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# Andrographolide ameliorates diabetic retinopathy by inhibiting retinal angiogenesis and inflammation

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ABSTRACT

*Background:* Andrographolide (Andro) is the main compound distributed in medicinal herb *Andrographis paniculata*. This study aims to observe the amelioration of Andro on streptozotocin (STZ)-induced diabetic retinopathy (DR) in mice.
*Methods:* STZ-induced non-proliferative DR (NPDR) for 2 months and proliferative DR (PDR) for 5 month in C57BL/6 mice were used in this study, respectively. Retinal vessels were observed by immunofluorescence staining for cluster of differentiation 31 (CD31). Evans blue permeation assay was used to detect the breakdown of blood-retinal barrier (BRB). Real-time PCR and immune-blot were used to detect mRNA and protein expression. Enzyme-linked immunosorbent assay (ELISA) was used to detect serum tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β.
*Results:* Retinal immunofluorescence staining with CD31 showed that Andro reduced the increased retinal vessels in STZ-induced PDR mice. Evans blue permeation results demonstrated that Andro attenuated the breakdown of BRB in STZ-induced NPDR mice. In STZ-induced PDR mice, Andro decreased the increased vascular endothelial growth factor (VEGF) in serum and vitreous cavity, and reduced the increased retinal mRNA expression of VEGF and its receptors. In STZ-induced NPDR mice, Andro abrogated the nuclear translocation of nuclear factor κB (NF-κB) p65 and early growth response-1 (Egr-1), and reduced the increased phospho-NF-κBp65, -inhibitor of kappa B (IκB), and -IκB kinase (IKK). Andro also decreased the increased serum and retinal mRNA expression of TNF-α, IL-6, IL-1β, serpine1, and tissue factor (TF).
*Conclusions:* Andro ameliorates DR via attenuating retinal angiogenesis and inflammation, and VEGF, NF-κB, and Egr1 signaling pathways all play important roles in this process.



## 1. Introduction

With the changing of lifestyle and the increasing of aging population, diabetes mellitus (DM) has been a serious and concerning health problem in the world. In China, the number of people who suffered from diabetes is about 98.4 million in the year 2013, which is the highest in the world [1]. DM is generally associated with severe complications such as diabetic retinopathy (DR) and diabetic nephropathy, which greatly reduce the quality of life and the survival of diabetic patients. DR, the chronic vascular complication due to the development of DM, is one of the most common and serious complications of DM [2,3]. Vision loss from DR has been a major and leading cause of blindness in adult. It is reported that nearly all persons with type 1 diabetes and about 60% of persons with type 2 diabetes will develop DR when living with DM for the first two decades [4,5].

The classification of DR mainly includes non-proliferative DR (NPDR) and proliferative DR (PDR) according to the International Clinical Diabetic Retinopathy Disease Severity Scale [6]. NPDR is characterized by selective loss of pericytes, the formation of acellular capillaries, the thickening of basement membrane, the increased vascular permeability, and capillary occlusion [5,7]. The resulting ischemia due to capillary non-perfusion leads to the increased secretion of various growth factors including VEGF, which promotes neoangiogenesis in retina, and retinal angiogenesis is the hallmark of PDR [5,7]. Thus, anti-inflammation and anti-angiogenesis have been considered as the potential therapeutic strategies for DR [8].

*Abbreviations:* Andro, Andrographolide; DR, diabetic retinopathy; STZ, streptozotocin; NPDR, non-proliferative DR; PDR, proliferative DR; CD31, cluster of differentiation 31; BRB, blood-retinal barrier; VEGF, vascular endothelial growth factor; ELISA, enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor-α; IL, interleukin; NF-κB, nuclear factor κB; Egr-1, early growth response-1; IκB, inhibitor of kappa B; IKK, IκB kinase; TF, tissue factor; DM, diabetes mellitus; FITC, fluorescein isothiocyanate; HMGB1, high-mobility group box-1; RAGE, receptor for advanced glycation end products; OPN, osteopontin; tPA, tissue plasminogen

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**Table 1**
Sequences of primers used for real-time RT-PCR.

| Target | Primer | Sequence annealing temperature (°C) |
|---|---|---|
| Actin | FP | 5′-TTCGTTGCCGGTCCACACCC-3′ 61 °C |
| | RP | 5′-GCTTTGCACATGCCGGAGCC-3′ |
| VEGFA | FP | 5′-GCTACTGCCGTCCGATTGAG-3′ 60 °C |
| | RP | 5′-ACTCCAGGGCTTCATCGTTACAG-3′ |
| FLT-1 | FP | 5′-CCTGATGGGCAAAGAATAACAT-3′ 60 °C |
| | RP | 5′-ATTTGGACATCTAGGATTGTATTGG-3′ |
| KDR | FP | 5′-GTGGTAAGTTGCGATTGTTGTG-3′ 60 °C |
| | RP | 5′-TGAACATTCGCCTTCTTTGATA-3′ |
| IL-1β | FP | 5′-AAAAAAGCCTCGTGCTGTCG-3′ 60 °C |
| | RP | 5′-GTCGTTGCTTGGTTCTCCTTG-3′ |
| IL-6 | FP | 5′-ACAAAGCCAGAGTCCTTCAGAGAG-3′ 62 °C |
| | RP | 5′-TTGGATGGTCTTGGTCCTTAGCC-3′ |
| TNFa | FP | 5′-CTGAACTTCGGGGTGATCGGT-3′ 62 °C |
| | RP | 5′-TCCTCCACTTGGTGGTTTGCTAC-3′ |
| Egr-1 | FP | 5′-GGCGATGGTGGAGACGAGTTAT-3′ 58 °C |
| | RP | 5′-CAAAGTGTTGCCACTGTTGGGT-3′ |
| TF | FP | 5′-ACACAAACCTTGGACAGCCAGTAA-3′ 59 °C |
| | RP | 5′-CTTTCCCGTGCTTGAGCCTTT-3′ |
| Serpine1 | FP | 5′-ACAGCTCATGCCCTCCGCCA-3′ 62 °C |
| | RP | 5′-CACCAGGCGTGTCAGCTCGTC-3′ |

Andrographolide (Andro), a natural diterpenoid lactone, is the main compound isolated from traditional medicinal herb *Andrographis paniculata* Nees (Acanthaceae) [9]. *A. paniculata* Nees is well-known for clearing away heat and toxic materials, and has been widely used for centuries in Asian countries like China, India, and Thailand for the treatment of sore throat, flu and upper respiratory tract infections [9]. There are various reports about the anti-inflammatory activity of andrographolide in experimental models of asthma, cigarette smoke-induced lung injury, pulmonary fibrosis, inflammatory bowel disease, etc. [10–13]. In addition, andrographolide is also reported to inhibit tumor angiogenesis in vivo and in vitro [14–16]. As andrographolide has obvious anti-inflammatory and anti-angiogenic activity, it may have potential therapeutic activity against DR. The present study aims to observe the amelioration of andrographolide on DR (including NPDR and PDR), and further explore the engaged mechanisms from inhibiting retinal inflammation and angiogenesis.

## 2. Materials and methods

### 2.1. Chemical compounds and reagents

Andrographolide (Andro), its purity is above 98.5%, was purchased from Nanjing TCM Institute of Chinese Materia Medica (Nanjing, China). Cluster of differentiation 31 (CD31) antibody and fluorescein isothiocyanate (FITC) conjugated anti-Rat IgG were purchased from BD Biosciences (Franklin Lakes, NJ). Enzyme-linked immunosorbent assay (ELISA) kit for VEGF was obtained from R&D (Minneapolis, MN), and other ELISA kits were purchased from RapidBio (West Hills, CA). Trizol reagent was purchased from Life Technology (Carlsbad, CA). PrimeScript® RT Master Mix and SYBR® Premix Ex Taq™ were purchased from Takara (Shiga, Japan). NE-PER® nuclear and cytoplasmic extraction reagents and Pierce® BCA Protein Assay Kit were purchased from Thermo Scientific (Bremen, Germany). NF-κBp65, phospho-NF-κBp65, phospho-IκB, phospho-IKK, Egr1, β-actin, and LaminB antibodies were all purchased from Cell Signaling Technology (Danvers, MA). Peroxidase-conjugated goat anti-Rabbit IgG (H + L) and peroxidase-conjugated goat anti-Mouse IgG (H + L) were purchased from Jackson ImmunoResearch (West Grove, PA). Other reagents unless indicated were purchased from Sigma Chemical Co. (St. Louis, MO).

### 2.2. Experimental animals

The C57BL/6 mice (18–22 g) were purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). The animals were maintained under controlled temperature (23 ± 2 °C), humidity (50%), and lighting (12 h light/12 h dark). The animals were fed with a standard laboratory diet and given free access to tap water. All animals received humane care according to the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine.

### 2.3. Establishment of the mice model of STZ-induced NPDR

Thirty-five mice were administered intraperitoneally (i.p.) with 55 mg/kg STZ for 5 consecutive days, while the other sixteen mice were injected (i.p.) with physiological saline and served as control animals. The concentration of serum glucose was measured 7 days after the last injection, and the mice with high glucose concentration (>16.5 mmol/L) were considered as diabetic mice. In this experiment, the glucose concentration of 33 mice was >16.5 mmol/L, and those mice were randomly divided into two groups: NPDR model (n = 17) and NPDR + Andro (10 mg/kg) (n = 16), respectively. At 1 month after the injection of STZ, the mice were administered intraperitoneally (i.p.) with Andro (10 mg/kg per day) consecutively for 1 month. At 2 months after the injection of STZ, 6 mice of each group were used for the measurement of BRB breakdown by using Evans blue. The other mice were anesthetized by sodium pentobarbital (30 mg/kg, i.p.), the blood samples were taken from the abdominal aorta, and the eyes were removed immediately.

### 2.4. Establishment of the mice model of STZ-induced PDR

Twenty-two mice were administered intraperitoneally (i.p.) with 55 mg/kg STZ for 5 consecutive days, while the other ten mice were injected (i.p.) with physiological saline and served as control animals. The concentration of serum glucose was measured 7 days after the

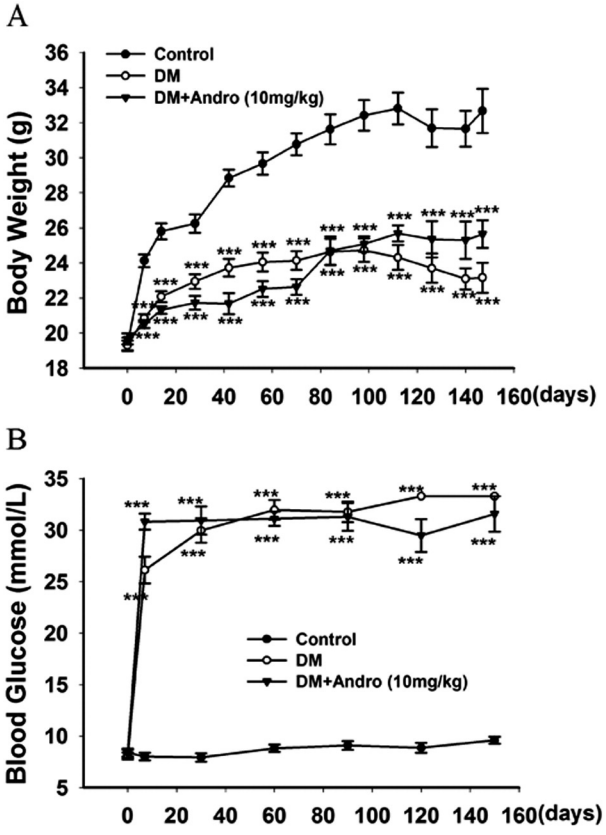


**Fig. 1.** Analysis of body weight and blood glucose level. (A) Body weight; (B) blood glucose level. Data = Means ± SEM (n = 9 for control, n = 10 for DM, n = 8 for Andro). ***$P < 0.001$ compared to control.