·Basic Research·

Effect of ginsenoside -Rg3 on the expression of VEGF and TNF-α in retina with diabetic rats

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Abstract

• AIM: To investigate the effect of ginsenoside-Rg3 on the expression of vascular endothelial growth factor (VEGF) and tumor necrosis factor-α (TNF-α) in retina with diabetic rats and its roles in preventing neovascularization in diabetes.

• METHODS: Sixty male Wistar rats were divided into 3 groups randomly: negative control group, diabetic control group and ginsenoside-Rg3 treatment group (5mg/kg, 0.2mg/mL) followed by establishing diabetic model. The expression of VEGF and TNF-α were measured after 8 weeks.

• RESULTS: There were significant differences among negative control group, diabetic control group and ginsenoside-Rg3 treatment group in the expression of VEGF and TNF-α (F =129.363, 211.992; all the P<0.01). VEGF and TNF-α expression were significantly higher in diabetic control group and ginsenoside-Rg3 treatment group than that in negative control group (P <0.01), with a significant reduction in ginsenoside-Rg3 treatment group than that in diabetic control group (P<0.01).

• CONCLUSION: Ginsenoside-Rg3 can down-regulate the expression of VEGF and TNF-α in retina, which may interfere in the development of diabetic retinopathy.

• KEYWORDS: ginsenoside-Rg3; diabetic retinopathy; vascular endothelial growth factor; tumor necrosis factor-α

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INTRODUCTION

Diabetic retinopathy (DR) is one of the most significant microvascular complications of diabetes, of which the mechanism is very complicated. Change of cytokine is widely considered as one of the most important reason. Vascular endothelial growth factor (VEGF) is a kind of cytokine which can act specially on vascular endothelial cells which leads to mitosis and neovascularization, consequently, promote angiogenesis [1]. Tumor necrosis factor-α (TNF-α) is assumed to be an inderect inductor of angiogenesis that increase VEGF expression. Ginsenosides, tetracyclic triterpenoid saponins, extracted form Panax ginseng, are physiologically and pharmacologically active ingredients that inhibit the protein synthesis and the proliferation of the cell. Some experiment findings indicated that ginsenoside Rg3 is a powerful antigenic inhibitor, which can down-regulate the expression of gene bFGF in vascular endothelial cells. Ginsenoside-Rg3 has biological effects of protection of ischemia reperfusion injury, extraction on oxygen radicals, antioxidation and block voltage-dependent channels (*e.g.* Ca^{2+}, K^+ and Na^+ channels)[2,3]. We studied the effect of ginsenoside-Rg3 on expression of VEGF and TNF-α in the diabetic mice retinas which induced by streptozotocin (STZ), and explored the possible mechanism.

MATERIALS AND METHODS

Materials Sixty male Sprague-Dawley (SD) rats (Qingdao Laboratory for the Control of Drugs), weight 230-250g, were given free access to standard rat food and drinking water and cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Then they were divided randomly into negative control group, diabetic control group and ginsenoside-Rg3 treatment group, with 20 rats in each group. Diabetic models were induced by intraperitoneal injection with streptozotocin (STZ, 60mg/kg) (Sigma, America), which was dissolved in 0.01mol/L citrate buffer, pH 4.6. The same volume of buffer solution was given to those in negative control group. All the animals were fasted for 2 hours. Blood samples collected from the tail vein were tested with glucometer (Model: Kyoto SUPER GLUCOCARD II, ARKRAY, Japan) after 72 hours. The one whose fasting plasma glucose concentration was higher than 16.7mmol/L was defined as diabetic models, which were divided randomly into diabetic control group and ginsenoside-Rg3 treatment group, including 20

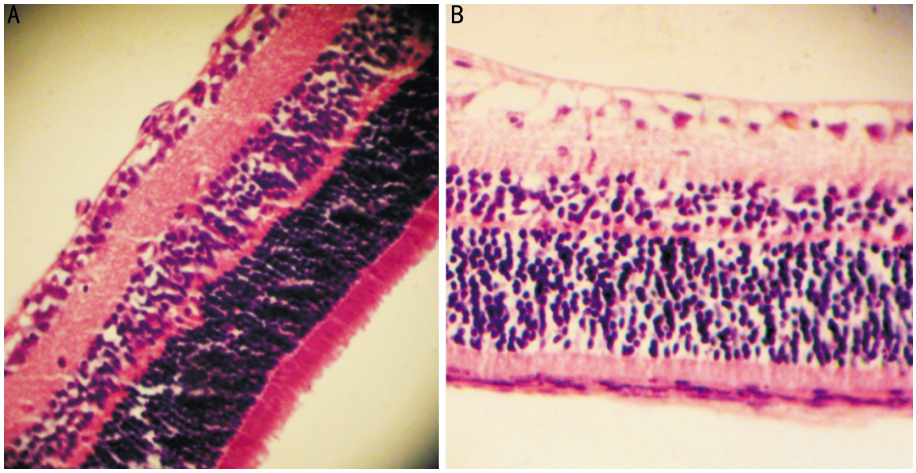

Figure 1 HE staining A: diabetic control group; B: ginsenoside-Rg3 treatment group (×400)

rats respectively. The one whose fasting plasma glucose concentration was lower the 5.6mmol/L was defined as negative control group. Negative control group received intragastric administration of ginsenoside-Rg3 (5mg/kg•d, 0.2mg/mL, Dalian Tianfu Research Institution of Drug) whereas diabetic and negative control groups received the same volume of salt solution for 8 weeks. The glucose concentration and the weight of the rats were tested once a week. Eight weeks later, the rats were anesthetized with an intraperitoneal injection of 350mg/kg chloral hydrate with the concentration of 10%. After killed, both eyes from each rat were enucleated after being perfused with 40g/L paraformaldehyde. Fixed in 40g/L neutral buffered formalin, dehydrated with alcohol and embedded in paraffin to make tissue sections (6μm) stained with hematoxyline-eosin (HE) and immunohistochemistry staining for light study and immunohistochemistry analysis. The sections were parallel to the sagittal plane of the cornea and optic papilla.

Immunohistochemistry The sections were dehydrated, and subsequently used for immunohistochemistry. In brief, after dewaxing, sections were treated in a microwave oven at low power for 10 minutes in 10mmol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase was inactivated using 3% hydrogen peroxide in methanol for 20 minutes. The sections were then incubated in protein-blocking agent for 30 minutes followed by incubation with a monoclonal mouse antibody to VEGF overnight at 4℃. Biotinylated rabbit anti-rat immunoglobulin G was used as the secondary antibody. Sections were then incubated with horseradish peroxidase-conjugated streptavidin. Peroxidase conjugates were localized by 3,3'-diaminobenzidine tetrahydrochloride (WuHan Boster Biological Technology, Ltd.) as a chromogen. Sections were counterstained with hematoxylin. Negative controls were stained with PBS instead. VEGF and TNF-α were observed in light microscope. The positive response showed the brown granules. Five sections were observed in each group. Five pictures of each slice were collected at random by the digital camera. The mean OD value of the cell was analyzed by Quantity One. Experimental data were described with mean value.

Table 1 Expression of VEGF 与 TNF-α in each group

Groups	*n*	VEGF	TNF-α
Negative control	20	72.5010±3.19227	174.4830±2.49524
Diabetic control	20	85.4695±2.36676	193.8080±3.96866
Ginsenoside-Rg3 treatment	20	81.2075±2.11482	185.6385±2.15837
F	/	129.363	211.992
P	/	<0.01	<0.01

Statistical Analysis The data were described with mean± SD. Statictical analysis was performed with the statistics program SPSS 13.0 for Windows using *t* test and analysis of variance (ANOVA) with multiple comparisons between groups.

RESULTS

HE Retinal cells were normal and distributed in order in each layer in negative control group; cell arranged loosely and distributed disorderly in external granular layer, vacuolar degeneration was observed in ganglion cell layer and granular layer in diabetic control group (Figure 1A); cells were normal and distributed in order with vacuolation in granular layer in ginsenoside-Rg3 treatment group (Figure 1B).

Immunohistochemistry VEGF and TNF-α showed faint expression in ganglion cell layer and inner nuclear layer in negative control group, while strong positive expression in diabetic control group (Figure 2A and 2C). The expression of VEGF and TNF-α were decreased dramatically in ginsenoside-Rg3 treatment group (Figure 2B and 2D), no significant differences compared with negative control group and significant differences compared with diabetic control group ($P<0.05$, Table 1).

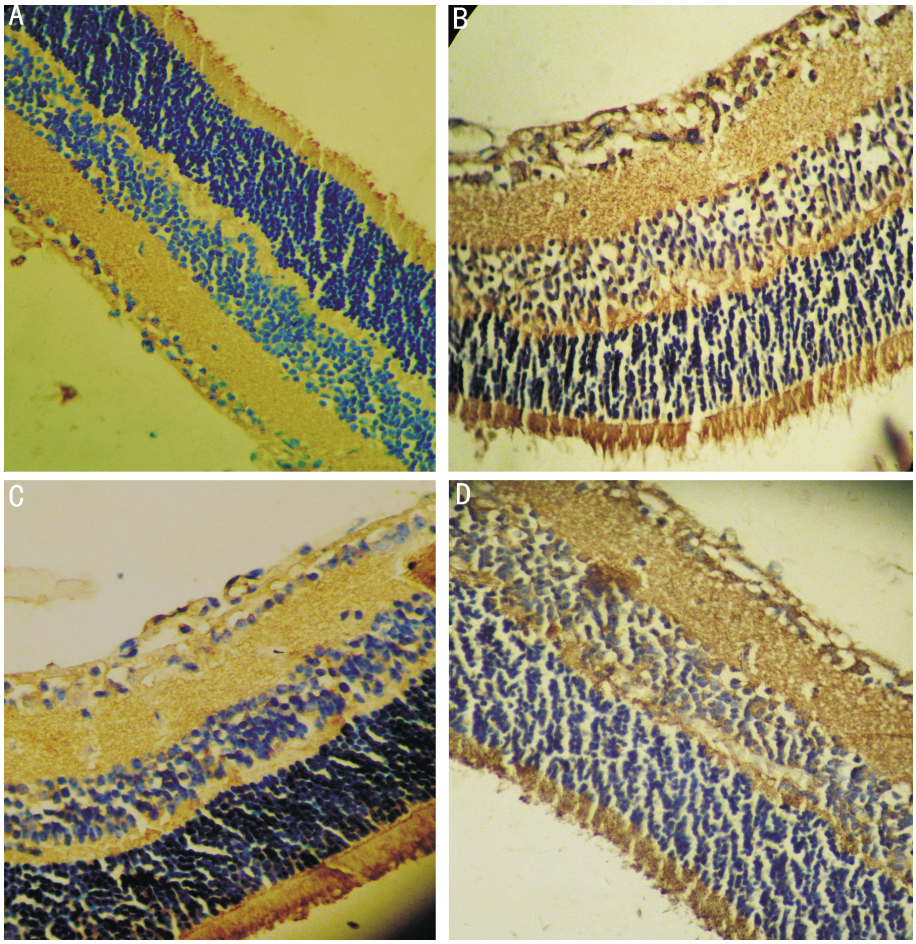

Figure 2 Immunohistochemistry staining A:expression of VEGF in diabetic control group; B: expression of VEGF in ginsenoside-Rg3 treatment group; C: expression of TNF-α in diabetic control group; D: expression of TNF-α in ginsenoside-Rg3 treatment group (×400)

DISCUSSION

The exact pathological mechanism of DR has not yet fully understood. Retinal neovascularization is formed due to the stimulation of retinal ischemia or hypoxia that augments the mRNA expression in VEGF. Clinical and animal experiments have confirmed that the major source of VEGF in diabetic eyes was the retina. It has been reported that the development of DR is related to the abnormal function of blood retinal barrier (BRB). VEGF eases the process of the injure to the blood retinal barrier destrcting its imperviousness and stimulates the neovascularisation process by up-regulating the gene expression of intercellar adhesion molecule-1 (ICAM1). Abnormal function of BRB causes retinal exudation, hemorrhage and edema (diabetic macular edema), inducing by leukocytes adhesion to the retinal vessels, which due to the improved level of mRNA and protein [4]. On the other hand, VEGF can stimulate the mitosis of vessel endothelial cells specifically. A series of signal transduction pathway has been activated by the combination of VEGF and its specific receptor, engendering the proliferation and migration and vessels lumen neogenesis eventually.

Vsacular endothelium is a maior traget of actions of TNF-α and the activation by nuclear factor (NF) enhances expression of cell surface adhesion molecules，including the intercellular adhesion molecules and vascular cell adhesion molecule, which in turn facilitates the attachment of blood leukocytes to endothelial surfaces [5,6]. TNF-α can increase the VEGF, PDGF as well as EGF and the proliferation, enhance the local inflammatory process with IL-8 produced by macrophages. In addition, TNF-α attenuates the tyrosine phosphorylation of IRS-1 in insulin signaling pathway, facilitates the lipolysis, augments the free fatty acid, induces and aggravates the insulin resistance, which promotes the development of diabetes and its complications.

Ginsenosides-Rg3, a tetracyclic triterpenoid saponins, which chemical stucture is $C_{42}H_{72}O_{13}$, relative molecular weight is 784, was found by Japanease researcher, and has been proved to be a inhibitor of tumor growth and metastasis[2,7]. It has been reported by Mochizuki *et al.*[8] that is an anti-angiogenesis agent. We found that treatment with ginsenosides-Rg3 reduced VEGF and TNF-α expression compared with diabetic control group, eclucidating that it may play an role in the development of DR by attenuating

the VEGF and TNF-α expression. Geng *et al.* [9] speculated that the effect of ginsenosides-Rg3 on inhibition of vascular endothelial cell proliferation and anti-angiogenesis may be relate to regulating of metabolism, blocking the combination of VEGF and its receptor, inhibiting the endothelial cell migration as well as down-regulating the growth factor receptor of vescular endothelial cells in proliferative phase.

Therefore, neovasculiration in diabetes can be inhibited by ginsenosides-Rg3, protecting from the DR. However, VEGF and TNF-α expression lower in ginsenosides-Rg3 treatment group compared with diabetic control group, wherase higer compared with negative control group, illustrating that ginsenosides-Rg3 can not prevent the development of DR radically. We conclude that it is not enough to prevent DR only by ginsenosides-Rg3, prevention from various aspects is necessary.

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