Original Paper

Protective Effects of *Radix Pseudostellariae* Extract Against Retinal Laser Injury

Guo Rui^{a,e} Wei Wei^{a,e} Wang Yuliang^{b,e} Li Kai^b Cui Xiaobing^c Zhu Changle^d
Shen Longshu^d

^aDepartment of Ophthalmology, Nanjing University of Chinese Medicine, ^bDepartment of Ophthalmology, the First Affiliated Hospital of Nanjing University of Chinese Medicine, ^cCollege of Pharmacy, Nanjing University of Chinese Medicine, ^dDepartment of Pathology, the First Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, China; ^eThese authors contributed equally to this work (co-first author)

Key Words

Radix Pseudostellariae • Frequency-doubled 532 nm Nd:YAG laser • Retina

Abstract

Background: This study aimed to analyze the protective effects of a saponin extract from *Radix Pseudostellariae* (RP) on retinal laser injury based on a retinal photocoagulation model.

Methods: Fifty-eight rabbits were randomly divided into three groups: Group A (saponin extract orally), Group B (physiological saline), and Group C (control). The animals were sacrificed 1 day, 7 days, 14 days, and 30 days after photocoagulation and lesions were evaluated with fundus photography, light microscopy, and electron microscopy. Superoxide dismutase (SOD) and malondialdehyde (MDA) levels were measured, and expression levels of c-fos and Bax genes were also determined. **Results:** The lesion sizes in Group A were smaller than in Group B. The levels of SOD in Group B were significantly lower than in groups A and C ($P < 0.001$) at all time points. The MDA levels were significantly lower than in groups B and C ($P < 0.001$) at the 1 month point, while the apoptosis rate of Group A was significantly lower than that of Group B at all time points. The expression levels of the c-fos gene in Group B were significantly higher than that in groups A and C, and expression levels of the Bax gene in Group A were significantly lower than that in groups B and C. **Conclusion:** The saponin extract of RP can inhibit oxidative stress, downregulate the levels of c-fos and Bax gene expression, and inhibit apoptosis in the retina after photocoagulation.

Copyright © 2014 S. Karger AG, Basel

Introduction

The broad use of lasers has resulted in an increasing number of patients who have visual problems due to occupational eye injuries [1-3]. Photocoagulation is also routinely used as a major treatment method for many retinal disorders. In clinical practice, injuries can also occur in the adjacent normal tissue. Therefore, many visual problems appear, such as retinal scarring, visual field defects and night vision loss. It has been reported that laser scars increase by 50% in size within approximately 2 years [4]. Laser-induced injuries mainly include necrosis in the area caused by laser irradiation and apoptosis in the adjacent area due to a thermal effect [4-6]. How to reduce side effects is an urgent topic that needs to be explored. Furthermore, it is necessary to study mechanisms and protection methods against laser-induced injuries.

Traditional Chinese medicine (TCM) has been successful in improving health, energy, and vitality all over the world for thousands of years. It has been suggested that TCM has many pharmacologic effects, such as an anti-apoptosis effect, anti-cancer effect, and anti-inflammatory effect [7-9].

In ophthalmology clinics, there are no significantly efficient treatment methods for many fundus diseases. Chinese herbal medicine therapy has been developed empirically over many years. *Radix Pseudostellariae* is one of the most widely used formulae in TCM. *Radix Pseudostellariae* is also known as *Pseudostellaria*. In the past decades, *Radix Pseudostellariae* has been reported to exert a wide range of functions and indications on the spleen and lungs. It is widely used as treatment for Qi deficiency. However, the underlying mechanisms of action on a molecular or system level are still unknown. Recent studies have shown that polysaccharides isolated from *RP* have an antioxidant effect in the neuroendocrine and immune systems [10-12].

In the current study, we created a retinal laser damage model in rabbits. Then, through stomach intervention, the effects of *RP* ginseng saponin extracts were compared using the model and controls. The experimental results showed that the *RP* extract prohibits an anti-apoptosis effect.

Materials and Methods

Experimental animals

Fifty-eight healthy adult Chinchilla rabbits (29 males and 29 females; 116 eyes) were selected that weighed 2.5-3.0 kg. The animals were provided by the animal experimental center of Nanjing Medical University. Before the experiment, an examination showed that the anterior segment and fundus of the eyes were normal. The animals were fed ad lib and maintained on a 12-hour light-dark cycle. All experimental protocols were approved by the Animal Care and Use Committee of Nanjing University of Traditional Chinese Medicine.

Animal models

Pupillary dilation was achieved with Mydrin-P eye drops (Otsuka Pharmaceutical Co. Ltd, Japan). Rabbits were anesthetized by an injection of 20% urethane (Shanghai Hengyuan Biological Technology Co. Ltd, China), and topical anesthesia with Benoxil (Otsuka Pharmaceutical Co. Ltd, Japan) was applied 5 minutes later. A standard retinal laser contact lens (Ocular Instruments, Bellevue, WA, USA) was used to focus the laser on the rabbit fundus. Laser irradiations were created using a diode laser (Lumenis, USA) coupled with a slit lamp microscope (Topcon SL-8Z, Japan) at a wavelength of 532 nm. The laser beams were focused on the macular area in a 1 disk-size diameter below the rabbit's retinal optic disk with a beam size of 200 μ m for 50 ms. Laser lesions were produced in 6 rows with 10 lesions in each row and a 1-lesion diameter space between lesions. All lesion reaction grades were Tso III [13, 14], and the area was marked at four vertices by Tso IV lesions (Fig. 1).

Fifty-eight experimental rabbits were randomly divided into three groups: 1) experiment Group A—23 rabbits received ginseng saponin extract gastric perfusion after laser irradiation; saline Group

Fig. 1. Photograph of post laser irradiation (Topcon SL-D7, Japan).

B—23 rabbits received the same volume of saline after laser irradiation; and the control Group C—12 rabbits did not receive laser irradiation. The rabbits were sacrificed 1 day, 7 days, 14 days or 30 days after laser irradiation. Eleven rabbits died during the course of the study; therefore, there were 4 rabbits in the treatment group and 3 rabbits in the control group at each time point but 5 rabbits in Group A and 6 rabbits in Group B at 1 month.

RP saponin extract

RP (native to Fujian herbal medicines planting base, China) was purchased from the pharmacy of Jiangsu Province Hospital of Traditional Chinese Medicine (Jiangsu, China). The authenticity of the plant was confirmed by Dr YF Zhu, a botanist at Jiangsu Province Hospital of Traditional Chinese Medicine (Jiangsu, China). Heterophylla 500 g was obtained, and 10 times the amount of 70% ethanol reflux was added and extracted 2 times, each time for 1 h. The alcohol extracts were combined and decompressed to obtain the evaporation residue. The residue was dissolved in 500 ml of water and transferred to a separatory funnel. Water-saturated butanol (500 ml of an n-butanol solution) extraction was performed 3 times. After each extraction, the butanol fraction was recovered by vacuum to obtain the total saponin in RP. The crude drug concentration was 1 g/ml.

Administration method

The intragastric volume was calculated according to the equivalent dose conversion ratio of the body surface area of humans and animals using the formula: $(10 \times 0.07/1.5) \times \text{rabbit body weight (kg)}$. The gavage was carried out immediately after laser irradiation and repeated daily in groups A and B. A semi-transparent silicone catheter, gag and rabbit clip were used to gavage.

Method for making specimens

Fundus photography was performed 1 day, 7 days, 14 days, and 30 days after laser irradiation to observe fundus changes. The rabbits were then sacrificed with a phenobarbital overdose, and their eyes were enucleated and fixed in 2% glutaraldehyde. Using a surgical microscope, dyed posterior segments of the eyes were dissected into tissue samples and pruned into rectangular specimens.

One rabbit was randomly selected from each group for other uses and three were chosen for light microscopy observation, immunohistochemistry tests and PCR detection. The specimens from random eyes of the rabbits were divided into two parts and then placed in liquid nitrogen for preservation; one part was for the measurement of SOD (superoxide dismutase) and MDA (malondialdehyde) levels, the other part was for real-time fluorescence quantitative PCR detection. The other specimens were marked for light microscopy and TdT-mediated dUTP nick end labeling (TUNEL) examination.

Light microscopy

The specimens were fixed in 10% formalin for 48 h. The specimens were obtained along the marked direction (RM2235 Leica, Germany) and then paraffin embedded (Tissue-Tek TEL SAKURA, Japan). Three continuous sections were obtained every 40 μm (2 slices per section, one for light microscopic observation and the other for TUNEL) for routine H&E staining and were observed under a light microscope using 4 $\times$ and 10 $\times$ magnification settings. The three widest lesion diameters of each slice were measured.

Table 1. The related sequences of primers

Primer name	Sequence (5'-3')	length (bp)
c-fos-forward	CTAGCGGCATGGAGCTGAAGGC	224
c-fos-reverse	TAATGTGCAGCTTGGGTACAGGTG	
BAX-forward	GCGCATTGGCGACGAACTGG	254
BAX-reverse	GCAGCCGCTCTCGGAGGAAG	
β -actin-forward	GAGTCGGGCCCTCCATCGT	75
β -actin-reverse	AGAGAGGGTGTCCGCCACTG	

Retinal lesion size measurement

Image-pro Plus 6 software (Version 6.0.0. 260, Media Cybernetics Inc.) was used for image analysis. The outer nuclear layer (ONL) changes in photocoagulation were taken as the reference layer.

Detection of apoptotic retinal cells

The specimens were fixed in paraffin sections, and the TUNEL method was used to detect apoptotic retinal cells. Briefly, for microwave repair, the samples were washed 3 times with PBS, and for digestion, proteinase K (50 μ L) was added to the samples for 15 minutes. The reaction containing the enzyme and nucleotides were incubated at a constant temperature (37 °C) for 1 h. After incubation, the samples were washed with PBS and subsequently incubated with an HRP-conjugated antibody at 37 °C for 30 min. The samples were stained with DAB, and microscopic observations were conducted. Near the nucleus, 5 randomly selected views were screened for lesions with a diameter of 1. The number of cells within the lesions were counted, and the total number of apoptotic cells photoreceptor cells, as indicated by staining, were obtained; the average photoreceptor apoptosis rate was calculated from these values.

Determination of MDA and SOD levels

The levels of SOD and MDA in all groups were measured 1 day, 7 days, 14 days, and 30 days after photocoagulation using a kit (Nanjing JianCheng Biological Engineering Institute, Nanjing). The protein content of the tissue was used as a reference, and the absorbance of a blank tube was used as a control.

Real-time PCR detection

Total RNA was isolated using the TRIzol reagent (Grand Island, NY) as described by the manufacturer. Briefly, the tissues of treated rabbits and controls were homogenized and resuspended in 1 ml of TRIzol. The suspension was then extracted with 0.2 ml of chloroform. After centrifugation, the aqueous phase was mixed with 0.5 ml of isopropyl alcohol. The resulting pellet was washed with 0.7 ml of 75% ethanol and then resuspended in 50 μ L of RNase-free water. The RNAs were used in subsequent steps when their OD₂₆₀/OD₂₈₀ values were between 1.8 and 2.0. All qualified 500 ng RNA samples were synthesized into cDNA using a reverse transcription reagent kit and were kept at -80°C until further use.

Expression levels of target genes were analyzed with an ABI 7500 fast real time PCR System and Power SYBR Green PCR Master Mix. The primers for the rabbit Bax, c-fos and β -actin genes were designed using Primer Express, as shown in Table 1. The optimized concentrations for real-time PCR were 0.2 μ M for both primers in a 10- μ L reaction volume. Rabbit β -actin expression was used as an internal control. Each sample was tested in triplicate. Cycle threshold (ct.) values were obtained graphically for the target genes and β -actin. The difference in ct. values between β -actin and the target genes are represented as Δ Ct values. $\Delta\Delta$ Ct values were obtained by subtracting Δ Ct values of the control samples from those of the treated samples. The relative fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$.

Statistical analysis

The data are expressed as the mean $\pm$ SD. A two-tailed, unpaired Student's t-test was used to analyze the significance of differences between lesion sizes at the same time points for two groups; ANOVA was used to analyze the significance of differences in lesion sizes within the same group and was also used to analyze the levels of SOD and MDA, apoptosis rates, expression levels of c-fos and bax within the same group and within the same time point. $P < 0.05$ was considered to represent a statistically significant difference.

Table 2. Lesion sizes in Groups A and B at different time points (unit: μm). Note: * $P < 0.05$ vs. 2 week; $^{\$}P < 0.05$ vs. 1 month within the same group. $^{\Delta}P < 0.05$ within the same time point

	1 day	1 week	2 week	1 month
Group A	159.54 $\pm$ 35.67 $^{\$}$	158.65 $\pm$ 24.39 $^{\$}$	131.24 $\pm$ 35.71	111.18 $\pm$ 31.60
Group B	181.13 $\pm$ 28.40 $^{*\$}$	165.67 $\pm$ 33.24	144.67 $\pm$ 41.37	138.79 $\pm$ 31.16
<i>t</i>	1.583	1.282	0.334	2.750
<i>P</i>	0.152	0.785	0.744	0.015 $^{\Delta}$

Fig. 2. Lesion size changes with time in different groups.

Results

Reduced lesion size with Radix Pseudostellariae treatment

Lesion sizes were significantly different at each time point between groups A ($F=6.106$, $P=0.002$) and B ($F=3.679$, $P=0.020$). Lesion sizes at 1 month were smaller than at 1 day and 1 week ($P=0.001$, $P=0.002$, respectively) in Group A. The lesions sizes at 1 day were larger than at 2 weeks or 1 month ($P=0.026$, $P=0.004$, respectively) in Group B (see Table 2). The lesion sizes at 1 day, 1 week and 2 weeks in Group A were smaller than in Group B, but the difference was not significant ($t=1.583$, $P=0.152$; $t=0.661$, $P=0.527$; $t=0.582$, $P=0.576$, respectively). However, the differences became significant at 1 month ($t=2.750$, $P=0.015$). As shown in Fig. 2, the lesion sizes in both groups A and B were largest at 1 day and then decreased with time. The lesion sizes in Group B stabilized at 2 weeks, while lesions sizes of Group A still had a downward trend at 1 month.

Enhanced SOD levels after RP treatment

As shown in Table 3 and Fig. 3, there were significant differences in the levels of SOD at different time points after photocoagulation in Group A ($F=34.386$, $P<0.001$). The SOD level reached the highest at 1 day and then decreased at 1 week, 2 weeks and 1 month ($P<0.001$). There were also significant differences in the levels of SOD at different time points in Group B ($F=39.203$, $P<0.001$). The SOD levels were highest at 1 day and then decreased at 1 week, 2 weeks and 1 month ($P<0.001$). The levels at 1 week were significantly lower than at 1 day ($P=0.001$) and higher than at 2 weeks and 1 month ($P=0.002$, $P=0.002$, respectively). There were no significant differences in Group C at different time points ($F=3.084$, $P=0.083$). The SOD levels in Group B were significantly lower than in groups A and C at 1 day, 1 week, 2 weeks, and 1 month ($P=0.001$, $P=0.002$, $P<0.001$, $P<0.001$, respectively). The SOD levels in Group A were significantly higher than in Group C ($P<0.001$) at 1 day and then decreased and were lower than Group C at 1 week ($P=0.015$). There were no significant differences between the two groups at 2 weeks and 1 month ($P=0.065$, $P=0.159$, respectively).

Table 3. SOD levels in different groups at different time points. Note: * $P<0.05$ vs. 1 week; # $P<0.05$ vs. 2 week; \$ $P<0.05$ vs. 1 month within the same group. ▲ $P<0.05$ vs. Group A; * $P<0.05$ vs. Group C within the same time point

	1 day	1 week	2 week	1 month
Group A	175.65±9.81*#\$\$	102.00±15.52*	102.64±8.15	108.35±8.42
Group B	113.58±10.50*#▲	70.36±9.49#*▲	31.77±7.70▲*	32.73±15.16▲*
Group C	146.63±8.13	133.30±7.52	123.30±15.83	121.39±13.04

Table 4. MDA levels in different groups at different time points. Note: * $P<0.05$ vs. 1 week; # $P<0.05$ vs. 2 week; \$ $P<0.05$ vs. 1 month within the same group. * $P<0.05$ vs. Group B; □ $P<0.05$ vs. Group C within the same time point

	1 day	1 week	2 week	1 month
Group A	7.12±0.48*#\$\$	4.42±0.44#*□	1.85±0.93*□	1.57±0.51*□
Group B	10.53±0.86#\$	10.73±0.79#\$	4.89±0.60	2.63±1.04
Group C	8.17±0.62	7.81±0.56	8.14±0.67	7.56±0.87

Fig. 3. SOD (Superoxide dismutase) levels in different groups after laser irradiation. **Fig. 4.** MDA (malondialdehyde) levels in different groups after laser irradiation.

Decreased MDA levels after RP treatment

MDA levels were also determined after RP treatment. As shown in Fig. 4 and Table 4, there were significant differences in the levels of MDA at different time points after photocoagulation in Group A ($F=58.186$, $P<0.001$). The MDA level reached the highest at 1 day and then decreased at 1 week, 2 weeks and 1 month ($P<0.001$). The level at 1 week was significantly lower than at 1 day ($P<0.001$) and was higher than at 2 weeks and 1 month ($P=0.001$, $P<0.001$, respectively). There were also statistically significant differences in the levels of MDA at different time points in Group B ($F=89.111$, $P<0.001$). The levels at 2 weeks and 1 month were lower than at 1 day and 1 week ($P<0.001$). There were no significant

Table 5. Comparison of cell apoptosis rates at different time points. Note: * $P < 0.05$ within the same time point. $\Delta P < 0.05$ vs. 1 day; $\$P < 0.05$ vs. 1 week; $\$P < 0.05$ vs. 2 week; $\square P < 0.05$ vs. 1 month within the same group

	1 day	1 week	2 week	1 month
Group A	0.140±0.023 $\Delta\$\square$	0.116±0.022	0.119±0.197	0.115±0.015
Group B	0.212±0.269 $\square$	0.239±0.039 $\$ \square$	0.180±0.045 $\Delta\$\square$	0.145±0.032 $\Delta\$\$$
Group C	0.034±0.011	0.034±0.011	0.033±0.011	0.032±0.009
F	133.802	149.082	64.169	78.263
P	0.000*	0.000*	0.000*	0.000*

Table 6. Expression levels of c-fos, multiple comparisons. Note: * $P < 0.05$ vs. 1 week; $\#P < 0.05$ vs. 1 d; $\$P < 0.05$ vs. 1 month within the same group. $\Delta P < 0.05$ vs. Group A; $\square P < 0.05$ vs. Group C within the same time point

	1 day	1 week	2 week	1 month
Group A	0.753±0.150*	1.111±0.165	0.946±0.188	1.044±0.109
Group B	1.906±0.143 $\Delta\$\square$	1.733±0.188 $\Delta\$\square$	2.338±0.111 $\Delta\#\$\square$	2.003±0.130 $\Delta\$\square$
Group C	1.000±0.196	1.143±0.069	1.119±0.027	1.096±0.183

Fig. 5. Cell apoptosis rates in different groups after laser irradiation.

Fig. 6. c-fos expression levels in different groups.

differences at the other time points ($P=0.167$). There were no significant differences in Group C at different time points ($F=0.587$, $P=0.639$). The MDA levels in Group A were significantly lower than in Group B at 1 day, 1 week, 2 weeks, and 1 month ($P=0.005$, $P<0.001$, $P<0.001$, $P<0.001$, respectively). The differences in MDA levels were not significant in groups A and C at 1 day ($P=0.105$), while there were significant differences at 1 week, 2 weeks, and 1 month ($P=0.001$, $P=0.002$, $P=0.002$, respectively).

Table 7. Expression levels of Bax at different time points. Note: * $P < 0.05$ vs. 1 week; # $P < 0.05$ vs. 2 week within the same group. * $P < 0.05$ vs. Group B; □ $P < 0.05$ vs. Group C within the same time point

	1 day	1 week	2 week	1 month
Group A	0.715±0.073*#*□	0.355±0.069*□	0.468±0.063*□	0.712±0.041*#*□
Group B	1.118±0.089	1.075±0.166	1.067±0.347	1.290±0.186
Group C	1.003±0.162	1.186±0.024	0.997±0.170	1.081±0.133

Fig. 7. Bax expression levels in different groups.

Reduced optic cell apoptosis rates after RP treatment

There were significant differences in Group A at each time point ($F=4.101$, $P=0.012$). The apoptosis rate at 1 day was significantly higher than at other time points ($P=0.011$, $P=0.024$, $P=0.003$, respectively). The difference was also significant in Group B at each time point ($F=15.304$, $P<0.001$). The apoptosis rate at 1 month was significantly lower than at 1 day, 1 week, and 2 weeks ($P<0.001$, $P<0.001$, $P=0.021$, respectively). The apoptosis rate in Group C showed no significant differences at each time point ($F=0.086$, $P=0.967$). The apoptosis rates in Group B were significantly higher than in Group A ($P<0.001$, $P<0.001$, $P=0.006$, $P=0.012$, respectively), and rates in Group A were significantly higher than in Group C ($P<0.001$, $P<0.001$, $P<0.001$, $P<0.001$, respectively; Table 5, Fig. 5).

Reduced c-fos and Bax mRNA levels after RP treatment

The results for groups A and C showed no significant differences at different time points ($F=3.004$, $P=0.095$; $F=0.611$, $P=0.627$, respectively). The results for Group B at different time points were significantly different ($F=9.136$, $P=0.006$), which at 2 weeks were higher than at 1 day, 1 week, and 1 month ($P=0.007$, $P=0.001$, $P=0.022$, respectively). There were significant differences in the three groups at 1 day, 1 week, 2 weeks and 1 month. The levels for Group B were higher than for groups A and C, while the results in groups A and C showed no significant differences (Fig. 6).

According to Table 6, there were no significant differences in groups B and C at different time points ($F=0.683$, $P=0.587$; $F=1.268$, $P=0.349$, respectively). The results for Group A at different time points were significantly different ($F=24.899$, $P<0.001$), which at 1 day were higher than at 1 week and 2 weeks ($P<0.001$, $P=0.001$, respectively). The results for 1 month were also higher than for 1 week and 2 weeks ($P<0.001$, $P=0.001$, respectively). There were significant differences among the three groups at 1 day, 1 week, 2 weeks, and 1 month. The levels of Group A were lower than those of groups B and C, while levels in groups B and C showed no significant differences (Fig. 7, Table 7).

Discussion

Large doses of hormones are used as a treatment method for retinal laser injuries [15, 16], but this treatment has only a short-term effect and has many side effects [17]. MK-801, which is a non-selective NMDA receptor antagonist, can also be used [18], but it has been shown to exert a certain toxicity in the human body. At present, there have been few studies on traditional Chinese medicine interventions for retinal laser injury. In previous studies, *RP* saponin extract was suggested to have antioxidant activity [11]. Our study showed that *RP* saponin extract could reduce the size of the retinal laser lesion, inhibit oxidative stress reactions, downregulate c-fos and Bax gene expression levels, and inhibit optic cell apoptosis, thereby exhibiting a protective effect on retinal laser injury.

The animal we chose to use was the chinchilla, mainly because its retina is rich in pigment and because the macular structure is similar to that of humans. As the cone density of the fovea was low, visual function was low [19]. Taking into account the combined magnifications of the contact lens and a rabbit's eye, the aerial images of 200 μm would be 132 μm on a retinal spot [20]. From morphologic comparison, light microscopy results showed that there were no significant differences in lesion sizes in the *RP* group and the saline group. Lesion sizes for both groups decreased gradually 1 month after photocoagulation, which was similar to the findings of Yannis [21-23]. However, Maeshima suggested that lesion size increased with time [24, 25]. We propose that there may be several reasons for this discrepancy: the observation period was insufficient; there was influence from the basic diseases; the lesion size measurement method was based on a different reference plane; we selected the ONL layer as baseline; and there were species differences. However, the lesion sizes of the *RP* group were lower than those in the saline group at all time points, and significant differences were demonstrated at 1 month. We suggest that the saponin extract of *RP* can reduce the size of lesions.

For the measurement of SOD and MDA levels and for the measurement of the expression levels of c-fos and Bax genes, we did not separate the retinal and choroidal layers because the separation manipulation might cause measurement error and because laser damage may affect both retinal and choroidal tissues [26, 27]. Previous studies on the mechanisms of laser induced-retinal damage showed that the damage was mainly due to thermal effects, which could produce oxidative stress, induce free radical generation, and cause the formation of lipid peroxide, thereby causing necrosis and apoptosis of cell photoreceptors with progressive enlargement of the laser lesion size, with reduced SOD levels and increased MDA levels [23, 24, 28, 29].

SOD is the most important substance in organisms to scavenge free radicals. There are more than 60 diseases caused by oxygen free radicals. SOD can convert O_2^- into H_2O_2 and exert influences against or block the damage to cells due to oxygen free radicals. MDA is the product of lipid peroxidation. In our study, SOD levels were significantly decreased in the saline group after 1 day, and stabilized at 2 weeks; while MDA levels reached a peak at 1 week and then decreased and stabilized by 2 weeks. We suggest that the retinal oxidative stress reaction after photocoagulation reached a steady state at 2 weeks. SOD levels were significantly decreased in the *RP* group after 1 day and then stabilized at higher levels at 1 week because short-term SOD compensation may be increased after laser photocoagulation. In addition, MDA levels decreased gradually after 1 day and stabilized at 2 weeks.

The SOD levels in the saline group were significantly lower than in the *RP* group and the control group at 1 day. The SOD levels in the *RP* group remained at the baseline level after 2 weeks. The MDA levels in the *RP* group were significantly lower than in the saline group at 1 day. We suggest *RP* saponin extract can inhibit the decrease in SOD concentrations and decrease MDA levels. *RP* extract has been postulated to exert antioxidant activity through the elimination of $-\text{OH}$, O^{2-} , H_2O_2 and increases in the activity of antioxidase. Therefore, *RP* extract may be an effective free radical scavenger and play an important role in the prevention and treatment of free radical related diseases.

Many previous studies have suggested that there is a direct relationship between oxygen free radicals and apoptosis [30-33]. It has been suggested that the upregulation of oxygen free radicals or the depletion of endogenous antioxidants could induce cell apoptosis. Endogenous or exogenous antioxidants can inhibit apoptosis. TUNEL is the most common and specific method for detecting apoptosis. Our study demonstrated that the apoptosis rate in the *RP* group at each time point was significantly lower than in the other two groups, suggesting that the saponin extract of *RP* had anti-apoptotic effects.

The *c-fos* gene has been suggested to induce neural cells apoptosis. In this study, the gene expression levels of *c-fos* reached a peak at 2 weeks in the saline group and showed significant differences at other time points. In comparison, *c-fos* gene expression levels were basically the same in the *RP* group and the control group at each time point. The expression levels of the *c-fos* gene in the saline group were higher than in the *RP* group and control group at all time points. We suggest that apoptosis in the retina after laser photocoagulation peaked at 2 weeks, and the saponin extract of *RP* can inhibit the expression level of the *c-fos* gene. The *Bax* gene has been shown to have a pro-apoptotic effect. In this study, our results showed that *Bax* gene expression levels at 1 week and 2 weeks in the *RP* group were lower than at 1 day and 1 month, presenting a 'U' shape change. In comparison, the expression levels of the *Bax* gene were almost the same in the saline and control groups at each time point. The protein levels of the *bax* gene in the *RP* group were lower than in the saline and control groups at all time points. We suggest the saponin extract of *RP* can inhibit the expression of the *bax* gene after laser photocoagulation, especially at 1-week and 2-week time points.

In conclusion, *RP* saponin extract can significantly reduce the size of lesions produced by a frequency-doubled 532-nm laser within 1 month after retinal photocoagulation. *RP* can inhibit the reduction of SOD levels after laser photocoagulation and render it to be maintained at the baseline level. Furthermore, it reduced MDA levels and achieved stability at 2 weeks. Through increasing *c-fos* levels and reducing *Bax* protein levels, the saponin extract of *RP* can inhibit cell apoptosis. Thus, *RP* saponin extract exhibits a protective effect on retinal laser injuries.

Acknowledgements

This work was supported by Chinese Medicine Administrative Bureau of Jiangsu Province(No:LZ11188).

References

- 1 Fowler BJ: Accidental industrial laser burn of macula. *Ann Ophthalmol* 1983;15:481-483.
- 2 Stuck BE, Zwick H, Molchany JW, Lund DJ: Accidental human laser retinal injuries from military laser systems. *SPIE* 1996;2674:7-20.
- 3 Wolbarsht ML: Permanent blindness from laser exposure in laboratory and industrial accidents. *SPIE* 1996;2674:21-24.
- 4 Brancato R, Pece A, Avanza A, Radrizzani E: Photocoagulation scar expansion after laser therapy for choroidal neovascularization in degenerative myopia. *Retina* 1990;10:239-243.
- 5 Dastgheib K, Bressler SB, Green WR: Clinicopathologic correlation of laser lesion expansion after treatment of choroidal neovascularization. *Retina* 1993;13:345-352.
- 6 Rutledge BK, Wallow IH, Poulsen GL: Sub-pigment epithelial membranes after photocoagulation for diabetic macular edema. *Arch Ophthalmol* 1993;111:608-613.
- 7 Liu C, Li J, Meng FY, Liang SX, Deng R, Li CK, Pong NH, Lau CP, Cheng SW, Ye JY, Chen JL, Yang ST, Yan H, Chen S, Chong BH, Yang M: Polysaccharides from the root of *Angelica sinensis* promotes hematopoiesis and thrombopoiesis through the PI3K/AKT pathway. *BMC Complement Altern Med* 2010;10:79.

- 8 Yin H, Guo R, Xu Y, Zheng Y, Hou Z, Dai X, Zhang Z, Zheng D, Xu H: Synergistic antitumor efficiency of docetaxel and curcumin against lung cancer. *Acta Biochim Biophys Sin* 2012;44:147–153.
- 9 Hoo RL, Wong JY, Qiao CF, Xu A, Xu HX, Lam KSL: The effective fraction isolated from *Radix astragali* alleviates glucose intolerance, insulin resistance and hypertriglyceridemia in db/db diabetic mice through its anti-inflammatory activity. *Nutr Metab* 2010;7:67.
- 10 Sheng R, Xu X, Tang Q, Bian D, Li Y, Qian C, He X, Gao X, Pan R, Wang C, Luo Y, Xia Y, Dai Y: Polysaccharide of *Radix Pseudostellariae* improves chronic fatigue syndrome induced by poly I: C in mice. *Evid Based Complement Alternat Med* 2011;2011:840516.
- 11 Xiong H, Pang J, Xie Z: Antioxidant activities of extracts from *Radix Pseudostellariae*. *Acta Scientiarum Naturalium Universitatis Nankaiensis* 2009;42:37–41. [article in Chinese]
- 12 Chen Z, Li S, Wang X, Zhang CL: Protective effects of *Radix Pseudostellariae* polysaccharides against exercise-induced oxidative stress in male rats. *Exp Ther Med* 2013;5:1089–1092.
- 13 Tso MOM: Retinal photocoagulation therapy: Clinical application and biological basis of therapeutic effects; in Tso MOM (ed): *Retinal Diseases*. Philadelphia, Lippincott, 1988, pp. 246.
- 14 Weingeist TA, Sneed SR: *Laser Surgery in Ophthalmology. Practical Applications*. Norwalk, CT, Appleton & Lang; 1992, 15–27.
- 15 Takahashi K, Fu J, Lam TT, Tso MO: The effect of high-dose methylprednisolone on laser-induced retinal injury primates: An electron microscope study. *Graefes Arch Clin Exp Ophthalmol* 1997;4:35–39.
- 16 Naveh N, Weissman C: Corticosteroid treatment of laser retinal damage affects prostaglandin E2 response. *Invest Ophthalmol Vis Sci* 1990;31:9–13.
- 17 Rosner M, Turetz J, Solberg Y, Belkin M: Neuroprotective therapy for argon laser induced retinal injury. *Exp Eye Res* 1997;65:485–495.
- 18 Solberg Y, Rosner M, Turetz J, Belkin M: MK-801 has neuroprotective and antiproliferative effects in retinal laser injury. *Invest Ophthalmol Vis Sci* 1997;38:1380–1389.
- 19 Teng Y, Song H, Zheng: Retinal function of healthy pigmented rabbits. *Acta Laboratorium Animalis Scientia Sinica* 2007;15:198–202. [article in Chinese]
- 20 Birngruber R: Choroidal circulation and heat convection at the fundus of the eye; in Wolbarsht ML (ed): *Laser Applications to Medicine and Biology*. New York, NY, Plenum Press, 1991, pp. 277–361.
- 21 Paulus YM, Jain A, Gariano RF, Stanzel BV, Marmor M, Blumenkranz MS, Palanker D: Healing of retinal photocoagulation lesions. *Invest Ophthalmol Vis Sci* 2008;49:5540–5545.
- 22 Hongxia Chen YG, Fanguang L: A study of Rabbit retinal effects from the frequency-doubled Nd:YAG Laser. *Chin J Laser Med Surg* 2005;14:69–73.
- 23 Lina H, Ying G, Fanguang L: Observation of biological effect on the retina and choroid of 532 nm consecutive Laser. *Acta Laser Biology Sinica* 2005;14:56–59. [article in Chinese]
- 24 Maeshima K, Utsugi-sutoh N, Otani T, Kishi S: Progressive enlargement of scattered photocoagulation scars in diabetic retinopathy. *Retina* 2004;24:507–511.
- 25 Mainster MA, Reichel E: Transpupillary thermotherapy of age-related macular degeneration: Long-pulse photocoagulation, apoptosis and heat shock proteins. *Ophthalmic Surg Lasers* 2000;31:359–373.
- 26 Marshall J, Mellerio J: Pathological development of retinal laser photocoagulation. *Exp Eye Res* 1964;6:303.
- 27 Marshall J, Hamilton AM, Bird AC: Histopathology of ruby and argon laser lesions in monkey and human retina. *Br J Ophthalmol* 1975;59:610–630.
- 28 Davor G, Lovro B, Kajo B, Karlica D, Lesin M, Znaor L: The role of oxidative stress after retinal Laser photocoagulation in Nonproliferative diabetic retinopathy. *Coll Antropol* 2011;35:835–840.
- 29 Jennings PE, MacEwen CJ, Fallon TJ, Scott N, Haining WM, Belch JJ: Oxidative effects of laser photocoagulation. *Free Radic Biol Med* 1991;11:327–330.
- 30 Kehrer JP: Free radicals and reactive oxygen species; in *Comprehensive Toxicology*, 2nd ed. New York, Pergamon and Publishing House, 2010, pp 277–307.
- 31 Cadenas E, Davies KJA: Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 2000;29:222–230.
- 32 Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB: Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal* 2014;20:1126–1167.
- 33 Ahn HJ, Il Kim K, Kim G, Moon E, Yang SS, Lee JS: Atmospheric-pressure plasma jet induces apoptosis involving mitochondria via generation of free radicals. *PLoS One* 2011;6:e28154.