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Effect of *Salvia miltiorrhiza* on retinopathy

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ABSTRACT

Objective: To explore the effect of *Salvia miltiorrhiza* on diabetic retinopathy (DR). **Methods**: Diabetic mice of natural incidence type with monogenic inheritance were selected. Alloxan was injected into the caudal vein of mice once to induce DR. The structural changes of retina tissue in normal mice, DR mice and mice with high, medium and low dose of *Salvia miltiorrhiza* injection were observed under microscope. Then the blood glucose concentration and malonaldehyde (MDA) content were detected. **Results**: There were some microaneurysms in retina of DR group, number of gangliocyte was decreased significantly, and cells were sparse and in disorder. After modeling, the blood glucose level of high-dose *Salvia miltiorrhiza* group (SM Ⅲ group) was significantly different from DR group (P<0.01). Till the tenth week, the blood glucose level of all SM groups was decreased significantly compared with DR group (P<0.01). The effective rates of three SM groups were 93.8%, 76.4% and 50.3%, respectively. After ten weeks, MDA content of DR group was significantly higher than those of the normal control group and SM group (P<0.01), and medium and low dose SM groups had significantly higher MDA than that of normal control group (P<0.01). **Conclusions**: *Salvia miltiorrhiza* had certain protective effect on DR mice through the blood-ocular barrier.

1. Introduction

Diabetic retinopathy (DR) is one of the most common and severe microvascular complications of diabetes mellitus (DM), and it indicates metabolic disturbance and damage of endocrine system and hematological system. The retina damage of optic nerve caused by DM can cause permanent vision disorder. Its incidence rate increases as the development of DM with 44.4% within 5a and 56% after 7a[1].

The pathological features of DR is abnormal tissue oxygenation in early stage, which firstly causes changes in microvascular function and than led to retinal hemangiectasis. Long-term hemangiectasis can cause the structural changes of microineorysm and vascellum, that is, pericyte denaturation, thickening basement membrane and endothelial cell hyperplasia. Because of the injury and disappearance of pericyte, the integrity of blood capillary is destroyed, and the blood-retina barrier is damaged, which causes a series of pathological changes (endothelial cell hyperplasia in blood capillary, thickening basement membrane). This then causes lumen coarctation and blood flow changes, and promotes ischemia retinae and new vessel generation in the anaphase of DR. The generation of new vessel is the symbol of proliferative diabetic retinopathy (PDR). PDR is characterized by retinal neovascularization and fibrosis, and its generation and development are related to many factors[2], such as vascular endothelial growth factor, Ang2, NP1, RSR, *etc*. The generation and development of neovascularization is a clinical problem because the appearance of new vessels can cause the loss of the vitreous integrity, tractional detachment of retina and damage in visual function. Neovascularization is the result of the interaction of retinal vessel and vitreous body, and this effect enhances the stimulation of neovascularization

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ability.

Salvia miltiorrhiza (SM) is perennial herb belonging to labiatae, salvia. The dried root and rhizome of SM taste bitter and are slightly cold in property, belonging to two meridians heart and liver meridian. They can promote blood circulation, remove heat to cool blood, tranquilize and sedate the mind, promote Guan pulse circulation and so on. It contains more than 30 kinds of chemical composition, which are divided into two parts: one part with quinoid red and yellow substance as most liposoluble constituent, mainly tanshinone; the other with tanshinol and protocatechualdehyde as most water-soluable components. SM is mainly used in the treatment of angiocarpy and blood diseases in clinic, while in recent years it is found that SM has unique curative effect on the treatment of ophthalmology diseases. Modern pharmacological studies show that SM has the functions such as protecting vascular endothelial, antiarrhythmia, anti-artherosclerosis, improving microcirculation, protecting the myocardium, suppressing and releasing platelet aggregation, increasing coronary flow, improving the body hypoxia tolerance, inhibiting the generation of collagenous fiber and promoting fibrin degradation, antiinflammation, resisting lipid peroxidation, scavenging free radical, protecting liver cells, anti-pulmonary fibrosis and others[3].

In recent years, many animal experiments have showed that SM is safe in retinal ischemia-reperfusion and is of little injury[4-6]. Retina is the peripheral part of central nervous system. To prove if SM can protect neuroretina from ischemic injury through blood-eye barrier, from April 2009 to April 2010 we observed the morphological changes of retinal tissues of diabetic mouse, and explored the effect of SM by randomized reference method.

2. Material and methods

2.1. Experimental animals and groups

Fifty diabetic mice of natural incidence type with monogenic inheritance, which were fed with high fat diet, and ten normal mice were selected. The mice were provided by the animal laboratory of Zhengzhou University Experimental Center and raised under standard general-level environment, weighting (50±2) g and without sex limitation. Alloxan (200 mg/kg) was injected into the vena caudalis of all mice to induce DM. The blood glucose concentration was detected, and excluded mice with glucose below 16.7 mmol/L. The rest mice were randomly divided into the DR group and SM group, and the normal control group. SM groups were then divided into high, medium and low dose groups. The low dose group (SMⅠ) was given dosage as 0.06 mL/kg, the medium group was 0.6 mL/kg and the high dose group was 1.4 mL/kg for ten weeks.

2.2. Reagents and instruments

Compound Danshen injection (Shanghai First Pharmaceutical Factory), alloxan (American Sigma Company), glucose oxidase kit (Shanghai ZhongSuo Reagent Co.Ltd), MDA kit (provided by Nanjing JuLi Biomedical Research Institute), continuous wavelength scanning microplate reader (TECAN Safire, America), Biofuge primo R desk-top low-temperature centrifuge (Shanghai Medical Analytical Instrument Factory).

2.3. Method

2.3.1 Production of light microscopy specimen

Under room temperature, mice had lumbar injection of 2% sodium pentobarbital (35 mg/kg) for anesthesia and 0.5% tropicamide for mydriasis. All mice were killed, and their eyes were removed immediately and washed away by brine ice. The eyeball was cut along the tie line of optic nerve and corneal vertex and put into paraformaldehyde fixation fluid for 24 h. They had gradient dehydration in 70%, 90% and 100% alcohol, embedded in routine paraffin and was cut into 4 μ slice continuously. All slices were dewaxed by dimethyl benzene and hydrated, then washed to remove hematoxylin for staining. They were soaked by 1% hydrochloric acid alcohol for twice and washed by water. After stained by eosin for three minutes they were taken out, under gradient dehydration by alcohol, then under transparence by dimethyl benzene. Neutral gum was used to mountand they were observed under light microscope (200×).

2.3.2 Determination of blood glucose and MDA

Blood glucose was determined before modeling and on the 1st d, 1st week and 10th week after modeling. A total of 0.1 mL blood was obtained by reduce tailing method and centrifuged for 3 min at 10 000 rpm/min. After the removal of precipitant red cells, the blood serum was obtained, and the absorbency was determined after the working fluid was added. The glucose concentration= the absorbency of measurement tube/the absorbency of calibration tube×5.6.

The measurement of MDA was as following: the mice were decapitated and their eyes were removed immediately. In the ice environment the corneosclera of mice was cut off in ring shape and the prosthomere and vitreous body were dropped. The wall of eye ball was evaginated and retina was peeled under dissecting microscope. Thio-barbituric acid method was used to determine MDA content of retina.

2.4. Statistical methods

SPASS16.0 statistical software was adopted to make the statistical analysis. The data information of experimental test index was expressed by mean±sd; completely random multi-level single-factor analysis of variance was used to compare the mean of blood sugar level and MDA level between control group, diabetes group and SM protection group; SNK-q test was adopted to make the comparison between groups. $P<0.05$ implied differences and having statistical significance.

3. Results

3.1. Changes of light microscope

The normal control group: The staining of outer nuclear layer was distributed evenly, retinal structure was clear and complete and tissues were arranged orderly (Figure 1). DR group: Hydrops could be seen in ganglion cell layer of retina; the granular layer became thinner; cell nucleus were loosened with vacuolation; microaneurysm of new vessels emerged in the external plexiform layer; gangliocyte was reduced significantly; cells were sparse with disorder and the boundaries disappeared; the staining of outer nuclear layer was distributed extremely unevenly (Figure 2). SM Ⅰ group: outer segment of optic nerve was tumid, sparse with vacuole; internal granular layer was tumid; the staining of outer nuclear layer was distributed extremely unevenly; microaneurysm of new vessels emerged in the external plexiform layer; gangliocyte was reduced significantly (Figure 3). SM Ⅱ group: the staining of outer nuclear layer was distributed unevenly; the surface of each layer was tumid and obvious pigment epithelium changes appeared; microaneurysm of new vessels emerged in the external plexiform layer; gangliocyte was reduced lightly (Figure 4). SM Ⅲ group: the staining of outer nuclear layer was distributed evenly; the structure of each layer in retina was clear and complete; microaneurysm of new vessels emerged in the external plexiform layer; was reduced lightly (Figure 5).

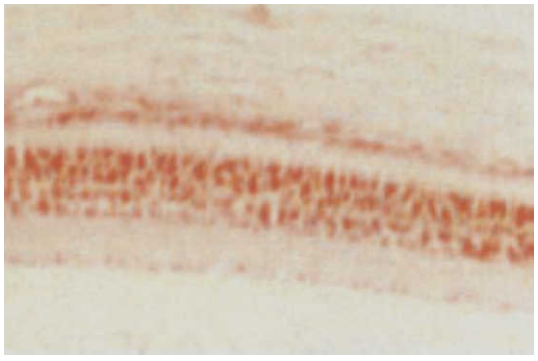

Figure 1. Retina of normal mice arranged in order and with clear structure (×200).

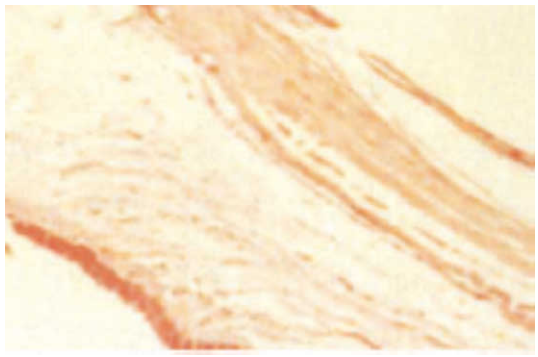

Figure 2. Retina of mice in DR group arranged in disorder with boundaries disappearing and uneven staining (×200).

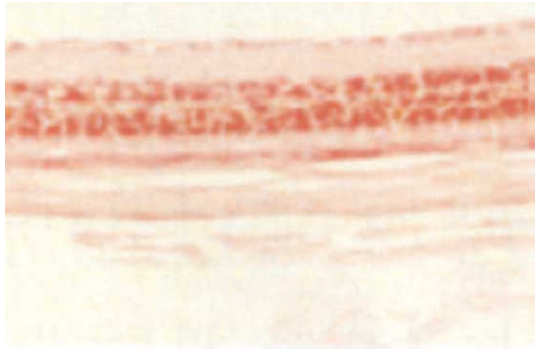

Figure 3. Retina of mice in SM Ⅰ group (×200).

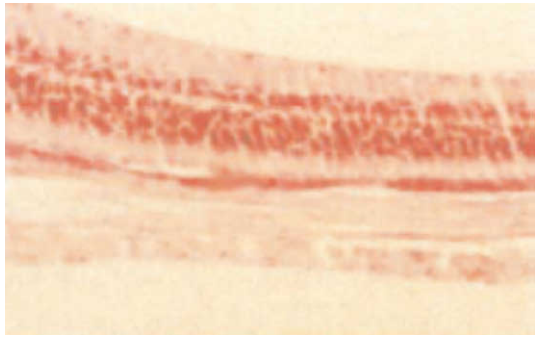

Figure 4. Retina of mice in SM Ⅱ group (×200).

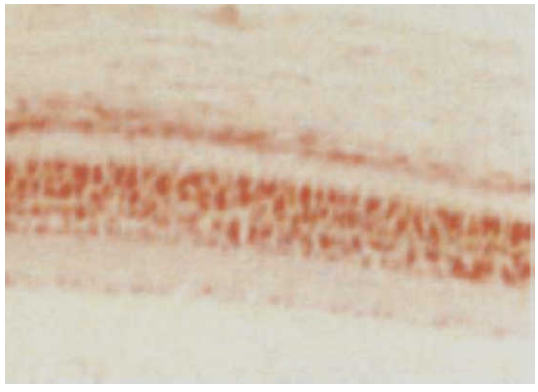

Figure 5. Retina of mice in SM Ⅲ group (×200).

3.2. Blood sugar changes

The blood sugar levels of mice in DR group and SM

Table 1
Changes of blood sugar levels in each experimental group ($\bar{x}$±sD) (mmol/L).

Groups	Blood sugar level		
	1 d	1st week	10th week
Normal control	4.79±0.10	4.78±0.14	4.90±0.18
DR group	20.19±1.66[a]	22.68±1.97[a]	27.48±1.54[a]
SM Ⅰ group	20.21±1.82[a]	18.91±1.76[ab]	13.88±1.34[ab]
SM Ⅱ group	20.15±0.93[a]	18.10±0.83[ab]	10.51±1.08[ab]
SM Ⅲ group	19.74±1.10[a]	17.47±0.97[ab]	7.51±0.99[ab]
F	230.15	214.54	489.10

Note: a indicated the comparison of DR group, SM group and normal control group, $P<0.01$; b indicated the comparison of SM group and DR group, $P<0.01$.

protection groups were significantly higher than that of normal group. After Compound Danshen injection for one week, the blood sugar levels of SM groups declined compared with DR group, but the differences among low-dose, medium-dose groups and DR group were not significant while there were significant differences between high-dose group and DR group ($P<0.01$). Till the tenth week, the blood sugar levels of low-dose, medium-dose and high-dose groups decreased greatly compared with DR group, and there was statistical significance ($P<0.01$). The efficiencies of high-dose, medium-dose and low-dose groups were 93.8%, 76.4% and 50.3%, respectively.

3.3. MDA contents

MDA content of DR group [(7.54±0.96) μmol/L] was significantly higher than those of the normal control group [(3.38±0.44) μmol/L] and SM protection groups ($P<0.01$); MDA of low-dose 5.70±0.84 and medium-dose groups [(4.37±0.72) μmol/L] were significantly higher than that of normal control group ($P<0.01$); but there was no significant difference of MDA between high-dose group [(3.93±0.70) μmol/L] and normal control group ($F=39.22$).

4. Discussion

DR is one of the serious complications of diabetes, and retinopathy will cause great decreas in patients' acuity, even severe blindness. DR is caused by microangiopathy in microcirculation of retina accompanied with microthrombus[7]. The long-term chronic hyperglycemia is the basis of DR invasion and numerous studies show that oxidative stress involves in DR pathogenic process and played an important role[8]. When the concentration of blood sugar is high, a series of saccharification oxide reactions cause more reactive oxygen species and the endogenous antioxidant protection mechanism weakens, the coactions increases the oxidative stress reactions[9]. Oxygen free radical can make epicyte occur lipid peroxidation and generate cross-linking reaction, improve the permeability of membrane. By attacking membrane protein and the intracellular enzyme system and nucleic acid, oxygen free radical can extend the cell generation circle and induce cell apoptosis. When DR occurr, free radicals in retina increase, lipid peroxides and malonaldehyde increase while superoxide dismutase decreases[10].

Under Chinese medicine theory, it is said that the basic pathological processes of blood stasis include three aspects, "stasis within the knot", "blood away from channels" and "blood filthy". The pathophysiological changes are reflected in the aspect "blood walk", expressed as "blood loss" and "blood barrier". Modern medicine believes that syndrome of blood stasis has a close relationship with microcirculatory disturbance in western medicine and therapy of activating blood to resolve stasis in traditional Chinese medicine is closely relative to microcirculatory improvement in western medicine, and they have similar regularity, which show that the major pathological basis of syndrome of blood stasis is circulatory disorder. SM, the representative drug which can promote blood circulation by removing blood stasis, plays a significant role in improving the microcirculation, anti-coagulation, thrombolysis, lipid lowing, anti-inflammation, anti-free radical oxidation and so on. In the treatment of ophthalmology disease, SM injection is effective from many aspects. SM injection has such functions as improving microcirculation, increasing ocular blood flow, reducing inflammatory exudation and promoting tissue repair, so it can inhibit growth stimulator of new vessels after corneal alkali burns to promote wound healing[10]. In the treatment of DR, SM injection can not only improve microcirculation, reduce blood viscosity, increase blood supply of retinal artery, and promote the absorption of congestion, but also effectively decrease LPO content in blood plasma of DR patient, improve the activity of SOD in red cells and has antioxidation action as good exogenous oxygen radical scavenger[11,12].

This study showed that the blood sugar and MDA levels

in DR group were significantly higher than that in normal control group and hyperglycemia was the basis of the pathogenesis of DR. Retina is highly aerobic tissue. There are a lot of dense mitochondria in the inner section of visual cells and complex chemical reactions happen there, which need a large amount of oxygen. The outer segment of retina, overlapped by many disk membranes, contains a higher level of unsaturated fatty acid, and can react with OH- to produce lipid radicals, cause a series of free radical chain reactions, which cause irreversible damages to the lipide in disk membrane, mitochondria and endoplasmic reticulum intramembrane. The final product, MDA could also cause severe injuries to membrane tissues. The major changes are decreased long-chain unsaturated fatty acid in retina and accumulated lipid peroxidation products. DR mice with salvia injection had lower blood sugar and MDA level than those mice without treatment, which showed that SM could scavenge free radicals and had certain protective effect on DR by regulating blood sugar level.

Light microscope observed that there was microaneurysms generation in DR mouse retina. Gangliocyte cells were reduced significantly, cells were sparse in disorder and the boundaries disappeared. After SM injection, the structure of each layer of mouse retina in high-dose group was clear and complete and got obvious improvement. DR pathological process was inhibited in the three groups, but the effectiveness of medium-dose and low-dose groups were not as good as high-dose group.

Currently, most researchers believed that blood-retina barrier was mainly located in retinal capillary endothelium and retinal pigment epithelium. Retinal capillary endothelium and its connection formed the inner barrier of retina and retinal pigment epithelium formed the outer barrier of retina. This study indicated that the thickening capillary basement membrane is the major injury of blood capillary ischemia in DR and the sign is the changes in structure. When active oxygen radicals could not be removed in time in DR after ischemia, the permeability of membrane is destroyed and lipid peroxidation of nerve cell membrane is stimulated to cause edema, forming the chain reaction. SM mechanism of action for DR of experimental mice is that when SM injection enters into the retinal hypoxia-ischemia tissues, SM can improve the recovery of blood oxygen transport and thus promote the absorption of retinal hemangioma. It relieves the damage of cell structure in morphology, and has strong protection effect on visual improvement. It contributes to a better control of blood sugar. This study indicats that SM injection can pass through the blood-ocular barrier and enter into retina to improve microcirculation.

According to our study, SM treatment is an effective treatment for DR.

Conflict of interest statement

We declare that we have no conflict of interest.

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