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# Effect of mitomycin combined with Nd−YAG laser on cell proliferation and invasion as well as MEK/ERK signaling pathway in obstructive lacrimal duct model

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ABSTRACT

**Objective:** To study the effect of mitomycin (MMC) combined with Nd-YAG laser on cell proliferation and invasion as well as MEK/ERK signaling pathway in obstructive lacrimal duct model. **Methods:** New Zealand rabbits were selected as experimental animals and divided into model group, laser group and MMC + laser group; obstructive lacrimal duct model was established, then laser group were given Nd-YAG laser intervention, and MMC + laser group were given Nd-YAG laser combined with mitomycin intervention. 2 months after intervention, the expression of proliferation molecules, invasion molecules and MEK-ERK signaling molecules in lacrimal duct tissue were measured. **Results:** TGF-β, CTGF, PCNA, Ki-67, Col-I, Col-III, MEK, ERK1/2, MMP2 and MMP9 protein levels in lacrimal duct tissue of laser group were significantly higher than those of model group while TSG-6, Cthrc1 and TIMP1 protein levels were significantly lower than those of model group; TGF-β, CTGF, PCNA, Ki-67, Col-I, Col-III, MEK, ERK1/2, MMP2 and MMP9 protein levels in lacrimal duct tissue of MMC + laser group were significantly lower than those of laser group while TSG-6, Cthrc1 and TIMP1 protein levels were significantly higher than those of laser group. **Conclusion:** Mitomycin can inhibit cell proliferation and invasion as well as MEK/ERK signaling pathway activation in obstructive lacrimal duct model after Nd-YAG laser treatment.

## 1. Introduction

Lacrimal duct obstruction disease is a common ophthalmological disease, and the common therapies include lacrimal duct probing, insertion of thread and polyethlene tube, Nd-YAG laser and so on[1]. The Nd-YAG laser has the advantages of short time, easy operation and less damage, and has been extensively applied in recent years[2]. However, there are different levels of inflammation and edema in the wound surface after laser treatment, which can stimulate fibroblast proliferation and increase the risk of further lacrimal passage stenosis and obstruction again. The enhancement of cell proliferation and invasion behaviors as well as the activation of MEK-ERK signaling pathway plays an important role in the process of fibroblast proliferation. Mitomycin C (MCC) is a kind of antimetabolite that has the effect of inhibiting cell proliferation and invasion[3]. It is not clear about its treatment value for the obstructive lacrimal duct re-stenosis after laser treatment. In the following studies, we analyzed the effect of mitomycin combined with Nd-YAG laser on cell proliferation and invasion as well as MEK/ERK signaling pathway in obstructive lacrimal duct model.

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## 2. Materials and methods

### 2.1 Experimental materials

Experimental animals were a total of 30 male New Zealand rabbits, animal license number was SCXK (Sichuan) 2013-17, animal experiments passed the hospital ethical review, and the animal experiments and processing after death were done according to the rules. Mitomycin C was purchased from Sigma Company, Nd-YAG laser lacrimal duct apparatus was from Huada Electronics Co., Ltd., protein lysis buffer RIPA was bought from Shanghai Beyotime Company, and enzyme-linked immunosorbent assay kit was purchased from Shanghai Westang Biotechnology Company.

### 2.2 Experimental methods

#### 2.2.1 Model establishing

30 New Zealand rabbits were randomly divided into model group, laser group and MMC + laser group, and the obstructive lacrimal duct model was established according to the following method: eye disinfection was done after intraperitoneal injection of 0.2 mL/kg Su-mian-xin injection for anesthesia, lacrimal probe was used to probe into the lacrimal duct, then lacrimal duct expansion pipe was used for expansion, balloon catheter containing guidewire with diameter of 2 mm was imbedded, the length was tagged, the balloon was inflated to make the pressure reach 6-8 atmospheric pressure, then the guidewire was pulled back and forth to make the balloon rub lacrimal duct, 3 times was repeated, each time interval was 1 minute, and then the modeling was completed.

#### 2.2.2 Intervention

Model group received no special intervention; laser group received Nd-YAG laser intervention, energy 180 MJ, frequency 10 times/second, for 20 min, twice every day; MMC+ laser group: the same method as that of laser group was referred for Nd-YAG laser intervention, the sponge probe with 0.2 g/L mitomycin C was probed into the lacrimal duct and taken out after 3 min, once daily. All three groups were intervened continuously for 2 months.

#### 2.2.3 Molecule expression detecting

After 2 months of intervention, the rabbits were executed, eyelid was cut, lacrimal duct was peeled, proper amount of lacrimal duct tissue was collected and added in RIPA lysis buffer to separate the protein, and enzyme-linked immunosorbent assay kit was used to determine TGF-β, CTGF, PCNA, Ki-67, TSG-6, Cthrc1, TIMP1, Col-I, Col-III, MEK, ERK1/2, MMP2 and MMP9 contents.

### 2.3 Statistical methods

SPSS 19.0 software was used to input and analyze data, measurement data comparison among three groups was by variance analysis, pair-wise comparison was by LSD-t test and $P<0.05$ indicated statistical significance in differences.

## 3. Results

### 3.1 Proliferation molecule expression

Analysis of proliferation molecules TGF-β (pg/mL), CTGF (pg/mL), PCNA (ng/mL) and Ki-67 (ng/mL) expression in lacrimal duct tissue among three groups of animals was as follows: TGF-β, CTGF, PCNA and Ki-67 protein levels in lacrimal duct tissue of laser group were significantly higher than those of model group, and TGF-β, CTGF, PCNA and Ki-67 protein levels in lacrimal duct tissue of MMC + laser group were significantly lower than those of laser group.

### 3.2 Invasion molecule expression

Analysis of invasion molecules TSG-6 (ng/mL), Cthrc1 (pg/mL), TIMP1 (pg/mL), Col-I (ng/mL) and Col-III (ng/mL) expression in lacrimal duct tissue among three groups of animals was as follows: TSG-6, Cthrc1 and TIMP1 protein levels in lacrimal duct tissue of laser group were significantly lower than those of model group while Col-I and Col-III protein levels were significantly higher than those of model group; TSG-6, Cthrc1 and TIMP1 protein levels in lacrimal duct tissue of MMC + laser group were significantly higher than those of laser group while Col-I and Col-III protein levels were significantly lower than those of laser group.

### 3.3 MEK/ERK signaling molecule expression

Analysis of MEK/ERK signaling molecules MEK (ng/mL), ERK1/2 (ng/mL), MMP2 (pg/mL) and MMP9 (pg/mL) expression in lacrimal duct tissue among three groups of animals was as follows: MEK, ERK1/2, MMP2 and MMP9 protein levels in lacrimal duct tissue of laser group were significantly higher than those of model group; MEK, ERK1/2, MMP2 and MMP9 protein levels in lacrimal duct tissue of MMC + laser group were significantly lower than those of laser group.

Table 1.

Comparison of proliferation molecule expression in lacrimal duct tissue among three groups of animals.

| Groups | $n$ | TGF-β | CTGF | PCNA | Ki-67 |
|---|---|---|---|---|---|
| Model group | 10 | 85.41±10.25 | 63.86±8.51 | 2.18±0.35 | 1.31±0.17 |
| Laser group | 10 | 231.36±33.29* | 213.65±27.86* | 6.57±0.78* | 5.38±0.73* |
| MMC + laser group | 10 | 152.38±18.69# | 121.35±14.86# | 3.73±0.55# | 2.77±0.36# |

*: compared with model group, $P<0.05$; #: compared with laser group, $P<0.05$.

Table 2.

Comparison of invasion molecule expression in lacrimal duct tissue among three groups of animals.

| Groups | $n$ | TSG-6 | Cthrc1 | TIMP1 | Col-I | Col-III |
|---|---|---|---|---|---|---|
| Model group | 10 | 1.76±0.22 | 264.61±33.26 | 187.64±20.35 | 0.93±0.13 | 0.77±0.09 |
| Laser group | 10 | 0.73±0.10* | 103.41±13.67* | 75.41±8.96* | 2.65±0.35* | 2.31±0.35* |
| MMC + laser group | 10 | 1.34±0.16# | 174.65±22.35# | 132.16±16.86# | 1.58±0.20# | 1.44±0.17# |

*: compared with model group, $P<0.05$; #: compared with laser group, $P<0.05$.

Table 3.

Comparison of MEK/ERK signaling molecule expression in lacrimal duct tissue among three groups of animals.

| Groups | *n* | MEK | ERK1/2 | MMP2 | MMP9 |
|---|---|---|---|---|---|
| Model group | 10 | 1.85±0.22 | 1.03±0.15 | 103.57±14.68 | 142.52±16.87 |
| Laser group | 10 | 5.42±0.67* | 3.27±0.46* | 325.22±42.64* | 462.51±56.53* |
| MMC + laser group | 10 | 2.83±0.35# | 1.75±0.22# | 172.41±20.35# | 244.21±32.58# |

*: compared with model group, $P<0.05$; #: compared with laser group, $P<0.05$.

## 4. Discussion

Lacrimal duct obstruction disease is the eye disease characterized by lacrimal duct fibrous connective tissue proliferation and extracellular matrix accumulation, persistent inflammation, ectopia and other stimuli are the common causes of disease, and local lacrimal duct mucosa is damaged under the action of the above causes and develops granulation tissue hyperplasia and lacrimal duct stenosis or obstruction. Nd-YAG laser is a common method to clinically treat lacrimal obstructive disease. It has the characteristics of short treatment time, simple operation and small injury to surrounding tissue[4,5]. However, there are different degrees of inflammation and edema in the wound surface after laser treatment, which will promote the proliferation of fibroblasts and re-stenosis in the local wound. Related studies have shown that the restenosis rate after laser treatment of lacrimal obstructive disease is about 10%-30%. Therefore, inhibiting the fibroblast proliferation and reducing the occurrence of lacrimal restenosis after laser treatment is a hot topic in the present study. Mitomycin C is an anti-metabolite that can be cross-linked with the DNA double helix structure, which can affect the replication and transcription of DNA to prevent cell cycle and inhibit cell proliferation[6]. Studies have reported the value of mitomycin C for the treatment of lacrimal duct obstruction disease[7], but there is no report about the effect of the drug on lacrimal duct restenosis as well as fibroblast proliferation and invasion after laser treatment.

Excessive fibroblast proliferation is an important pathological link in the process of lacrimal duct restenosis after laser therapy. TGF-β is an important regulatory molecule that promotes the proliferation of fibroblasts. The molecule can activate CTGF through the downstream Smad2/3 pathway, and then promote fibroblast proliferation by the biological effect of CTGF[8–10]. PCNA and Ki-67 are the antigen molecules that reflect cell proliferation activity, and they are increasingly expressed and play regulatory effect in cell mitosis and cell cycle progression[11]. In the study, analysis of above proliferation molecule expression in lacrimal duct tissue after laser treatment showed that TGF-β, CTGF, PCNA and Ki-67 protein levels in lacrimal duct tissue of laser group were significantly higher than those of model group. This shows that laser treatment can promote the expression of proliferation molecules in the lacrimal duct to some extent, then induce cell proliferation and increase the risk of restenosis. Further analysis of the effect of mitomycin on the proliferation molecule expression in lacrimal duct tissue showed that TGF-β, CTGF, PCNA and Ki-67 protein levels in lacrimal duct tissue of MMC + laser group were significantly lower than those of laser group. This means that mitomycin combined with laser treatment can reverse the up-regulatory effect of laser treatment alone on proliferation molecule expression in lacrimal duct tissue, and help to suppress the excessive proliferation of fibroblasts after laser treatment of obstructive lacrimal passage.

In the process of lacrimal restenosis, the proliferation of fibroblasts is also accompanied by the excessive accumulation of collagen and the invasive growth of cells. TSG-6 is a kind of hyaluronic acid-binding protein, which participates in the regulation of extracellular matrix remodeling process and inhibits the role of collagen synthesis[12,13]; Cthrc1 is an active molecule highly expressed in fibroblasts, which has the effect of inhibiting collagen deposition; TIMP1 is a specific inhibitory molecule of various MMPs, which has inhibiting effect on cell invasion[14]. In the study, analysis of above invasion molecule expression in lacrimal duct tissue after laser treatment showed that TSG-6, Cthrc1 and TIMP1 protein levels in lacrimal duct tissue of laser group were significantly lower than those of model group while Col-I and Col-III protein levels were significantly higher than those of model group. This indicates that laser therapy can promote the formation of collagen and the invasive growth of fibroblasts in lacrimal passage. Further analysis of the effect of mitomycin on the invasion molecule expression in lacrimal duct tissue showed that TSG-6, Cthrc1 and TIMP1 protein levels in lacrimal duct tissue of MMC + laser group were significantly higher than those of laser group while Col-I and Col-III protein levels were significantly lower than those of laser group. This means that mitomycin combined with laser therapy can reverse the regulatory effect of laser therapy alone on invasion molecule expression in lacrimal duct tissue, and help to suppress the deposition of collagen and the invasive growth of fibroblasts after laser treatment of obstructive lacrimal duct.

The activation of MEK/ERK signaling pathway plays a vital role in the process of fibroblast proliferation and invasion as well as abnormal collage deposition in local tissue. The activation of MEK can cause phosphorylation of ERK1/2, which can further activate the expression of MMP2 and MMP9 and promote cell invasion by hydrolysis of extracellular matrix[15]. In the study, analysis of above

signaling pathway molecule expression in lacrimal duct tissue after laser treatment showed that MEK, ERK1/2, MMP2 and MMP9 protein levels in lacrimal duct tissue of laser group were significantly higher than those of model group. This shows that laser treatment can cause the activation of MEK/ERK pathway in lacrimal passage to a certain extent, thus promoting the proliferation and invasion of fibroblasts. Further analysis of the effect of mitomycin on above signaling pathway molecule expression in lacrimal duct tissue showed that MEK, ERK1/2, MMP2 and MMP9 protein levels in lacrimal duct tissue of MMC + laser group were significantly lower than those of laser group. This means that mitomycin combined with laser treatment can reverse the activating effect of laser treatment alone on MEK/ERK pathway in lacrimal duct tissue, and help to suppress the fibroblast proliferation and invasion after laser treatment of obstructive lacrimal duct.

In the study, the animal experiments were done to clarify the value of mitomycin for obstructive lacrimal duct restenosis after laser treatment, and the preliminary conclusions are as follows: pure laser treatment of obstructive lacrimal duct can promote fibroblast proliferation and invasion as well as collagen deposition, and thus increase the risk of restenosis after treatment; mitomycin can inhibit the proliferation and invasion of fibroblasts as well as the activation of MEK/ERK signaling pathways after laser treatment of obstructive lacrimal duct.

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