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Inhibitory effect of Telmisartan on STZ-induced early retinopathy in diabetic mice

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Abstract: **To investigate the effect of Telmisartan intravitreal injection on diabetic retinopathy and its protective mechanism. 54 adult male C57B/6L mice were included in this study. All the mice were randomly divided into blank control group (8), solvent control group (8) and diabetic group (36). Diabetic mice was intraperitoneally injected Streptozotocin (STZ), and sodium citrate buffer was intraperitoneally injected in to the solvent control group. According to the drugs given in the vitreous cavity of the left eye after modeling, diabetic mice were randomly divided into Telmisartan group, Conbercept group and model control group. Western blot was applied to detect the protein expression of VEGF-A, RAGE and TNF-α. HE staining was used to observe the changes of retinal morphology in each group under an optical microscope. The expression levels of VEGF-A, RAGE and TNF-α were significantly increased in the model control group compared with the blank control group (P<0.01). The expression levels of VEGF-A, RAGE and TNF-α in the left eye of Telmisartan group were significantly lower than that in the right eye ((P<0.01). The expression levels of VEGF-A, RAGE and TNF-α in the left eye of Conbercept group was lower than that in the right eye (P<0.001 for all). There was no statistically significant difference in VEGF-A, RAGE and TNF-α (P<0.01) between the solvent control group and the blank control group. There was no statistically significant difference in VEGF-A, RAGE and TNF-α (P<0.01) between the left eye and the right eye of the model control group. HE staining results showed that the retinal cells of the normal group had clear boundaries, normal morphology and neat arrangement of nerve cells. In the model control group, the retinal boundary was not clear, the cells were vacuolated and the nerve cells were not arranged in order. Intravitreal injection of Telmisartan can improve early diabetic retinopathy in mice by inhibiting the expression of VEGF-A, RAGE and TNF-α.**

Keywords: Diabetic retinopathy; Telmisartan; Vascular endothelial growth factor A (VEGF-A); Terminal products of late glycosylation; Tumor necrosis factor α (TNF-α)

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1. Introduction

Diabetic retinopathy (DR) is the primary cause of acquired blindness in people during working age[1]. Studies have shown that renin-angiotensin system (RAAS) is not only involved in the regulation of systemic blood pressure, but also interacts with hyperglycemia in diabetic patients[2-3]. RAAS system is an important humoral regulatory system that consists of a series of peptide hormones and corresponding enzymes. Its main function is to regulate and maintain the balance of human blood pressure, water and electrolytes, and maintain the relative stability of human internal environment. Excessive activation of RAAS system can increase blood pressure with pathological conditions. It has been proved that there is an independent renin-angiotesin system and its related receptors in the eye[4]. It has attracted attention that how the RAAS system and its receptors affect DR, and whether the inhibition of the RAAS system has an impact on the occurrence and development of DR. This studies was to analyze and observe the effect of Telmisartan on the expression of VEGF-A, RAGE (Receptor for advanced glycosylation end product), TNF-α (Tumor necrosis factor receptor-α), the influence on DR retinal tissue microstructure, and retinal blood perfusion status. Basis on establishing a diabetic mice model and giving Telmisartan intravitreal injection intervention therapy, our work would to further explore the mechanism of DR and the prevention role of Telmisartan on DR.

2. Materials and methods

2.1. Preparation of animal

C57BL/6J mice, SPA level, 6 months, male, 19.88±2.31g, were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd.

2.2. Medicines and reagents

The reagents included Telmisartan purchased form Solarbio (analytically pure). In a sterile environment, 10mg Telmisartan was dissolved in 100μL NaOH solution, and was diluted with normal saline twice to a concentration of 0.5 $g.L^{-1}$, and then was titrated with hydrochloric acid at a pH of 8.3[5]. Conbercept ophthalmic injection was Kanghong Langmu pharmaceutical product. Rabbit anti-rat VEGF-A antibody, rabbit anti-rat RAGE antibody, rabbit anti-rat TNF-α (Abcam, batch Numbers ab52917,

ab216329, ab6671, respectively), Streptozotocin (STZ; Sigma,USA).

2.3. Instruments

5μL pipette with 33 G needle (Hamilton, Swiss), Johnson glucose meter (Johnson&Johnson medical equipment Co., Ltd USA), dissecting microscope (Zeiss, Germany), microscopic instruments (Suzhou medical instrument factory), BH-2 optical microscope (Olympus, Japan), pathological tissue bleach and drying treatment apparatus (Shanghai heli industry Co., LTD.), RM2016 slicer (Shanghai medical equipment Co., LTD.), optical microscope (Zeiss, Germany).

2.4. Animal modeling and grouping

All C57BL/6J mice were randomly divided into blank control group (8e), solvent control group (8) and diabetes model group (36). Mice in blank group were not treated. 36 diabetic model mice were fed with high sugar and fat diet for a month, and were intraperitoneally injected with STZ solution (STZ was dissolved in 0.1 mol·L^{-1} with a pH value of 4.2 as citrate sodium buffer) after fasting for 12h, and were continuously injected with 50mg·kg-1 for 5d. Mice with fasting blood glucose (fasting time > 12h) and fasting blood glucose > 13.9mmol/L were included in the diabetes model 1 week after STZ injection. Mice in the solvent control group were intraperitoneally injected with the same dose of sodium citrate solution. After the successful induction of the model, the diabetic mice were randomly divided into three groups according to the intervention method, Telmisartan group (12), conbercept treatment group (12), and model control group (12). During the experiment, at 1 month of diabetes, 2 mice died in the model control group and conpercept group, and 1 mouse died in the Telmisartan group at 2 months of diabetes, and the death model was supplemented timely.

2.5. Medication

Basis on the model, intervention was given at the 17th week. All left eyes of diabetic mice were selected as the experimental eye, and the right eyes were not treated. The model control group was injected with normal saline (2.5μL). In the conbercept group, 2.5μL of 1 g·L^{-1} conbercept solution (containing 2.5μg conbercept) was injected into the vitreous cavity of the left eye. The left eyes in the Telmisartan group were injected with 2.5μL of 0.5 g·L^{-1} telmisartan solution (containing Telmisartan 1.25g). All right eyes were negative controls.

2.6. Specimen collection

Diabetic mice treated with the intervention were fed for 4 weeks after administration. Blood glucose and body weight were measured after 12h on an empty stomach. In treated group, blank control group and solvent group, a certain number of mice were randomly selected to be sacrificed by decervation after anesthesia, and their eyes were quickly removed to prepare for subsequent western blot detection and HE staining.

2.7. Western Blot

After the treatment 4 weeks, 6 mice were randomly selected in each group. Both eyeballs were extracted, and total retinal protein was extracted, and the protein concentration was measured by microplate. The solution volume with 30g of protein was calculated as the sample size. After mixing with protein buffer, the sample was heated for 5 min to make its degeneration. The prepared polyacrylamide gel was added for electrophoresis. After the electrophoresis, the protein was soaked in the membrane buffer for 5min to transfer to the PVDF membrane. After sealing and cleaning, rabbit antibody VEGF-A (1:10000), TNF-α (1:5000), RAGE (1:5000), β-actin (1:5000) were tested. Under 4℃ overnight, the film was washed, and then second antibody goat anti-rabbit IgG was added, and was incubated for 2.5h. The film was washed with TBST for 3 times, every 15min for 1 time. Color was developed by ECL chemiluminescene. The brand was scanned by absorbance integral. β-actin was used as the internal reference. The ratio of target protein absorbance to internal reference protein absorbance was used for comparison.

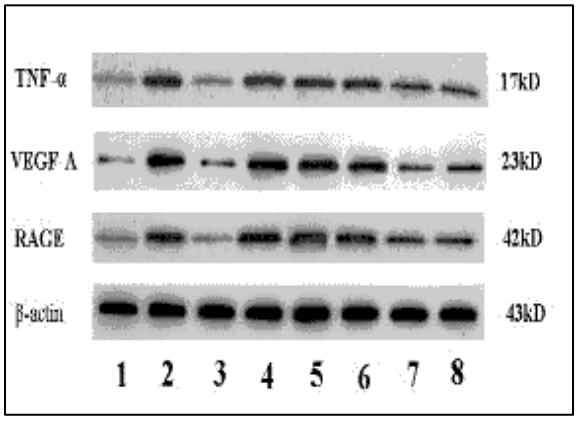

Figure 1. Supplementary description: 1. Telmisartan, left eye; 2. Telmisartan, right eye; 3. Conbercept, left eye; 4, Conbercept, right eye; 5. Model control group, left eye; 6. Model control group, right eye; 7. Blank control group; 8. Solvent group.

2.8. Histopathological examination

4 weeks after the treatment, mice were sacrificed after anesthesia, and both eyeballs were removed.

The eyeballs immediately removed were fixed in the prepared pathological fixative for more than 12h. The fixed eyeballs were dehydrated in alcohol with volume fractions of 55%, 65%, 75%, 85%, 95% and 99.99%, respectively. At the step of anhydrous alcohol, the lens was removed, and be made transparent in xylene. After that, the sample was embedded in 60℃ paraffin, and then was sliced. Finally, the sample was stained with hematoxylin-eosin and sealed with neutral gum to observe the morphology of retinal microstructure.

2.9. Statistical analysis

SPSS 16.0 statistical software was used for statistical analysis, and the data were marked by $\bar{x} \pm s$. One-way ANOVA and LSD-t were used. P<0.01 was considered statistically significant.

3. Results

3.1. Observation on the general situation of diabetic mice

Compared with the blank control group and the solvent control group, the diabetic mice in each group had general mental state, dull hair, reduced activity, slow reaction, significantly increased intake of food and water, and larger odor of pad materials.

3.2. Effects of retinal VEGF-A, RAGE, TNF-α protein expressioin in diabetic mice

The expression of VEGF-A, RAGE and TNF-α proteins in retinas of mice in each group were shown in Figure 1 and Table 1. The results showed that there were no significant differences in the expression levels of VEGF-A, RAGE and TNF-α proteins between the solvent control group and the blank control group (p>0.05). VEGF-A, RAGE and TNF-α protein expression levels were not significantly different in the left eye and right eye of the model control group (p>0.05). VEGF-A, RAGE and TNF-α protein expression levels in the model control group were significantly higher than those in the blank control group (p<0.05). VEGF-A, RAGE and TNF-α protein expression levels in the left eye of Telmisartan group were significantly lower than those in the right eye (p<0.05). VEGF-A, RAGE and TNF-α protein expression levels in the left eye of conbercept group were significantly lower than those in the right eye (p<0.05).

Table 1. The comparison of VEGF-A, RAGE, TNF-α protein expression in retina within each group ($\bar{x} \pm s$)

Group	Number of eyes (n)	VEGF-A	RAGE	TNF-α
Blank control group	6	0.48 ± 0.03^{a}	0.66 ± 0.05^{a}	0.59 ± 0.05^{a}
Solvent group	6	$0.55\pm0.03^{a\#}$	$0.64\pm0.05^{a\#}$	$0.61\pm0.04^{a\#}$
Model control group, left eye	6	$0.93\pm0.03^{*}$	$0.88\pm0.03^{*}$	$0.92\pm0.04^{*}$
Model control group, right eye	6	0.87±0.03	0.86±0.02	0.90±0.02
Telmisartan group, left eye	6	0.32 ± 0.02^{b}	0.37 ± 0.03^{b}	0.37 ± 0.02^{b}
Telmisartan group, right eye	6	0.93±0.03	0.95±0.02	0.92±0.03
Conbercept group, left eye	6	0.47 ± 0.02^{b}	0.38 ± 0.04^{b}	0.33 ± 0.02^{b}
Conbercept group, right eye	6	0.94±0.03	0.90±0.04	0.92±0.03

Note: The comparison with right eye of model control group, "a" P<0.05; The comparison with right eye of model control group, "*" P>0.05; The comparison with right eye, "b" P<0.05; The comparison with blank control group, "#" P>0.05.

3.3. Effects of retinal microstructure changes in diabetic mice

The results of HE staining showed that, compared with the blank control group, the retinal cells in the model control group were irregularly arranged in each layer, and the thickness became thinner, and the number of ganglion cells decreased, and capillary growth was observed in the ganglion cell layer. In the left eye of Conbercept group, compared with the right eye, cells in all layers were arranged in order, and retinal thickness was not significantly thinner, and no obvious vacuolar degeneration was observed in the nerve fiber layer, and cells in the inner and outer nuclear layer were arranged in order. Compared with the right eye, the retinal thickness of the left eye in Telmisartan group did not change significantly, a small amount of vacuolar degeneration could be seen in the nerve fiber layer, and the cells in the inner and outer nuclear layer were basically arranged in order.

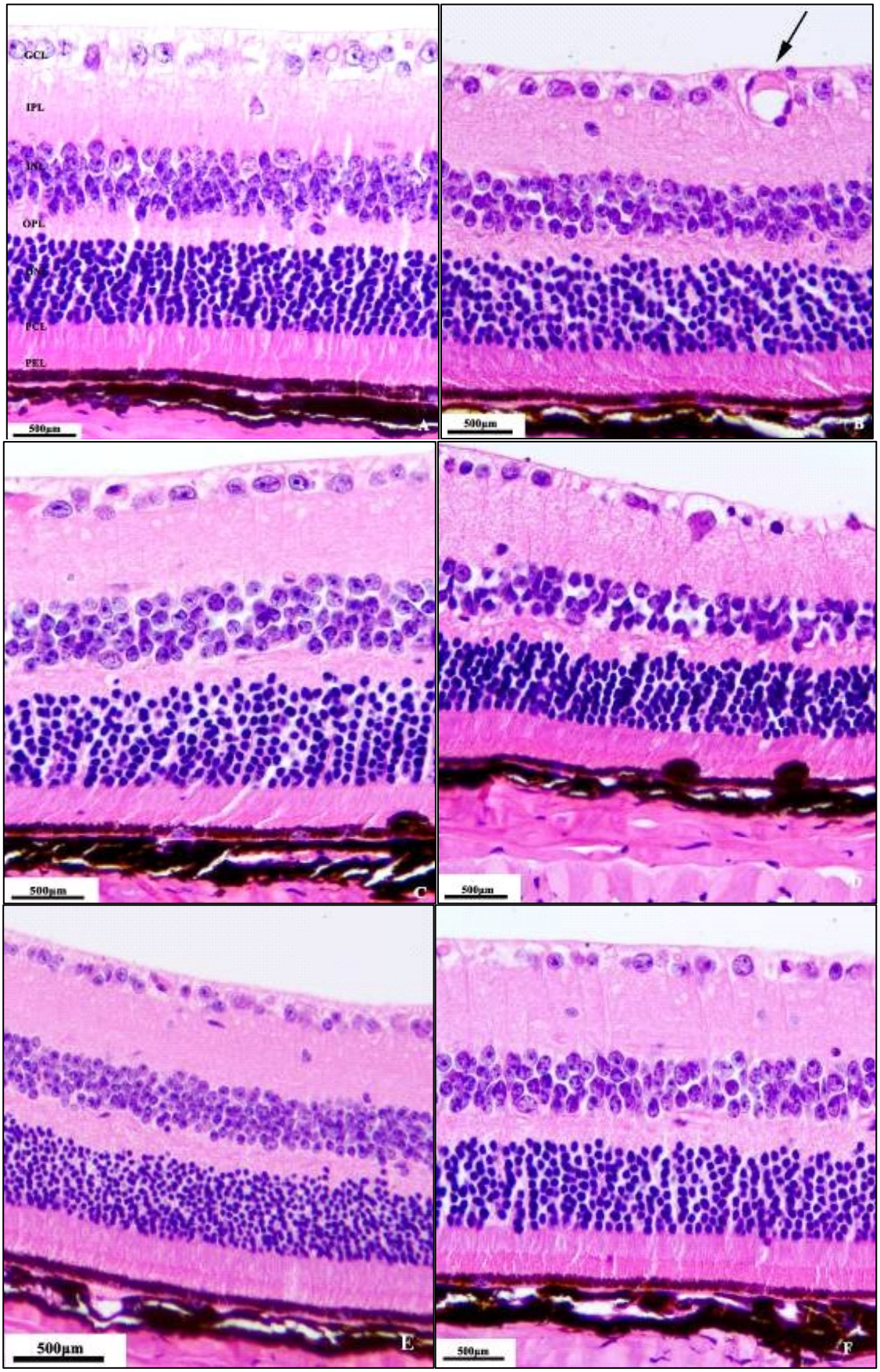

Figure 2. Retinal HE staining of mice in each group showed retinal tissue damage. A. Blank control; B. Model control; C. Conbercept, left eye; D. Conbercept, right eye; E. Telmisartan, left eye; F. Telmisartan, right eye. The arrow indicates neovascularization.

4. Discussion

In this study, small dose of STZ was used to induce DR lesions in diabetic mice many times to simulate the onset process of human diabetes. STZ can destroy pancreatic β cells and induce apoptosis. Through triggering the binding of antigens and antibodies in vivo, the cell damage effect is further amplified and the occurrence of DM is mediated [6]. In this experiment, the diabetic mice presented pathological changes of retina at the 5th month after successful modeling. And studies have found that the VEGF-A, RAGE and TNF-α expression was significantly increased in retina tissue of diabetic tissue. Compared with normal mice, the retinal microstructure of diabetic mice showed morphological changes. Studies have shown that the retinal tissue damage occurred as early as march in the STZ-induced diabetes model. The expression of VEGF-A was significantly increased. Small amount of retinal neovascularization was observed at 17th week[7,8]. The decomposition of Blood retina barria (BRB) is an important step in the progress of DR. Studies have shown that VEGF-A, TNF-α, a large number of pericytes and oxidative stress of cells play key roles in the mechanisms of BRB decomposition[9]. VEGE-F, as a powerful endothelial vascular growth inducer, the increase of it induces a decrease in tight junction proteins. TNF-α as one of the major inflammatory factors, it can destroy BRB through mediating the stagnation of retinal leukocytes and apoptosis. The decrease of tight junction proteins and the apoptosis of vascular endothelial cell will increase the permeability of retinal blood vessels significantly[10]. RAGE, as advanced glycosylation end product (AGEs), binds to receptor proteins plays a role on the cell surface. Increased AGEs can induce up-regulation of RAGE, promote oxidative stress, accelerate apoptosis of pericytes, and up-regulating VEGF to promote the formation of new blood vessels[11]. The above factors increase vascular endothelial permeability, apoptosis of pericytes, decomosition and destruction of BRB, which are clinically manifested as retinal hemorrhage, exudation, edema, hyperplasia of microangioma capillanisum and growth of new blood vessels.

At the gene level, Savaskan and Danser et al[4] proved that vitreous, retina, cornea, RPE layer and other local tissues can be synthesized independently and secrete angiotensin II (Ang II) to participate in the RAAS system[3,12,13]. Research has shown that Ang II, AS the end product of RAAS system, is elevated at the expression level of the DR patients. Its main mediated and conducted by Ang II type 1 receptor (AT1-R) signals[14]. The activated RAAS system overexpresses Ang II and activates the downstream NF-κB and PKC pathways in cells can promote NADPH oxidative induction, accelerate oxidative stress and inflammatory factor secretion, increase VEGF expression, promote proliferation and vascular remodeling of endothelial cell. These two processes not only increase retinal vascular permeability, but also lead to infiltration of inflammatory cells, adhesion of while blood cells, changes in retinal hemodynamics, and finally collapse of the blood-reinal barrier (BRB)[14-16]. In turn, high blood glucose levels can further promote the functioning of the RAAS system, and accelerate the process of inducing apoptosis, hypertrophy, angiogenesis, inflammation and fibrosis[17]. The results showed that the expression of VEGF-A, RAGE, TNF-α decreased after the treatment of Telmisartan in the left eye of diabetic mice. Intraocular injection of Telmisartan solution could inhibit the expression of VEGF-A, RAGE, TNF-α, and delay the microstructural morphological damage of retina. It was speculated that intravitreal injection of Telmisartan solution can selectively bind with intraocular Angiotensin receptor 1(ATR 1), and inhibit the over-activation of Ang II mediated RAAS system and delay the decomposition of BRB. Relevant studies abroad have shown that in mice with diabetic retinopathy, early intraperitoneal injection of Telmisartan solvent can control the occurrence and development of diabetic retinopathy[18,19]. In domestic clinical studies on Telmisartan, patients with hyertension were given oral treatment of Telmisartan and sulodite for 6 months. Follow-up results showed that retinal microangioma was alleviated and the rate of retinopathy progression was delayed[20,21]. As a selective angiotensin 1 receptor inhibitor, Telmisartan has attracted more and more attention in the prevention and treatment of DR. In this study, intraocular injection of Telmisartan blocked Ang II-mediated AT1R to make effects, which could be applied as a new idea for the treatment of DR[14].

Angiotensin II (Ang II) type 1 receptor inhibitor (ARB) has become a first-line treatment for diabetic nephropathy and cardiovascular complications. Telmisartan replace angiotensin II to combine with AT1 receptor subtypes (known angiotensin II loci) with high affinity. There is no partial agonist effect at the AT1 receptor site[22,23]. The binding effect is long-lasting and can delay the progress of DR effectively by blocking the RAAS system[24]. This study made intravitreal injection firstly to avoid adverse effects caused by Telmisartan systemic application, and improved the medicine concentration in the eye, and reduced the passage process of medicine through the blood-retina barrier. This study provided a new idea for the treatment of DR by ARB, and further clarified the pathogenesis of diabetic retinopathy. Due to the small sample size and limited observation time, the long-term effects of Telmisartan on the diabetic retinopathy need be further studied and observed. As a widely used antihypertensive medicine in clinical practice, the inhibitory effect on diabetic retinopathy makes us

more confident in the future research on Telmisartan and DR.

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