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

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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 vivo.

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, 200 mW/cm²; 60, 90, 120 J/cm²) 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 200 mW/cm² 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 200 mW/cm² (120 J/cm²) 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

INTRODUCTION

S cc 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 activates 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]. 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 [8-10]. 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. [11] reported that He-Ne laser irradiation of 10 J/cm² energy density could
damage normal and injured skin fibroblasts in vitro. Gross et al.\(^{(1)}\) reported that kidney epithelial proliferation was suppressed by He-Ne laser irradiation of 40mW/cm\(^2\) power density (energy density 11.9-142J/cm\(^3\)) 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\(^3\)) 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 \(^{(8)}\). 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.

Standard trabeculectomy was performed on the He-Ne laser irradiation site, a zone with the fornix-based conjunctival flap, a 3mm x 3mm two-thirds full-thickness scleral flap, a 1.5mm x 1mm trabeculum flap, a 1.5mm x 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 7\(^{th}\), 14\(^{th}\) and 28\(^{th}\) 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 \(\chi^2\) 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 7\(^{th}\) day postoperatively, decreasing dramatically on the 14\(^{th}\) and 28\(^{th}\) days (Figure 1). The ratio of

<table>
<thead>
<tr>
<th>He-Ne laser</th>
<th>PCNA</th>
<th>CTGF</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.8±0.6</td>
<td>3.9±0.80</td>
</tr>
<tr>
<td>100mW/cm(^2)</td>
<td>2.0±0.9</td>
<td>4.0±1.9</td>
</tr>
<tr>
<td>150mW/cm(^2)</td>
<td>1.6±0.6</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>200mW/cm(^2)</td>
<td>1.7±0.3</td>
<td>4.1±1.8</td>
</tr>
</tbody>
</table>

\(F\) value: 0.321, 0.166; \(P\) value: 0.810, 0.917
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 7th, 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/cm² 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)

<table>
<thead>
<tr>
<th>He-Ne laser</th>
<th>PCNA (%)</th>
<th>CTGF (%)</th>
<th>Collagen (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 7th day</td>
<td>26.7±1.8</td>
<td>24.6±4.0</td>
<td>-</td>
</tr>
<tr>
<td>14th day</td>
<td>20.4±1.9</td>
<td>12.7±3.1</td>
<td>10.5±2.4</td>
</tr>
<tr>
<td>28th day</td>
<td>3.0±0.6</td>
<td>4.7±1.7</td>
<td>21.3±4.7</td>
</tr>
<tr>
<td>He-Ne laser 7th day</td>
<td>18.8±1.5</td>
<td>17.1±5.0</td>
<td>-</td>
</tr>
<tr>
<td>14th day</td>
<td>12.0±1.1</td>
<td>4.8±1.5</td>
<td>7.8±0.5</td>
</tr>
<tr>
<td>28th day</td>
<td>2.8±1.4</td>
<td>1.5±0.8</td>
<td>15.7±1.4</td>
</tr>
</tbody>
</table>

*p<0.05 vs control; †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 myoblast and then undergo apoptosis following irradiation with a He-Ne laser. A 180J/cm² He-Ne laser induces alteration of the cell cycle, the ratio of DNA synthesis prophase cell increased while that of G2 phase decreased dramatically, and that He-Ne lasers may inhibit cell proliferation through induction of cell cycle arrest [19,20]. In other experiments, it is found that He-Ne lasers down- regulated the expression of type I procollagen 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 level 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 vitro. Our data shows that He-Ne lasers with a power density range of 100 to 200mW/cm² applied for ten minutes
a day for three consecutive days (60 to 120J/cm²) 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, 200mW/cm² of He-Ne laser irradiation for ten minutes a day for three consecutive days (120J/cm²) 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 fibroblast 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/cm², ten minutes a day for three consecutive days (120J/cm²) 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