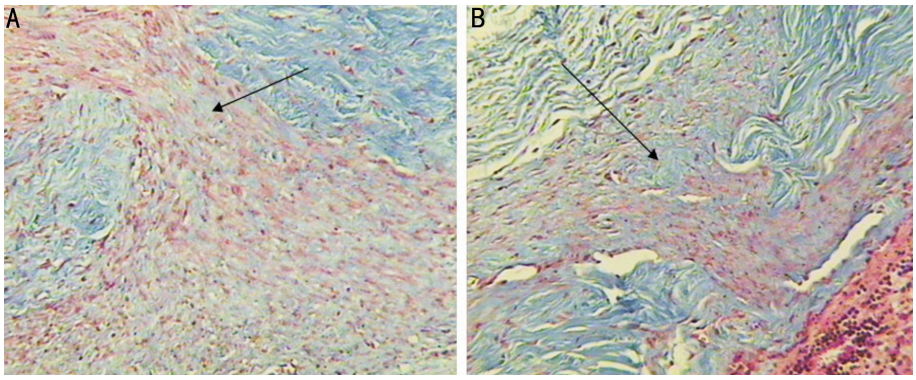

Figure 3 Collagen density in the filtration site after trabeculectomy in rabbit (SABC×400) A: control group on the 28th day; B: the 200mw/cm^2 He-Ne laser irradiation group on the 28th day

Table 2 Expression of PCNA, CTGF and collagen in rabbit after trabeculectomy (mean±SD, n=7)

He-Ne laser		PCNA (%)	CTGF (%)	Collagen (A)
Control	7th day	26.7±1.8	24.6±4.0	-
	14th day	20.4±1.9[c]	12.7±3.1[c]	10.5±2.4
	28th day	3.0±0.6[c]	4.7±1.7[c]	21.3±4.7[c]
He-Ne laser	7th day	18.8±1.5[a]	17.1±5.0[a]	-
	14th day	12.0±1.1[a,c]	4.8±1.5[c]	7.8±0.5[a]
	28th day	2.8±1.4[c]	1.5±0.8[a,c]	15.7±1.4[a,c]

[a]P<0.05 *vs* control; [c]P<0.05 *vs* the last time site

DISCUSSION

Trabeculectomy is one of the most effective anti-glaucoma surgeries, and is widely used around the world. Trabeculectomy controls the intraocular pressure (IOP) to create new outflow canal for the aqueous humor from the anterior chamber to the subconjunctival space, marked as functional filtration bulb. The trauma of surgery could elicit the normal healing response from the body-including inflammatory reaction, fibroblast cell activation, migration and proliferation and collagen synthesis-which will lead to scarring of the filtration canal and failure of the surgery. Currently, the average survival time of a successful filtration bulb is only about five years [1-6]. Antimetabolites such as mitomycin C (MMC) and 5-fluoro-2,4 (1H,3H) pyrimidinedione (5-Fu) could improve the outcome of trabeculectomy through suppression of the activation, migration and proliferation of fibroblast cells. Unfortunately, MMC and 5-Fu could also result in serious complications, especially if used unreasonably [19]. New safe and effective methods are needed to prevent filtration bulb scarring to improve the outcome of trabeculectomy.

Laser technology is widely used in diagnosis and treatment of disease. The mechanisms by which lasers produce these biological effects is not clear, though the heating and photobiological effects of lasers are well known. He-Ne lasers are a weak group of lasers, with a wavelength of 632.8nm. Numerous studies have shown that the influence of He-Ne lasers on biological tissue depends on the power and energy density, as discussed above: low power, low energy density He-Ne lasers promote proliferation and collagen synthesis of fibroblast cells, thus promoting healing of the wound, while high energy lasers may suppress proliferation and collagen synthesis, and even induce apoptosis [10-18]. It is still not clear how He-Ne lasers influence the proliferation and collagen synthesis of fibroblast cells. It is. found that fibroblast cell transform into myoblost and then undergo apoptosis following irradiation with a He-Ne laser. A 180J/cm^2 He-Ne laser induces alteration of the cell cycle, the ratio of DNA synthesis prophase cells increased while that of the S stage decreased dramatically, and that He-Ne lasers may inhibit cell proliferation through induction of cell cycle arrest[20,21]. In other experiments, it is found that He-Ne lasers down- regulated the expression of type I precollagen gene, while Hawkins[17] and Houreld[18] demonstrated damage to the DNA of fibroblast cells.

Proliferating cell nuclear antigen (PCNA) is a DNA polymerase-associated cytokine. The level of PCNA in cells changes with the cell cycle-maintaining low levels in the Go phase, rising in the early G1 phase, peaking in the S phase, and decreasing in G2 and M phase-so PCNA may be looked on as an index of cell proliferation activity. PCNA levels rise after trabeculectomy in rabbits, typically one to four weeks postoperatively[22]. Connective tissue growth factor (CTGF) is an important scarring-associated cytokine that was discovered only recently. CTGF is a polypeptide rich of cysteine that belongs to immediate early gene family. CTGF could promote cell proliferation and collagen synthesis, mediate cell adhesion and chemotasis, and induce new vessel and granulation tissue growth. The level of CTGF is positively correlated with the scarring of tissue[23,24]. Collagen is the major component of extracellular matrix, and collagen density often can be taken as an index of the extent of skin wound scarring. In this study, we tested the influence of He-Ne lasers of various power densities on rabbit fibroblast cells *in vivo*. Our data shows that He-Ne lasers with a power density range of 100 to 200mW/cm^2 applied for ten minutes

a day for three consecutive days (60 to 120J/cm^2) have no influence on the expression of PCNA and CTGF, and do not induce cell apoptosis, findings which differ from experiments conducted *in vitro*. However, 200 mW/cm^2 of He-Ne laser irradiation for ten minutes a day for three consecutive days (120J/cm^2) dramatically reduced scarring of the filtration site after trabeculectomy, corresponding with a reduction of fibroblast cell proliferation and collagen synthesis, possibly through down-regulation of PCNA and CTGF. The contrasting results from the non-surgery and trabeculectomy experiments indicate that the effect of He-Ne lasers on the fibroblaft is related not only to the power and energy density, but also to the status of the cells. He-Ne lasers make it difficult for the cells to be activated by surgery trauma.

We demonstrated *in vivo* that irradiation with a He-Ne laser with a power density of 200mW/cm^2, ten minutes a day for three consecutive days (120J/cm^2) as pretreatment before trabeculectomy, could prevent filtration bulb scarring in rabbits, possibly through suppression of fibroblast proliferation and collagen synthesis. However, further research is needed to determine the precise relationship between the effect and the He-Ne laser power density and energy density.

REFERENCES

1 Labbe A, Khammari C, Baudouin C. Wound healing modulation in glaucoma surgery. *J Fr Ophtalmol* 2007;30(6):631-646

2 Desjardins DC, Parrish RK II, Folberg R, Nevarez J, Heuer DK, Gressel MG. Wound healing after filtering surgery in owl monkeys. *Arch Ophthalmol* 1986;104: 1835-1839

3 Hitchings RA, Grierson I. Clinicopathological correlation in eyes with failed fistulizing surgery *Trans Ophthalmol Soc UK* 1983;103:84-88

4 Migdal C, Gregory W, Hitchings R. Long-term functional outcome after early surgery compared with laser and medicine in open-angle glaucoma *Ophthalmology* 1994;101:1651-1656

5 Cordeiro M, Bhattacharya S, Schultz G, Khaw PT. TGF-beta1, -beta2 and -beta3 *in vitro* biphasic effects on Tenon's fibroblast contraction, proliferation and migration. *Invest Ophthalmol Vis Sci* 2000;41:756-763

6 Lama PJ, Fechtner RD. Antifibrotics and wound healing in glaucoma surgery. *Surv Ophthalmol* 2003;48:314-346

7 Fuller JR, Bevin TH, Molteno ABC, Vote BJT, Herbison P. Anti-inflammatory fibrosis suppression in threatened trabeculectomy bleb failure produces good long term control of intraocular pressure without the risk of sight threatening complications. *Br J Ophthalmol* 2002;86:1352-1354

8 Mark Brian Sherwood, FRCS, FRCP, FRCOphth. A sequential multiple-treatment, targeted approach to reduce wound healing and failure of glaucoma filtration surgery in a rabbit model (an American ophthalmology society thesis). *Trans Am Ophthalmol Soc* 2006;104:478-492

9 Trabeculectomy Study Group, Khaw P, Grehn F, Holló G, Overton B, Wilson R, Vogel R, Smith Z. A phase III study of subconjunctival human anti-transforming growth factor beta (2) monoclonal antibody (CAT-152) to prevent scarring after first-time trabeculectomy. *Ophthalmology* 2007;114(10):1822-1830

10 Hawkins D, Abrahamse H. Biological effects of helium neon laser irradiation on normal and wounded human skin fibroblasts. *Photo med Laser Surg* 2005;23: 251-259

11 Gross AJ, Jelkmann W. Helium-neon laser irradiation inhibits the growth of kidney epithelial cells in culture. *Lasers Surg Med* 1990;10(1):40-44

12 AlWatban FA, Andres BL. The effect of He Ne laser (632.8nm) and solcoseryl *in vitro*. *Lasers Med Sci* 2001;16:267-275

13 Shu B, Wu ZY, Hao LL, Zeng DF, Feng GR, Lin YH. Experimental study on He-Ne laser irradiation to inhibit scar fibroblast growth in culture. *Chin J Traumatol* 2002;5(4):246-249

14 Shu B, Yu HP; Yu ZQ, Fang XQ, Ma XL, Wu ZY. Apoptosis of cultured fibroblasts derived from scar induced by He-Ne laser. *Chin J Clin Rehabil* 2005;9 (2):140-141

15 Yang HZ, Yang XC. Inhibitory effects of He Ne laser repeated irradiation on collagen synthesis in hypertrophic scar-derived fibroblasts in culture. *Chin J Clin Rehabil* 2002;6(2):296-297

16 Yasukawa A, Hrui H, Koyama Y, Nagai M, Takakuda K. The effect of low reactive-level laser therapy (LLLT) with helium-neon laser on operative wound healing in a rat model. *J Vet Med Sci* 2007;69(8):799-806

17 Hawkins DH, Abrahamse H. The role of laser fluence in cell viability, proliferation, and membrane integrity of wounded human skin fibroblasts following helium-neon laser irradiation. *Lasers Surg Med* 2006;38(1):74-83

18 Houreld N, Abrahamse H. *In vitro* exposure of wounded diabetic fibroblast cells to a helium-neon laser at 5 and 16 J/cm^2. *Photomed Laser Surg* 2007;25(2):78-84

19 Higginbotham EJ, Stevens RK, Musch DC, Karp KO, Lichter PR, Bergstrom TJ, Skuta GL. Bleb-related endophthalmitis after trabeculectomy with mitomycin C. *Ophthalmology* 1996;103:650-656

20 Pourreau-Schneider N, Ahmed A, Soudry M, Jacquemier J, Kopp F, Franquin JC, Martin PM. Helium-Neon laser treatment transforms fibroblasts into myofibroblasts. *Am J Pathol* 1990;137:171-178

21 Shu B, Hao LL, Jiang XH. Effects of He-Ne laser with various power densities on the growth of cultured fibroblasts in hypertrophic scar. *Modern Rehabilitation* 2001;5(9):150-151

22 Wei P, Wang AQ, Lei XW, Cao ZX, Li XL, Wang Q. Effects of Butyrylchitosan on expression of proliferating cell nuclear antigen in fibroblast proliferation of rabbit eyes after filtering operation. *Acta Academiae Medicinae Sinicae* 2006;28 (6):813-816

23 Daniels JT, Schultz GS, Blalock TD, Garrett Q, Grotendorst GR, Dean NM, Khaw PT. Medication of transforming growth factor-beta (1)-stimulated matrix contraction by fibroblasts: a role for connective tissue growth factor in contractile scarring. *Am J Pathol* 2003;163:2043-2052

24 Yamanaka O, Saika S, Ikeda K, Miyazaki K, Kitano A, Ohnishi Y. Connective tissue growth factor modulates extracellular matrix production in human subconjunctival fibroblasts and their proliferation and migration *in vitro*. *Jpn J Ophthalmol* 2008;52:8-15