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Microarray-based gene expression profiles in rabbit retina due to negative pressure suction

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ABSTRACT. We investigated a possible molecular pathogenesis involving retinal ganglion cell apoptosis following transient high intraocular pressure. Changes in the gene expression profiles of the retina were detected via gene chip methodology. Twelve New Zealand white rabbits were randomly assigned to control and 3-min negative pressure suction groups. The control group was treated only with a laser, and the experimental group was also treated with suction for 3 min, using a negative pressure generator. Total RNA was then extracted from the retinal tissue at different recovery stages to analyze gene expression profiles using the Agilent rabbit one-way gene chip. The groups were then compared. Immediately after negative pressure suction induction, 704 genes were differentially expressed. Among these, 485 genes were upregulated, and 219 were downregulated. Expression of the genes encoding CRYAA, CRYAB, and TLR3 genes, which are involved in apoptosis, was elevated. The KRT18 gene, which is involved in apoptosis, had reduced expression. Seven days after negative pressure suction, 482 genes were differentially expressed. Among these, 178 genes were upregulated, and 304 were downregulated. Expression of the genes encoding CRYAB, IL1-BETA and IL1R1, which are involved in apoptosis, was upregulated. Ten days after negative pressure suction, 402 genes were differentially expressed. Of these, 213 genes were upregulated, and 189

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were downregulated. Apoptosis genes CRYAB, CRYBA3, CRYBB2, IL1-BETA, and IL1R1 showed higher expression levels. We concluded that negative pressure suction for long periods of time (for example, 3 min) results in changes in gene expression. Genes with higher fold changes help protect retinal ganglion cells from apoptosis. We suggest that promoting the expression of these genes should be considered as a new means for treating ischemic-hypoxic retinopathy.

Key words: Transient high intraocular pressure gene chip; Laser *in situ* keratomileusis

INTRODUCTION

Negative suction during LASIK can lead to transient high intraocular pressure (IOP), which can reach 65 mmHg. Some studies (Wachtlin et al., 1998; Bradley et al., 2007) have demonstrated that IOP can reach 98-230 and 150-360 mmHg during lamellar corneal flap construction. It is known that the normal human IOP is 10-21 mmHg. A normal IOP is required to maintain the histological structure and physiological functions of the retina and optic nerve, and a substantial increase in IOP can result in a series of histopathological changes in the retina and optic nerve, as well as visual function impairment. We previously showed that the retina and optic nerve were not damaged upon negative pressure suction for 20 and 45 s (Liu, 2005; Zhao et al., 2005, 2008, 2009); however, the structure, function and protein expression of the retina and optic nerve were altered by negative pressure suction for 3 min, and retinal ganglion cell (RGC) apoptosis was observed. However, it was found that these changes could be reversed to normal levels with prolonged recovery periods. In the past, most studies on the mechanism of RGC apoptosis have focused only on the expression of certain proteins and cytokines, and there has been a lack of systematic research throughout the field, including research at the cellular and molecular level. The present study sought to analyze changes in retinal gene expression profiles at different times after 3 min of negative pressure suction, using gene chip technology. The gene chip was a 4*44 K single-channel gene chip made by Agilent.

MATERIAL AND METHODS

Experimental animals and grouping

Twelve healthy New Zealand white rabbits were obtained from the Animal Laboratory Center of Inner Mongolia Medical Colleges. The average weight of the rabbits was 3-4 kg; no eye disease was present, and both male and female animals were used. They were divided into the following groups: a control group and groups that were assessed immediately (0 days), 7 and 10 days after 3 min of negative pressure suction, with 3 rabbits in each group. Both eyes were used in the experiment.

Experimental models

To simulate the procedures used in LASIK surgery, after the rabbits were anesthetized, the eyes were submitted to negative pressure suction for 3 min. The corneal epithelium

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was then cut via phototherapeutic keratectomy, and the corneal stroma was scanned by an excimer laser according to -9.00 D standards. The following conditions were used: 193 nm laser wavelength, 125 mJ/cm^2 energy density, and 10 Hz pulse frequency. The control group was treated only with the excimer laser, and topical antibiotics were administered to the eyes of all animals in the surgery groups after LASIK.

Samples and specimens

The animals in the experiment groups were sacrificed immediately, 7 and 10 days after the operation via air embolism, and the eyeballs were then removed by conventional ophthalmectomy. The anterior segment and lens were then removed on a clean bench, the retina was peeled, and the samples were quickly placed in Eppendorf tubes pre-cooled by liquid nitrogen. The samples were then frozen and stored in a freezer after blast freezing in liquid nitrogen.

Gene chip detection

Total RNA of the retinal tissue was extracted using a one-step process using Trizol, concentrated with isopropanol precipitation, and then purified with the Total RNA Purification kit (QIAGEN RNeasy Mini Kit, USA). Finally, quantitative analysis by ultraviolet spectrophotometry (Beckman, USA) and quality control inspection on 1% agarose gel electrophoresis were performed. cDNA was synthesized by reverse transcription and used fluorescent dye labeling at the same time. DNA templates were dissociated by alkaline substances. Then, cRNA markers have been purified and hybridized.

Image collection and data analysis

Gene chips were scanned by a single-channel chip scanner made by Agilent (Santa Clara, CA, USA). Images were analyzed by the Agilent Feature Extraction image analysis software, and image signals were converted into digital signals. The data from the chip were then filtered, and weak signal points were removed. Normalization was then conducted using the quantile normalization algorithm of the Genespring software, and genes that showed a fold expression change greater than or equal to 2 were considered to be differentially expressed. Finally, the results were analyzed by means of systematic cluster analysis with Gene Cluster 3.0.

RESULTS

RNA extraction

The OD_{260}/OD_{280} values of total RNA from the retinal tissue were in the range of 1.95-2.01; 18S and 28S rRNA bands could be seen clearly by agarose gel electrophoresis, and the 28S band was 1- to 2-fold brighter than the 18S band (Figure 1).

Gene chip hybridization fluorescence labeling graphs

The control and experimental groups were both labeled with Cy3 (shown in green) in

the gene chip hybridization fluorescence labeling graphs (Figure 2). Each point in the graph represents one gene locus, indicated by the signal at a certain point, which will be green if a signal is present. A brighter color denotes a stronger signal, indicating greater expression.

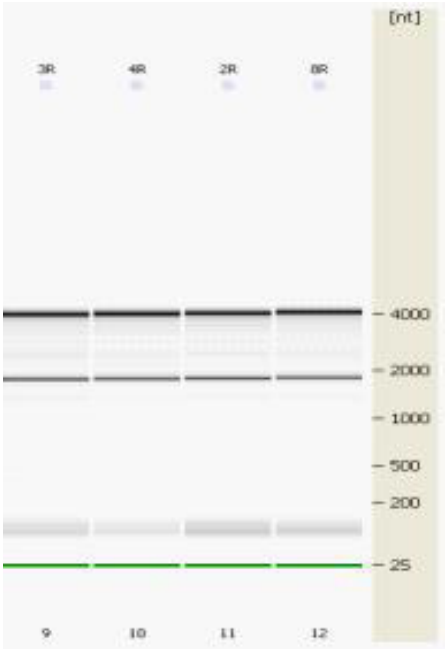

Figure 1. Agarose gel (1%) electrophoresis pattern.

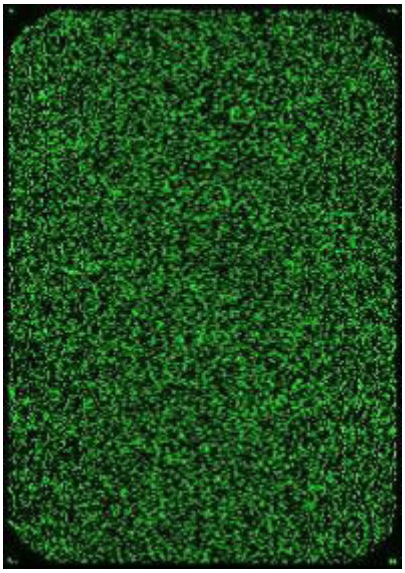

Figure 2. Fluorescently labeled gene chip graph.

Cluster analysis diagram

To show the differentially expressed genes directly and facilitate visualization of the data, we used hierarchical cluster analysis with the Cluster 3.0 software to analyze the genes and the TreeView software to display the data. Red in the graph represents upregulated genes, green represents downregulated genes, and black represents differentially expressed genes. Cluster analysis indicated that the genes CRYAA, CRYAB and CRYBB2 were upregulated and that IL1-BETA and IL1R1 were downregulated (Figures 3-6).

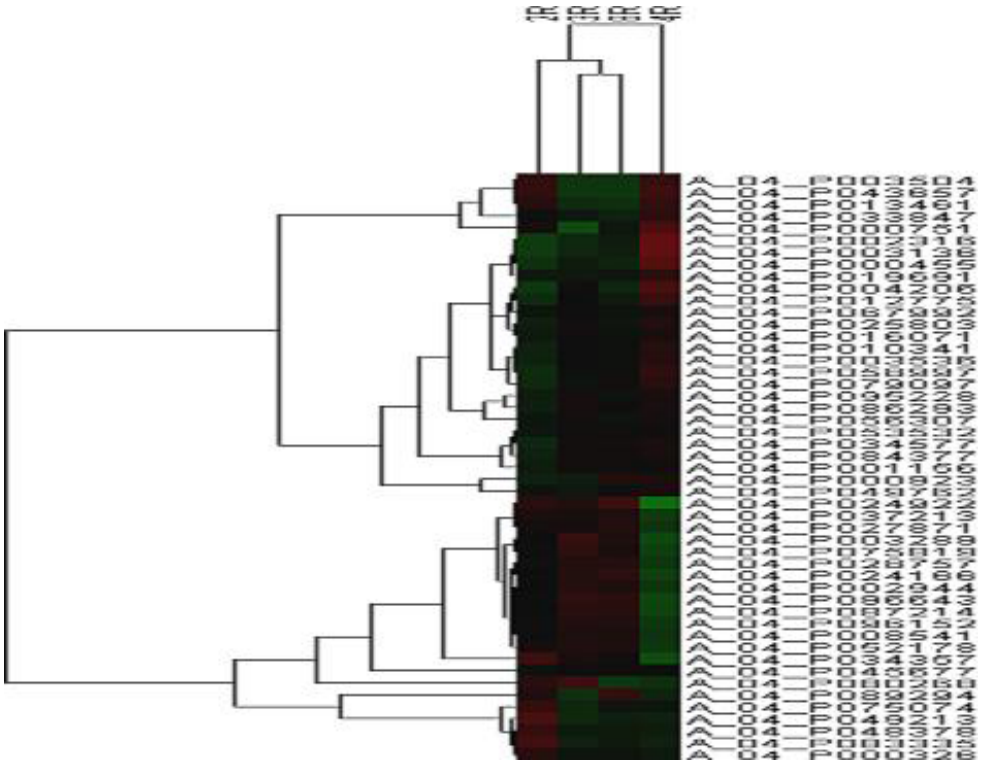

Figure 3. Cluster analysis diagram for the experimental and control groups.

Gene chip detection results

Differentially expressed genes immediately after negative pressure suction

Immediately after negative pressure suction, the number of differentially expressed genes was 704. Of these, 485 genes were upregulated, and 219 were downregulated. The number of apoptosis genes was 6; among them, 5 genes were upregulated, and 1 was down-regulated. The apoptosis genes showing the greater increase in expression were the CRYAA, CRYAB, TLR3 genes, and that showing lower expression was the KRT18 gene (Table 1).

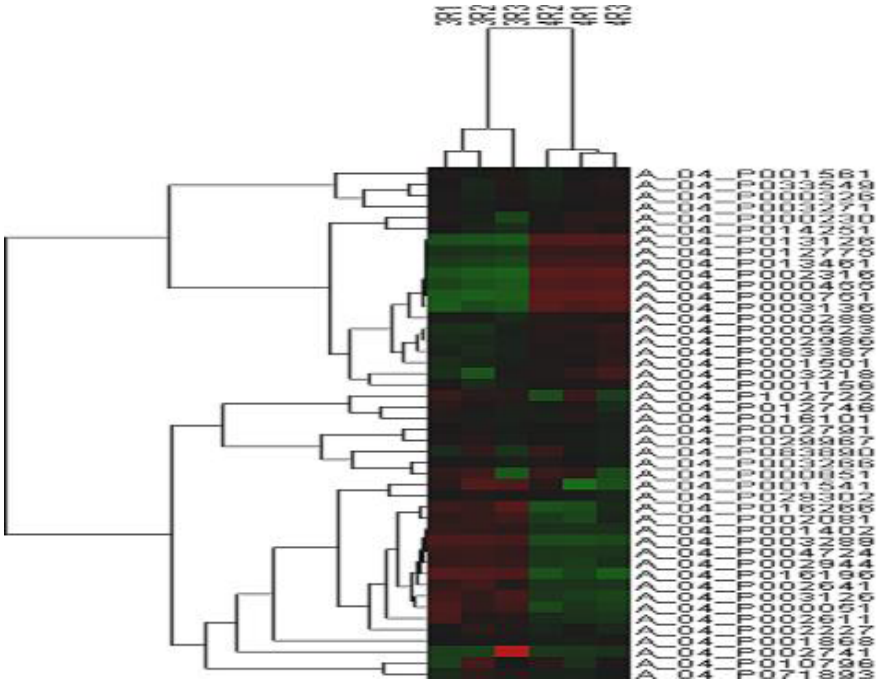

Figure 4. Cluster analysis diagram immediately after negative pressure suction for 3 min.

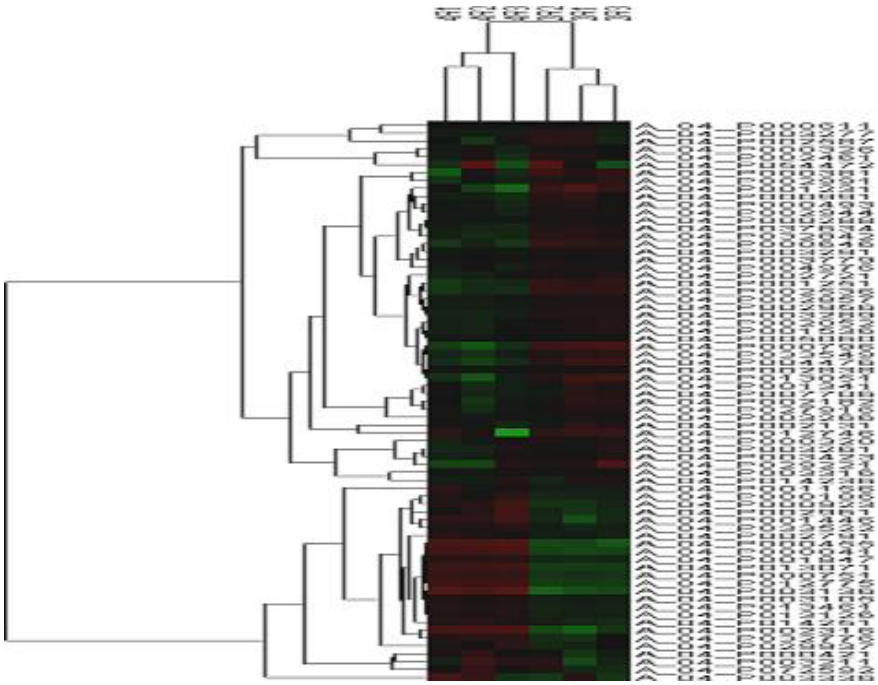

Figure 5. Cluster analysis diagram at 7 days after negative pressure suction for 3 min.

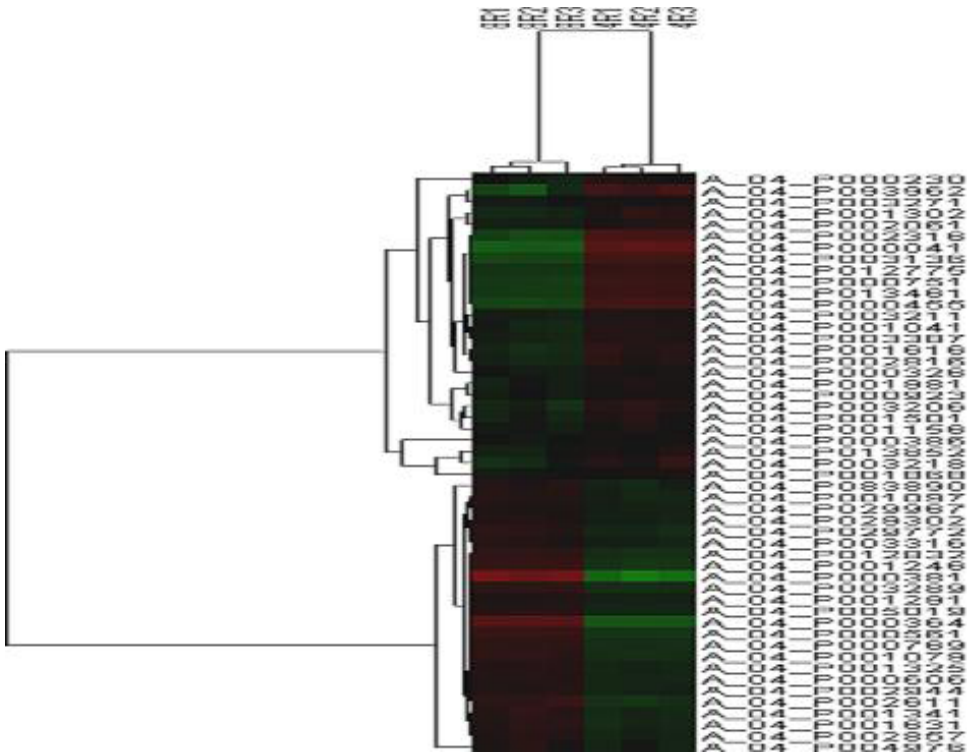

Figure 6. Cluster analysis diagram at 10 days after negative pressure suction for 3 min.

Table 1. Differentially expressed genes (fold change higher than 3) immediately after negative pressure suction.

Apoptosis genes					Other genes				
Gene symbol	GenBank accession	Fold change	Regulation	Function	Gene symbol	GenBank accession	Fold change	Regulation	Function
CRYAA	NM_001082406	14.5455	Up	Anti-apoptosis	GRO-A	NM_001082386	12.78805	Up	Chemotactic factor
TLR3	NM_001082219	3.990537	Up	Anti-apoptosis	SAA3P	CU465214	11.57556	Up	Biochemical factors
CRYAB	NM_001082407	3.81649	Up	Anti-apoptosis	NAT-2	NM_001082186	6.183721	Up	Anabolism-promoting factor
Caspase 14	NM_001099972	3.25066	Up	Mediates apoptosis	APOBEC1	NM_001082341	6.183721	Up	Nucleic acid synthesis
CD86	NM_001082208	3.13421	Up	Mediates apoptosis	MSR1	NM_001082248	6.137041	Up	Scavenger receptors
KRT18	AY099113	3.6306	Down	Anti-apoptosis	SLC	EU407478	4.315628	Up	Chemotactic factor
					VCAM-1	NM_001082152	3.391678	Up	Signal transduction factor, cell adhesion molecule
					IL18	NM_001122940	3.39155	Up	Cytokine
					EMP1	NM_001082357	3.221247	Up	Signal transduction factor
					PTGR1	NM_001082702	3.013181	Up	Oxidation reduction reaction
					GSTM2	NM_001082252	3.090303	Up	Anabolism factor
					MCP-2	M28884	39.88422	Down	Chemotactic and signal ransduction factor
					BPI	M28884	39.88422	Down	Immunological factor

Differentially expressed genes 7 days after negative pressure suction

Seven days after negative pressure suction, the number of differentially expressed genes was 482. Of these, 178 genes were upregulated, and 304 were downregulated. The number of apoptosis genes was 4; among them, 1 gene was upregulated, and 3 genes were downregulated, where the highest expression was seen in the CRYAB gene and the lowest in the IL1-BETA and IL1R1 genes (Table 2).

Table 2. Differentially expressed genes (fold change higher than 3) 7 days after negative pressure suction.

Apoptosis genes					Other genes				
Gene symbol	GenBank accession	Fold change	Regulation	Function	Gene symbol	GenBank accession	Fold change	Regulation	Function
CRYAB	NM_001082407	3.49816	Up	Anti-apoptosis	PTGR1	NM_001082386	7.048008	Up	Oxidation reduction reaction
IL1-BETA	NM_001082201	4.80228	Down	Pro-apoptosis	CDKL2	NM_001081999	5.240953	Up	Signal transduction factor
IL1R1	NM_001082770	4.158917	Down	Pro-apoptosis	GSTM2	NM_001082252	5.183841	Up	Anabolism factor
KRT18	AY099113	4.54469	Down	Anti-apoptosis	CASQ2	NM_001101691	4.403626	Up	Somatomedin
					DAF-2	AY339877	4.090386	Up	Glucose metabolism
					MRP-8	D17405	42.42799	Down	Scavenger receptors
					MCP-2	M28884	25.03051	Down	Chemotactic and signal transduction factor
					HBA	NM_001082389	9.064601	Down	Hemoglobin anabolism
					LGALS3	NM_001082338	3.922307	Down	Cell differentiation factor
					INMT	NM_001082043	3.094631	Down	Transaminase activity

Differentially expressed genes 10 days after negative pressure suction

Ten days after negative pressure suction, the number of differentially expressed genes was 402. Of these, 213 genes were upregulated and 189 were downregulated. The number of apoptosis genes was 6; among them, 4 genes were upregulated, and 2 genes were downregulated, where the highest expression was seen in the CRYAB, CRYBA3, CRYBB2 genes and the lowest in the IL1-BETA and IL1R1 genes (Table 3).

Table 3. Differentially expressed genes (fold change higher than 3) 10 days after negative pressure suction.

Apoptosis genes					Other genes				
Gene symbol	GenBank accession	Fold change	Regulation	Function	Gene symbol	GenBank accession	Fold change	Regulation	Function
CRYAB	NM_001082407	24.6942	Up	Anti-apoptosis	MIP	NM_001099961	7.466379	Up	Lens metabolism
CRYBA3	NM_001082130	14.13033	Up	Anti-apoptosis	THROMBIN	M81396	4.48396	Up	Coagulation factors
CRYBB2	NM_001089317	11.90117	Up	Anti-apoptosis	PTGR1	NM_001082702	3.546637	Up	Oxidation reduction reaction
CRYGS	NM_001099960	4.874096	Up	Anti-apoptosis	GSTM2	NM_001082252	3.370343	Up	Anabolism factor
IL1-BETA	NM_001082201	4.98262	Down	Pro-apoptosis	FMO4	NM_001082284	3.202386	Up	Oxidation reduction reaction
IL1R1	NM_001082770	3.9647	Down	Pro-apoptosis	APOD	NM_001082258	3.143393	Up	Fat metabolism
					MCP-2	M28884	39.95671	Down	Chemotactic and signal transduction factor
					PYGM	NM_001082184	4.243008	Down	Glucose metabolism
					KCNB	NM_001082087	4.23854	Down	Ion channel
					CX3CR1	EB375831	3.03786	Down	Signal

Transduction factor, cell adhesion molecules.

DISCUSSION

At present, much attention in corneal refractive surgery has been drawn to the fact that transiently high IOP is caused by negative pressure suction during LASIK surgery. Although transient high IOP has an influence on posterior polarity, the exact mechanism underlying this phenomenon is unclear. We conducted research at the protein level to explore this mechanism; however, it was difficult to fully understand the mechanism using traditional study methods. In contrast, gene chip technology has the following advantageous characteristics: 1) it is a high throughput technology, by which we can quantify gene expression, and 2) the level of protein in the cell in its working state is reflected by the quantity of mRNA measured. We used gene chip technology to analyze changes in retinal gene expression profiles after negative pressure suction for a long duration (3 min).

This study shows that immediately after negative pressure suction, the number of differentially expressed genes was 704. Among them, 485 genes were upregulated and 219 were downregulated. The number of apoptosis genes was 6. Five of these genes were upregulated and 1 gene was downregulated; the highest expressions among the apoptosis genes were in the CRYAA, CRYAB and TLR3 genes, and the lowest expression was in the KRT18 gene. Seven days after negative pressure suction, the number of differentially expressed genes was 482. Of these, 178 genes were upregulated, and 304 were downregulated. The number of apoptosis genes was 4; among these, 1 gene was upregulated and 3 genes downregulated. The highest expression among the apoptosis genes was seen in the CRYAB gene, and the lowest expressions were in the IL1-BETA and IL1R1 genes. Ten days after negative pressure suction, the number of differentially expressed genes was 402. Of these, 213 genes were upregulated and 189 were downregulated. The number of apoptosis genes was 6; among them, 4 were upregulated and 2 were downregulated. The highest expressions of apoptosis genes were in the CRYAB, CRYBA3 and CRYBB2 genes, and the lowest in the IL1-BETA and IL1R1 genes. The gene chip results were analyzed using systematic cluster analysis. The analysis showed that the CRYAB and CRYBB2 gene were overexpressed compared to controls and that their expression levels increased over time, whereas IL1-BETA and IL1RA genes were overexpressed but decreased in expression over time.

The CRYAB and CRYBB2 genes are members of the crystallin (cry) gene family. Cry was discovered by Mörner in 1893 and is regarded as one of the main structural proteins of the lens in vertebrates, keeping the lens transparent and refractive.

According to many studies (Whiston et al., 2008; Khan et al., 2010), the CRYAB gene plays a vital role in maintaining the normal function of the retina and protecting it from injury. Ashby et al. (2010) reported that CRYAB gene expression is upregulated in the retinas of highly myopic eyes because of cellular injury. In this study, the upregulation of CRYAB gene expression after negative pressure suction for 3 min could result from the transient high IOP caused by negative pressure suction. According to a report by Kumarapeli et al. (2008), the CRYAB gene has anti-apoptotic effects, protecting cells against ischemic injury. Therefore, the upregulation of CRYAB gene expression after negative pressure suction for 3 min can protect retinal ganglion cells from continuous hypoxic ischemic injury, demonstrating that the upregulation of CRYAB is benign. Ganguly et al. (2008) showed that the CRYBB2 gene promotes nerve differentiation. Liedtke et al. (2007) also reported that CRYBB2 gene expression is upregulated during retina regeneration. Therefore, the upregulation of CRYBB2 after

negative pressure suction for 3 min can promote retinal repair and reduce retinal injury from a transient high IOP.

Previous studies (Trubiani et al., 1995; An et al., 2004) found that IL-1β participates in the process of apoptosis, and that there is a positive correlation between its expression and apoptosis. Zhang and Hu (2005) and Relton and Rothwell (1992) reported that the apoptosis index increases with the increase in IL-1β expression, showing that IL-1β contributes to apoptosis. IL1R1 is one subtype of the IL-1 receptor; the domain of the peptide chain extending into the cytoplasm is longer than IL-1 and is involved in transmitting and activating signals. IL-1β binds to IL1R1, activating the apoptotic signaling pathway. In this study, we showed that the downregulation of IL1-BETA and IL1R1 played an anti-apoptotic role and protected RGCs from hypoxic ischemic injury.

In conclusion, the mechanism of RGC apoptosis by transient high IOP during LASIK surgery is complex, and thus, we studied gene expression profiles of retinas after negative pressure suction for 3 min using gene chip technology. The experimental results showed that the apoptosis genes with the highest expression levels were the CRYAB, CRYBB2, IL1-BETA, and IL1R1 genes. The protein products reduce retinal damage caused by negative pressure suction and protect RGCs from further hypoxic ischemic injury. Thus, these results also showed that although negative pressure suction induced RGC apoptosis, the process of apoptosis did not worsen over the period after negative pressure suction. In contrast, the apoptotic process could be reversed with the activation of a series of protective physiological and biochemical responses. This study has an important role in understanding the pathogenesis of retinopathy caused by high IOP and for determining prognosis and developing new treatments for this condition.

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