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Effects of Lycii Fructus and Salviae Miltiorrhizae on the Syndrome of Deficiency with Blood Stasis in RCS (rdy-/-, p-/-) Rats with Retinitis Pigmentosa: An Intervention Study

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ABSTRACT

Objective To investigate the effects of Lycii Fructus (LF, Gou Qi Zi, 枸杞子) and Salviae Miltiorrhizae Radix Ex Rhizoma (SM, Dan Shen, 丹参) on the syndrome of deficiency with blood stasis in the RCS (rdy-/-, p-/-) rats with retinitis pigmentosa (RP).

Methods A total of 32 RCS (rdy-/-, p-/-) rats were divided into 4 groups (equal amounts of female and male rats in each group): model group treated with 0.9% normal saline, LF group treated with LF formula granules, SM group treated with SM formula granules, and LF and SM (L·S) group treated with LF and SM formula granules. Eight RCS (rdy+/+, p+/+) rats (4 males and 4 females) were treated with 0.9% normal saline to serve as blank group. The contents of E2, PG, P-Selectin, plasma viscosity, whole blood relative index of the high shear rate and fibrinogen content in plasma, and the content of cAMP and cGMP in retinal homogenate were detected. The retina was evaluated by hematoxylin-eosin staining.

Results The contents of E2, PG, P-Selectin, plasma viscosity, whole blood relative index of the high shear rate, and fibrinogen content in the plasma of L·S group significantly differed from those of model group ($P < 0.01$), but were similar to those of blank group. The contents of cAMP and cGMP in the retinal homogenate of L·S group significantly differed from those in model group ($P < 0.01$) but were similar to those in blank group ($P > 0.05$).

Conclusions LF and SM can effectively treat retinitis pigmentosa by ameliorating the syndrome of deficiency with blood stasis.

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1 Introduction

Retinitis pigmentosa (RP) is a group of heterogeneous inherited retinal degeneration disease [1], with a globalprevalence of approximately 1/7000–1/3000 [2]. Today, an estimated 1.5 million RP patients have visual impairment, worldwide [3]. Therefore, the discovery of an effective intervention for RP is a very crucial requirement.

At present, stem cell therapy, gene therapy, cell transplantation, and retinal prosthesis from western medicine remain in the stages of basic research and animal experiment for RP treatment, warranting further confirmation via clinical research [4-7]. Traditional Chinese medicine (TCM) displays numerous advantages for improving visual acuity and visual field, and delaying the progression of RP. TCM has also been demonstrated to have a positive significance in the improvement of the quality of life of RP patients.

The etiology of RP in TCM can be summarized as a congenital deficiency [8]. Many clinical observations and mechanism studies in TCM have confirmed that the pathogenesis of RP patients involves the syndrome of deficiency with blood stasis [9]. Treating the deficiency and activating blood circulation thus serve as the main treatment methods/strategies. Clinical and basic studies have shown that cAMP and cGMP are important indicators of the deficiency syndrome in many diseases [10-12]. Deficiency syndrome is closely related to the content of cAMP and cGMP. Studies have reported that cAMP activity and PKA protein expression are reduced in the tissues associated with the deficient rat model [13]. Following the use of the corresponding Chinese material medical in the treatment of syndrome differentiation, the detection of cAMP, ATP content and PKA in the tissue, and both mRNA and protein expression were enhanced [14]. In this study, the representative Chinese herbal medicines that could treat the deficiency and activate blood circulation, Lycii Fructus (LF, Gou Qi Zi, 枸杞子) and Salviae Miltiorrhizae Radix Ex Rhizoma (SM, Dan Shen, 丹参), were used for treatments in the RP animal model. Based on the results of clinical observation and preliminary experimental studies, we can deduce that LF and SM have a definite effect on RP, and the use of TCM to treat RP deserves a wider clinical application.

To clarify the mechanism of LF and SM for the treatment of RP, the RCS (rdy-/-, p-/-) rat model of RP with the syndrome of deficiency with blood stasis was established in this study [15]. Some of the RCS (rdy-/-, p-/-) rat groups were treated with LF and SM. After 28 d of intragastric administration, the changes in cAMP, cGMP, cAMP/cGMP, PG, E2, P-Selectin and hemorheology were detected and applied to determinethe changes in the syndrome of deficiency with blood stasis of RCS (rdy-/-, p-/-) rats.

2 Methods

2.1 Animals and grouping

Thirty-two RCS (rdy-/-, p-/-) rats (16 females and 16 males ; weight, 140-160 g; age, 28-42 d) and 8 RCS (rdy+/+, p+/+) rats (4 females and 4 males ; weight, 200-240 g; age, 28-42 d (matched with the age of RCS (rdy-/-, p-/-) rats) were used in the present study. All rats were provided by the JOINN Laboratories (China) Co., Ltd., (SPF; quality certificate numbers: 201512444 and 201600559). Rats were housed in the SPF Room at the Animal Experiment Center of Hunan University of Chinese Medicine at aroom temperature of 22 ± 2 ℃, humidity of 50%, regular replacement of cushion, and free diet.

Thirty-two RCS (rdy-/-, p-/-) rats were randomly divided into the following 4 groups with 4 males and 4 females in each group (n = 8): model group, LF group, SM group, LF and SM (L·S) group. Eight RCS (rdy+/+, p+/+) rats (4 males and 4 females) were enrolled in blank group (n = 8).

All rats were fed adaptively for 1 week, and then given an intragastric administration for 28 d. This study was approved by the Institutional Review Board (IRB) of the Hunan University of Chinese Medicine.

2.2 Drugs

The drugs were purchased from the Pharmacy Department of the First Hospital of Hunan University of Chinese Medicine.

LF formula granule, 3.0 g per bag (equivalent to 10 g clinical dosage), was purchased from Guangdong Yifang Pharmaceutical Co., Ltd. (License: YUE20110214, Lot Number: 5103761). SM formula granule, 1.8 g per bag (equivalent to 10 g clinical dosage), was obtainedfrom Guangdong Yifang Pharmaceutical Co., Ltd. (Licence: YUE20110214, Lot Number: 5100631).

The dose of drugs was determined using the

following Human-animal surface area equivalent dose ratio conversion formula: W * adult dosage (g)/ animal weight (kg), conversion factor W = 0.018, adult dosages of LF and SM are 10 g each. The specific drugs in each group were:

Blank group: 0.9% normal saline was administered intragastrically (5.4 mL/kg·d).

Model group: 0.9% normal saline was administered intragastrically (5.4 mL/kg·d).

LF group: LF formula granules was administered intragastrically (1.08 g LF/kg·d). A solution equivalent to 10 g LF formula granule was prepared in 50 mL sterile purified water, with shaking at room temperature.

SM group: SM formula granules were administered intragastrically (1.35 g SM/kg·d). A solution equivalent to 10 g SM formula granules was prepared in 40 mL sterile purified water, with shaking at room temperature.

L·S group: L·S formula granule was administered intragastrically (1.08 g LF/kg·d and 1.35 g SM/kg·d). The solution preparation method involved the mixing of the solutions for LF group and SM group in a 1 : 1 volume configuration.

2.3 Index detection

2.3.1 Determination of E2, PG, and P-Selectin in plasma 1% phenobarbital sodium was injected intraperitoneally (3.5 mL/kg) into rats. After anesthesia, the abdominal aorta was separated and blood was collected. Within 30 min, the blood was centrifuged at 3000 rpm and the temperature of 4 degrees Celsius for 15 min. Thereafter, the supernatant was collected and stored in the refrigerator at – 80 ℃. The sampless were naturally thawed at normal atmospheric temperature. The contents of E2, PG, and P-Selectin in plasma were determined by ELISA. The operation and method were carried out according to the instructions of the kit.

2.3.2 Determination of cAMP and cGMP in retinal homogenate 1% phenobarbital sodium was injected intraperitoneally (3.5 mL/kg) into rats. After anesthesia, 75% alcohol was used to disinfect the eyelid, extirpate the eyeball, separate the retinal tissue, and prepare the 10% homogenate for storage in a refrigerator at – 80 ℃. The contents of cAMP and cGMP in the retinal homogenate were determined by ELISA. The operation and method were carried out according to the instructions of the kit.

2.3.3 Determination of hemorheology 1% phenobarbital sodium was injected intraperitoneally (3.5 mL/kg) into rats. After anesthesia, the abdominal aorta was separated and blood was collected. The volume of blood was approximately 3 mL, and thoroughly mixed blood and anticoagulant. At the end of blood collection, blood sampless were sent immediately for hemorheology examination, including plasma viscosity, blood viscosity, high and low shear relative index, and fibrinogen content.

2.3.4 Retinal histomorphometry 1% phenobarbital sodium was injected intraperitoneally (3.5 mL/kg) into rats. After anesthesia, 75% alcohol was used to disinfect their eyelid. Thereafter, the eyeball was extirpated and the retinal tissue was separated, immediately fixed in 4% paraformaldehyde, embedded in paraffin, sliced, and stained with HE. The morphological changes in the retinal layers of RCS (rdy-1-, p-/-) rats stained with HE were observed under an optical microscope.

2.4 Statistical analysis

Experimental data were analyzed using the SPSS 23.0 system software. All experimental data are expressed as mean ± standard deviation ($\bar{x} \pm s$). The multiple sets of comparisons satisfied the normality and the variance homogeneity, and the variance analysis was used. If the normality and homogeneity of variance were not satisfied, the rank sum test was employed. $P < 0.01$ was considered to indicate statistical significance.

3 Results

3.1 Determination of E2 and PG in plasma

The secretion of the sex hormone, E2, in the female RCS (rdy-/-, p-/-) LF group and female RCS (rdy-/-, p-/-) L·S group was increased after 28 d. Compared to model group, a significant difference was found in LF group and L·S group ($P < 0.01$) (Table 1 and Figure 1A).

The secretion of the sex hormone, PG, in the male RCS (rdy-/-, p-/-) L·S group was increased after 28 d, with a significant difference found between model group and L·S group ($P < 0.01$) (Table 1 and Figure 1B).

3.2 Determination of cAMP, cGMP and cAMP/ cGMP in retinal homogenate

Compared to model group, the content of cAMP and cGMP in the retinal homogenate of RCS (rdy-/-, p-/-) rats treated with LF and SM was decreased after 28 d ($P < 0.01$) to a level close to that of blank group of RCS (rdy +/ +, p +/ +) rats ($P > 0.05$) (Table 2, Figure 2A and 2B).

The value of cAMP/cGMP in the retinal homogenate of RCS (rdy-/-, p-/-) rats treated with LF and SM was decreased after 28 d compared to model group ($P < 0.01$); this value was close to that of blank group of RCS (rdy+/+, p+/+) rats (Table 2 and Figure 2C).

Table 1 Average plasma E2 content (pg/mL) of female rats and PG content (pg/mL) of male rats ($\bar{x} \pm s$)

Group	*n*	E2	PG
RCS (rdy+/+, p+/+) Blank	4	56.06 ± 7.05[a]	30.64 ± 9.59[d]
RCS (rdy-/-, p-/-) Model	4	25.81 ± 0.88	5.92 ± 0.89
RCS (rdy-/-, p-/-) LF	4	35.13 ± 3.54[b]	10.45 ± 2.98
RCS (rdy-/-, p-/-) SM	4	27.65 ± 1.55	7.88 ± 0.99
RCS (rdy-/-, p-/-) L·S	4	40.91 ± 3.77[c]	16.15 ± 1.04[e]

Compared to blank group, [a]$P < 0.01$, [b]$P < 0.01$, [c]$P < 0.01$; compared to model group, [d]$P < 0.01$,[e]$P < 0.01$.

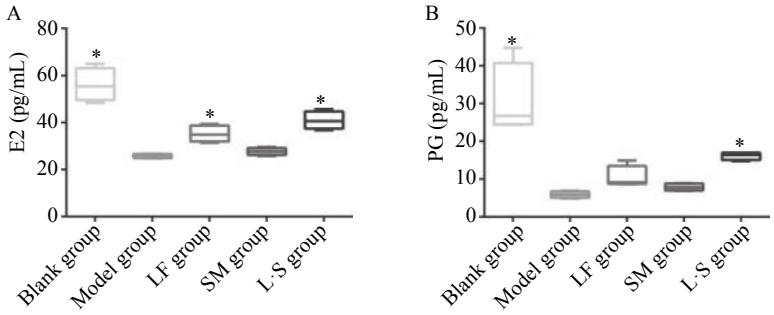

Figure 1 Average plasma E2 content (pg/mL) of female rats and PG content (pg/mL) of male rats

A, average plasma E2 content (pg/mL) of female rats. B, average plasme PG content (pg/mL) of male rats.

Table 2 The contents of cAMP (pg/mL), cGMP (pg/mL), cAMP/cGMP in the retinal homogenate ($\bar{x} \pm s$)

Group	*n*	cAMP	cGMP	cAMP/cGMP
RCS (rdy+/+, p+/+) Blank	8	22.78 ± 0.53[c]	6.11 ± 0.24[g]	3.73 ± 0.11
RCS (rdy-/-, p-/-) Model	8	37.07 ± 2.64	8.19 ± 0.36	4.52 ± 0.19
RCS (rdy-/-, p-/-) LF	8	36.04 ± 3.49	7.90 ± 0.49	4.56 ± 0.31
RCS (rdy-/-, p-/-) SM	8	33.04 ± 2.38[b]	7.25 ± 0.79[f]	4.58 ± 0.25
RCS (rdy-/-, p-/-) L·S	8	23.62 ± 1.23[ad]	5.90 ± 0.50[eh]	4.03 ± 0.43[i]

For cAMP, compared to model group, [a]$P < 0.01$, [b]$P < 0.01$,[c]$P < 0.01$, compared to blank group, [d]$P > 0.05$. For cGMP, compared to model group, [e]$P < 0.01$,[f]$P < 0.01$, [g]$P < 0.01$, compared to blank group, [h]$P > 0.05$. For cAMP/cGMP, compared to model group, [i]$P < 0.01$.

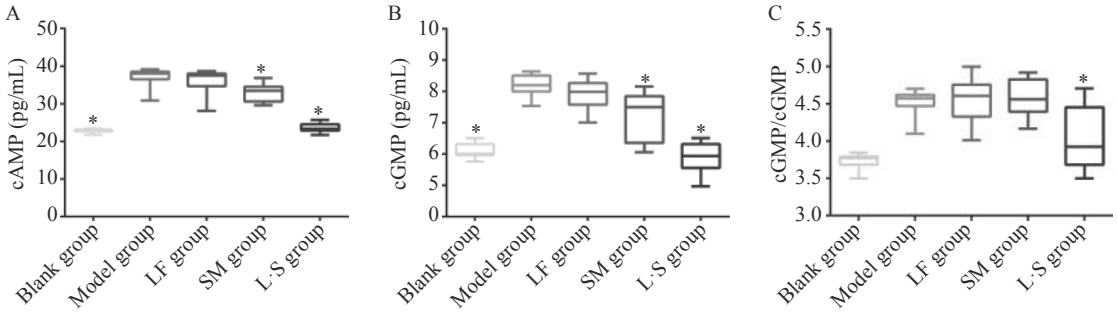

Figure 2 The contents of cAMP (pg/mL), cGMP (pg/mL), and cAMP/cGMP in the retinal homogenate

A, the contents of cAMP (pg/mL) in the retinal homogenate. B, the contents of cGMP (pg/mL) in the retinal homogenate. C, the contents of cAMP/cGMP in the retinal homogenate.

3.3 Determination of hemorheology

Compared to model group, the content of the whole blood relative index of the high shear rate in the plasma of RCS (rdy-/-, p-/-) rats treated with LF and SM was decreased after 28 d ($P < 0.05$) to a level close to that of blank group of RCS (rdy +/ +, p +/ +) rats ($P > 0.05$) (Table 3 and Figure 3A).

After 28 d of treatment, the contents of plasma viscosity and fibrinogen in the plasma of RCS (rdy-/-, p-/-) rats treated with LF and SM were decreased relative to those of model group ($P < 0.01$) (Table 3, Figure 3B and 3C).

3.4 Determination of P-Selectin in plasma

Compared to model group, the content of P-Selectin in the plasma of RCS (rdy-/-, p-/-) rats treated with LF and SM was decreased after 28 d ($P < 0.05$) to a level close to that of blank group of RCS (rdy +/ +, p+/+) rats ($P > 0.05$) (Table 4 and Figure 4).

3.5 Retinal histomorphometry

The retinal structure of RCS (rdy +/ +, p +/ +) rats in blank group appeared clear and normal, with evident nerve fiber layer, ganglion cell layer, inner plexiform

Table 3 Comparison of the whole blood relative index of the high shear rate, plasma viscosity (mPa.S) and fibrinogen content (g/L) in each group ($\bar{x} \pm s$)

Group	*n*	Relative index of high shear rate	Plasma viscosity	Fibrinogen content
RCS (rdy+/+, p+/+) Blank	8	3.60 ± 0.59	0.74 ± 0.21	1.58 ± 0.41
RCS (rdy-/-, p-/-) Model	8	7.71 ± 2.81	1.48 ± 0.11	3.10 ± 0.35
RCS (rdy-/-, p-/-) LF	8	7.22 ± 2.17	1.44 ± 0.25	3.03 ± 0.31
RCS (rdy-/-, p-/-) SM	8	4.72 ± 0.95	1.43 ± 0.16	3.10 ± 0.40
RCS (rdy-/-, p-/-) L·S	8	3.43 ± 0.37[ab]	1.05 ± 0.28[c]	2.27 ± 0.37[d]

For relative index of high shear rate, compared to model group, [a]$P < 0.05$, compared to blank group, [b]$P > 0.05$. For plasma viscosity, compared to model group, [c]$P < 0.01$. For fibrinogen content, compared to model group, [d]$P < 0.01$.

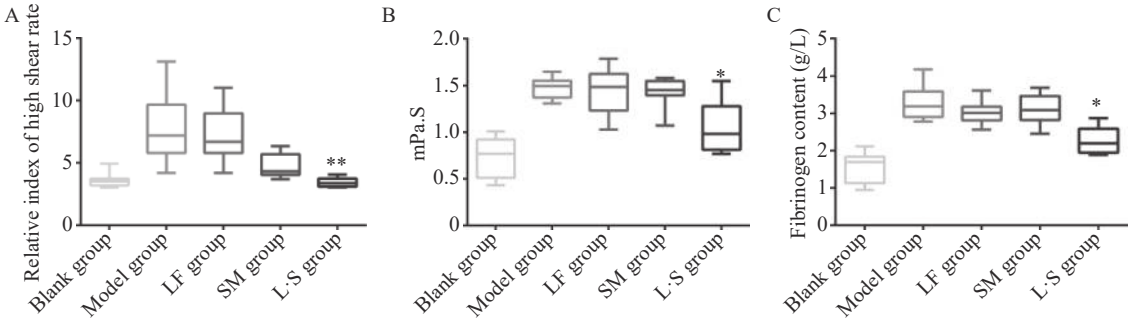

Figure 3 Comparison of the whole blood relative index of the high shear rate, plasma viscosity and fibrinogen content (g/L) in each group

A, comparison of the whole blood relative index of the high shear rate in each group. B, comparison of plasma viscosity in each group. C, comparison of fibrinogen content in each group.

Table 4 Average value of P-Selectin (ng/mL) in each group ($\bar{x} \pm s$)

Group	*n*	P-Selectin
RCS (rdy+/+, p+/+) Blank	8	6.65 ± 3.53[b]
RCS (rdy-/-, p-/-) Model	8	13.66 ± 3.66
RCS (rdy-/-, p-/-) LF	8	10.63 ± 2.58
RCS (rdy-/-, p-/-) SM	8	11.05 ± 2.25
RCS (rdy-/-, p-/-) L·S	8	8.54 ± 3.06[ac]

Compared to model group, [a]$P < 0.01$, [b]$P < 0.01$, compared to blank group, [c]$P > 0.05$.

layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, photoreceptor inner/outer segment layers, and retinal pigment epithelium (Figure 5A).

In the RCS (rdy-/-, p-/-) model group, the structure of each retinal layer was disordered, atrophied, and thinned, with evident changes in the outer plexiform layer, outer nuclear layer, photoreceptor inner/outer segment layers, and retinal pigment epithelium. The retinal pigment epithelium was lost, and the outer nuclear layer photoreceptor sensory cilium was completely atrophied and disappeared. The number of photoreceptor nuclei was reduced and its shape was irregular. Additionally, a small part of the photoreceptor nucleuswas stained and clustered, while other parts were not (Figure 5B).

In the RCS (rdy-/-, p-/-) LF group, the structure of each retinal layer was disordered, atrophied, and thinned, with evident changes in the outer plexiform layer, outer nuclear layer, photoreceptor inner/outer segment layers, and retinal pigment epithelium. However, the number of photoreceptor nuclei did not exceed that of the RCS (rdy-/-, p-/-) model group. The outer nuclear layer was also slightly thickened, and other structures appeared similar to those of the RCS (rdy-/-, p-/-) model group (Figure 5C).

In the RCS (rdy-/-, p-/-) SM group, the structure of each retinal layer was disordered, atrophied and thinned, with evident changes in the outer plexiform layer, outer nuclear layer, photoreceptor inner/outer segment layers, and retinal pigment epithelium. However, the number of photoreceptor nuclei was greater than that of the RCS (rdy-/-, p-/-) model group. The outer nuclear layer was slightly thickened, and the other structures appeared similar to those of the RCS (rdy-/-, p-/-) model group (Figure 5D).

Retinal thickness in the RCS (rdy-/-, p-/-) L·S group was significantly thicker than that in the RCS (rdy-/-, p-/-) model group. In addition, their retinal pigment epithelium was visible. The number of photoreceptor nuclei in the outer nuclear layer was greater than that in the RCS (rdy-/-, p-/-) model group and the outer plexiform layer, outer nuclear layer, and photoreceptor inner/outer segment layers were clearer and more thickened than those in the RCS (rdy-/-, p-/-) model group (Figure 5E).

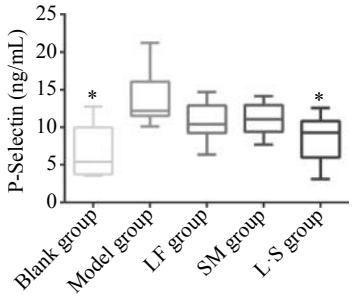

Figure 4 Average value of P-Selectin (ng/mL) in each group

4 Discussion

Based on the pathogenesis of deficiency with blood

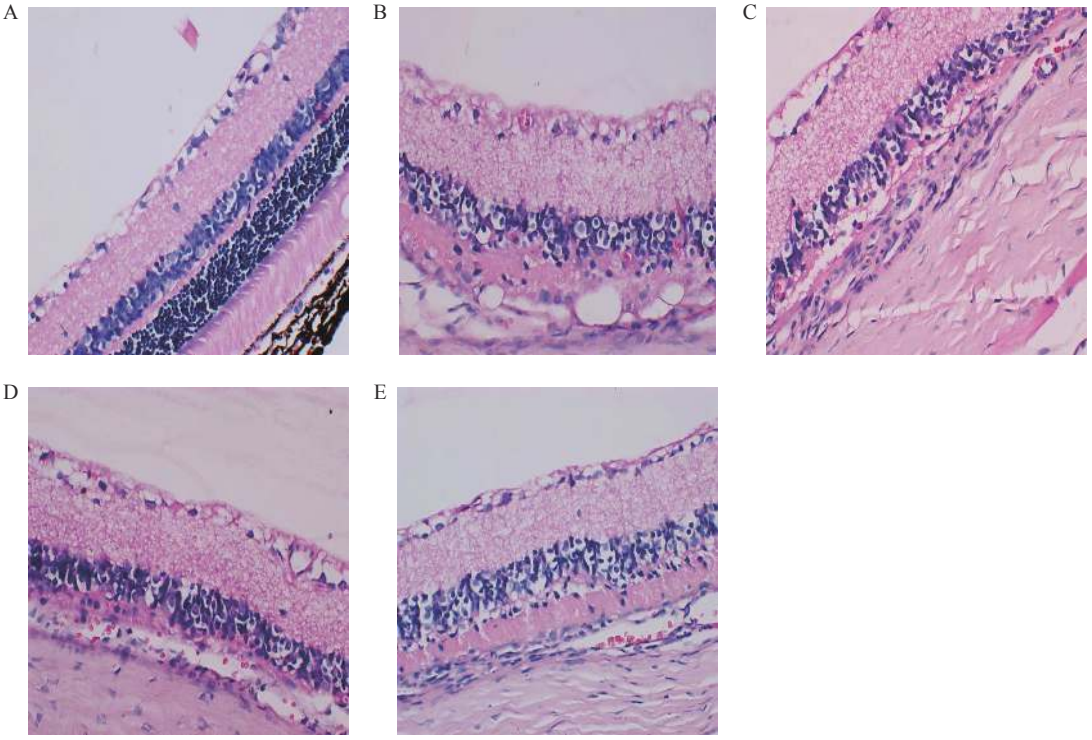

Figure 5 Comparison of the layers of the retina structure in each group (HE, × 400)

A, blank group. B, model group. C, LF group. D, SM group. E, L·S group.

stasis in RP, the method used to treat deficiency and activate blood circulation has become the favorable treatment. Therefore, we selected the Chinese herbal medicines, LF and SM, which areused to nourish the liver and kidney andactivate blood circulation, respectively, as interventions for RCS (rdy-/-, p-/-) rats with the syndrome of deficiency with blood stasis.

Following 28 d of intragastric administration, the sex hormone in female RCS (rdy-/-, p-/-) rats, E2, and that in male RCS (rdy-/-, p-/-) rats, PG, was increased in L·S group. In addition, a significant difference was found between L·S group and model group. LF and SM improved the syndrome of kidney deficiency in RCS (rdy-/-, p-/-) rats. The values for cAMP, cGMP, and cAMP/cGMP in the retinal homogenate of RCS (rdy-/-, p-/-) rats treated with L·S were decreased after 28 d to levels close to those found in blank group. L·S also improved the syndrome of Yin and Yang deficiency in RCS (rdy-/-, p-/-) rats. The content of plasma viscosity, the whole blood relative index of the high shear rate, fibrinogen content, and P-Selectin in plasma of RCS (rdy-/-, p-/-) rats treated with L·S were demonstrated to decrease after 28 d, achieving levels close to blank group. L·S can therefore decrease plasma viscosity, the whole blood relative index of thehigh shear rate, fibrinogen content, and P-Selectin to improve blood stasis. Through a study of retinal pathomorphology, we found that in the RCS (rdy-/-, p-/-) LF group and SM group, the structure of each layer of the retinal layer was disordered, atrophied and thinned, with evident changes in the outer plexiform layer, outer nuclear layer, photoreceptor inner/outer segment layers, and retinal pigment epithelium. However, the number of photoreceptor nuclei was greater than that in the RCS (rdy-/-, p-/-) model group and the outer nuclear layer was slightly thickened. Other structures were demonstrated to be similar to those in the RCS (rdy-/-, p-/-) model group. In the RCS (rdy-/-, p-/-) LF group, the structure of each retinal layer was disordered, atrophied, and thinned, with evident changes in the outer plexiform layer, outer nuclear layer, photoreceptor inner/outer segment layers, and retinal pigment epithelium. However, the number of photoreceptor nuclei was greater than that of the RCS (rdy-/-, p-/-) model group and the outer nuclear layer was slightly thickened. The other structures were found to be similar to those of the RCS (rdy-/-, p-/-) model group.

LF contains a variety of chemical components, including sugars, amino acids, trace elements, superoxide dismutase, alkaloids, and inorganic salts. Lycium barbarum polysaccharide (LBP) is the main chemical component of LF and has been implied to enhance or regulateimmune function, immunomodulation, antioxidant, anti-aging, and anti-tumor activity, reduce blood sugar and lipid, and protect the reproductive system [16]. Previously, LBP was demonstrated to exhibit anti-fatigue, anti-radiation, and blood pressure lowering effects, promote development, among other effects [17]. The cAMP/cGMP system was confirmed to be involved in the action of LBP, which is associated with the deficiency syndrome. In addition, the content of this system can be regulated by LBP [14, 18]. SM is widely used in the field of angiocardiopathy and through modern pharmacological research, it was revealed to repair the vascular endothelium, enhance antiplatelet aggregation, expand the coronary artery, improve microcirculation, anti-atherosclerosis, resist inflammation, and so on [19]. SM has remarkable effects on anticoagulant function, and its active ingredient, the tanshinone compound, can inhibitplatelet adhesiveness and aggregation, dilate blood vessels, and improve microcirculation. Herein, the combination of the 2 herbs (i.e., LF and SM) could treat the deficiency and activate blood circulation to treat RP.

In summary, LF and SM can improve the pathophysiological characteristics and ameliorate the syndrome of deficiency with blood stasis in RCS (ray-/-, p-/-) rats with RP.

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Competing Interests

The authors declare no conflict of interest.

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枸杞子配伍丹参对 RCS(rdy-/-, p-/-)视网膜色素变性大鼠虚中夹瘀证的干预研究

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【摘要】目的 探讨枸杞子和丹参对 RCS(rdy-/-, p-/-)视网膜色素变性大鼠虚中夹瘀证的影响。**方法** 将 32 只 RCS(rdy-/-, p-/-)大鼠分为四个组(每组雌雄各半),根据灌胃药剂不同分为:模型组给予生理盐水 0.9% 进行治疗,枸杞子(LF)组给予枸杞配方颗粒溶剂,丹参(SM)组给予丹参配方颗粒溶剂,枸杞和丹参(L·S)组给予丹参配方颗粒和杞子配方颗粒混合溶剂。将 8 只 RCS(rdy +/+, p +/+)大鼠(雌雄各 4 只)作为空白组,用 0.9% 生理盐水灌胃处理。灌胃 28 天后,我们检测了各组大鼠血液中 E2、PG、P-选择素的含量、血浆黏度、高剪切率全血相对指数和血浆中的纤维蛋白原含量,视网膜匀浆中的 cAMP、cGMP 含量,并通过 HE 染色评估视网膜。**结果** L·S 组血浆中 E2、PG、P-选择素的含量、血浆黏度、高剪切率全血相对指数和血浆纤维蛋白原含量与模型组相比有显着性差异($P < 0.01$),并且类似于空白组。L·S 组视网膜匀浆中 cAMP、cGMP 的含量与模型组差异有统计学意义($P < 0.01$),与空白组相似($P > 0.05$)。**结论** 枸杞子和丹参可改善虚中夹瘀证,有效干预视网膜色素性。

【关键词】视网膜色素变性;枸杞子;丹参;虚中夹瘀证;RCS(rdy-/-, p-/-)大鼠