

## Original Article

# Endotoxin-induced uveitis promotes retinal endothelial cell injury and Muller cell proliferation

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**Abstract:** Uveitis is one of the most common eye diseases that cause eye blind. It mainly occurs in young adults. The pathogenesis of uveitis has not been clarified, leading to great difficulties in the treatment. Rat endotoxin-induced uveitis (EIU) is an ideal animal model for the study of human endogenous uveitis. Retinal microvascular endothelial cells and Muller cells are responsible for the supply of retinal neurotrophic requirements. They play a key role in the visual protection through maintaining the blood-retinal barrier. However, the role and mechanism of EIU in retinal endothelial cell injury and Muller cell expression has not been reported. SD rats were randomly divided into two groups. The EIU animal model was established using Escherichia coli endotoxin (LPS). Retinal endothelial cells and Muller cells proliferation were detected by MTT assay. Cell apoptosis was evaluated by measurement of Caspase 3 expression. Real-time PCR and Western blot were used to test the vascular endothelial growth factor (VEGF) expression. Enzyme-linked immunosorbent assay (ELISA) was applied to assess the level of interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Our results showed that retinal endothelial cell and Muller cell proliferation was attenuated in EIU group. Caspase 3 activity was enhanced, with increased VEGF expression and IL-1 $\beta$  and TNF- $\alpha$  secretion in the EIU group compared with control group ( $P < 0.05$ ). In conclusion, EIU causes retinal endothelial cell damage and Muller cell expression reduction by promoting inflammatory factors secretion, facilitating VEGF expression, and inducing apoptosis.

**Keywords:** Endotoxin-induced uveitis, VEGF, inflammatory factors, retinal endothelial cell, Muller cell

## Introduction

As one of the common diseases in ophthalmology, uveitis is a T cell-mediated autoimmune disease leading to eye blind [1, 2]. The incidence of uveitis is relatively high as its incidence reaches 200/100,000 in developed countries and 700/100,000 in non-developed countries. Most patients are accompanied by conjunctivitis, visual impairment, and other serious eye complications. Among them, the number of blinding is up to a quarter [3, 4]. Uveitis is divided into anterior, intermediate, and posterior uveitis, most of which occurs in young adults [5, 6]. The repeatability and persistent characteristics of uveitis makes its treatment extremely difficulty [7]. Due to the effect of inflammation on the posterior segment in the visually impaired uveitis patients, it often leads to retinal vasculitis, choroidal inflammation, and retinal inflammation [8]. Abnormal vascular function caused

by uveitis can induce retinal ischemia and neovascularization disorders [9]. Retinal blood vessels are complicated structures which are composed of a variety of cells, of which retinal microvascular endothelial cells are responsible for the supply of retinal neurotrophic requirement. They play a crucial role in the visual protection through maintaining blood-retinal barrier, eliminating toxins, and alleviating inflammatory factors [10, 11].

Muller cells, also known as Muller fiber, were first discovered and named by Muller from Germany. They are stained deep in the inner core layer of retina with slender cell body. Except the pigment epithelium and emulsion layer, Muller cell occupies the other parts of the retina, thus covering most of the neurons in the retina [12]. Under physiological conditions, Muller cells exhibit the function of synthesis and storage of glycogen, thus providing nutritional support for

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retinal neuron. Moreover, they maintain the stability of the cell environment. Muller cells specialized foot plate and retinal capillaries participate in the composition of blood-retinal barrier [13, 14]. As the study of human uveitis is restricted by a number of factors, the current research is performed mainly using animal models. Rat EIU is an ideal animal model for the study of human endogenous panuveitis [15, 16]. However, the impact and mechanism of EIU on retinal endothelial cell injury and Muller cell expression have not been reported.

### Materials and methods

#### *Experimental animals*

Specific pathogen free (SPF) grade male Sprague-Dawley (SD) rats aged 2 months with a body mass at  $250 \pm 20$  g were purchased from the Experimental Animal Center of Heilongjiang. The feeding conditions contained constant temperature at  $21 \pm 1^\circ\text{C}$  and relative humidity at 50-70%. The day/night cycle was 12 h/12 h.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Daqing Oilfield General Hospital.

#### *Main materials and instruments*

Escherichia coli endotoxin (LPS) was purchased from Invitrogen (USA). Pentobarbital sodium and lidocaine were purchased from Zhpharma (Shanghai, China). IL-1 $\alpha$  and TNF- $\alpha$  ELISA kits were purchased from R&D (USA). PVDF membrane was purchased from Pall Life Sciences. EDTA was purchased from Hyclone. Western blot related chemical reagents were from Beyotime. ECL reagent was obtained from Amersham Biosciences. Rabbit anti-rats VEGF monoclonal and horseradish peroxidase (HRP) labeled IgG secondary antibodies were purchased from Cell Signaling (USA). Caspase 3 activity detection kit was purchased from Nanjing Jiancheng bio-engineering research institute. Surgical microscopes were purchased from Suzhou Medical Equipment Factory. RNA extraction and reverse transcription kits were purchased from Axygen (USA). Labsystem Version1.3.1 Microplate reader was purchased from Bio-rad. ABI 7700 Fast Fluorescence Quantitative PCR was purchased from ABI (USA). Clean bench was purchased from Suzhou Sutai Purification Equipment Co., Ltd. Other common reagents were purchased from Sangon (Shanghai, China).

#### *Grouping and treatment*

The SD rats were equally and randomly divided into two groups, control and EIU group. Lipopolysaccharides (LPS) were used to establish EIU rat model [15, 16]. LPS at 1 mg/ml were injected into the bilateral foot pad at 1 mg/kg.

#### *Retinal endothelial cells and Muller cells isolation and cultivation*

The rats were anesthetized with 2% lidocaine and the bilateral eyeballs were aseptically removed. The eyeball was washed by gentamicin saline and transferred to the DMEM medium. Next, the eyeball was circuit cut at 3 mm posterior to the corneal limbus to remove the anterior segment and vitreous. Then the tissue was digested by 0.25% trypsin at room temperature for 10 min to separate the retinal endothelial cell layer and Muller cell layer. The tissue was further digested by 0.25% trypsin at  $37^\circ\text{C}$  for 30 min. Then the cell suspension was centrifuged at 800 rpm for 10 min. At last, DMEM medium containing 100 U/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin was added into the cells and cultured at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .

#### *MTT assay*

20  $\mu\text{L}$  MTT was added into retinal endothelial cells and Muller cells at logarithmic phase and incubated for 4 h. Then, 150  $\mu\text{L}$  DMSO was added into the plate for 10 min followed by measuring the absorbance value at 570 nm. Each experiment was repeated for three times.

#### *Caspase 3 activity detection*

Caspase 3 activity was tested according to the manual instructions. The cells were digested by trypsin and centrifuged at 600 g at  $4^\circ\text{C}$  for 5 min. Next, the cells were lysed on ice for 15 min and centrifuged at 20,000 g for 5 min at  $4^\circ\text{C}$ . At last, 2 mM Ac-DEVD-pNA was added into the cells and the absorbance value at 405 nm was measured to calculate Caspase 3 activity.

#### *ELISA*

ELISA was used to test the inflammatory factor contents in the supernatant. The plate was tested at 450 nm to obtain the OD value. The OD value of standard substance was used to prepare the linear regression equation, which

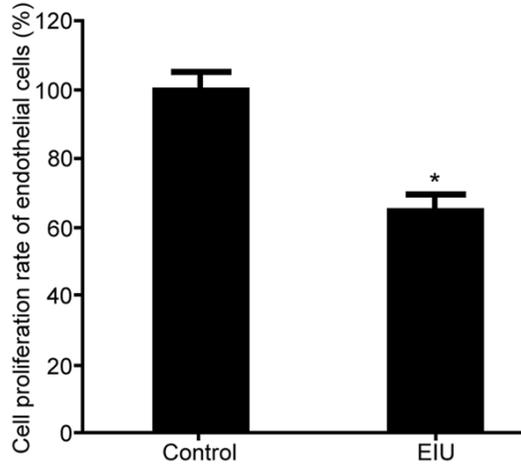
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**Table 1.** Primer sequences

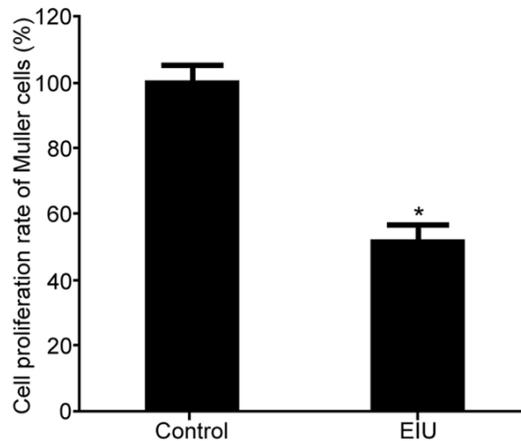
Gene	Forward 5'-3'	Reverse 5'-3'
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACCTTGCCGTGGGTAG
VEGF	AGCGGCTCATCTAAACAATGG	GGCGCACATTCTCTCCGTA

### Western blot

RIPA lysis buffer was added into the retinal endothelial cells and Muller cells and incubated on ice for 15-30 min. Next, the cells were treated with ultrasound at 5 s for 4 times and centrifuged at 10000 g for 15 min. The protein was transferred to new tube and quantified by Bradford method. The protein was separated on 10% SDS-PAGE and transferred to PVDF membrane at 100 mA for 1.5 h. After blocked with 5% skim milk for 2 h, the membrane was incubated with VEGF antibody (1:1000) at 4°C overnight. Then the membrane was incubated with goat anti rabbit secondary antibody (1:2000) at room temperature for 30 min. Next, ECL substance was added into the membrane for 1 min followed by exposure to observe the result. The film was scanned by Quantity One software and analyzed by protein image processing system. Each experiment was repeated for four times.



**Figure 1.** The effect of EIU on retinal endothelial cell proliferation. \*P < 0.05, compared with control.



**Figure 2.** The effect of EIU on Muller cell proliferation. \*P < 0.05, compared with control.

was adopted to calculate the concentration of samples.

### Real-time PCR

Total RNA was extracted from the retinal endothelial cells and Muller cells, and reversely transcribed into cDNA (**Table 1**). Real-time PCR was performed at 52°C for 1 min, followed by 35 cycles of 52°C for 1 min, 90°C for 30 s, and 72°C for 35 s. GAPDH was selected as an internal reference. The relative expression of mRNA was calculated by  $2^{-\Delta Ct}$  method.

### Statistical analysis

All data analyses were performed on SPSS16.0 software presented as mean  $\pm$  standard deviation and compared by one-way ANOVA. P < 0.05 was depicted as statistical significance.

## Results

### Effect of EIU on retinal endothelial cell proliferation

MTT assay was adopted to test the impact of EIU on retinal endothelial cell proliferation. EIU significantly inhibited the retinal endothelial cell proliferation compared with control group (P < 0.05) (**Figure 1**).

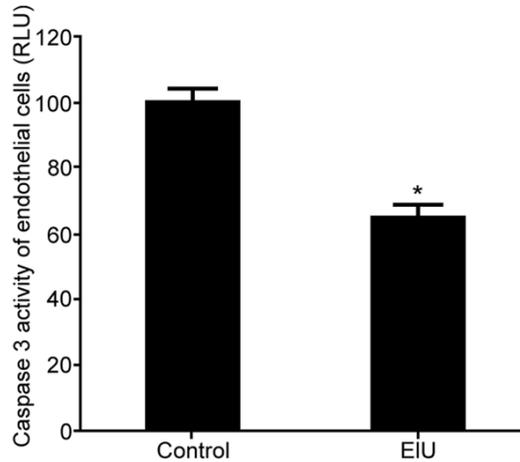
### Effect of EIU on Muller cell proliferation

MTT assay was used to detect the effect of EIU on Muller cell proliferation. EIU obviously suppressed Muller cell proliferation compared with control group (P < 0.05) (**Figure 2**).

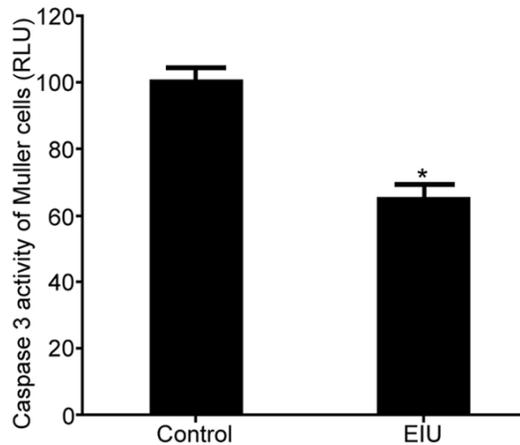
### Influence of EIU on the activity of Caspase 3 in retinal endothelial cells

The effect of EIU on the activity of Caspase 3 in retinal endothelial cells was analyzed using Caspase 3 activity detection kit. The results showed that EIU significantly promoted the activity of Caspase3 in retinal endothelial cells

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**Figure 3.** The influence of EIU on the activity of Caspase 3 in retinal endothelial cells. \* $P < 0.05$ , compared with control.



**Figure 4.** The influence of EIU on the activity of Caspase 3 in Muller cells. \* $P < 0.05$ , compared with control.

compared with control group ( $P < 0.05$ ) (**Figure 3**).

### *Influence of EIU on the activity of Caspase 3 in Muller cells*

The effect of EIU on the activity of Caspase 3 in Muller cells was analyzed by Caspase 3 activity detection kit. The results demonstrated that EIU significantly facilitated the activity of Caspase 3 in Muller cells compared with control group ( $P < 0.05$ ) (**Figure 4**).

### *Impacts of EIU on VEGF expression in retinal endothelial cells*

The effect of EIU on the expression of VEGF in retinal endothelial cells was assessed by Real-

time PCR and Western blot. The results revealed that the expression of VEGF mRNA and protein in retinal endothelial cells was significantly higher in EIU group than that in control group ( $P < 0.05$ ) (**Figure 5**).

### *Impacts of EIU on VEGF expression in Muller Cells*

The effect of EIU on the expression of VEGF in EIU cells was assessed by Real-time PCR and Western blot. The results revealed that the expression of VEGF mRNA and protein in Muller cells was markedly higher in EIU group than that in control group ( $P < 0.05$ ) (**Figure 6**).

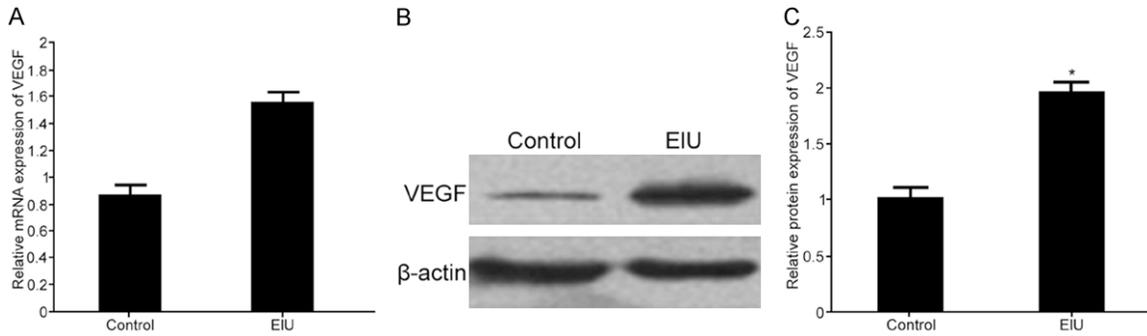
### *Function of EIU on IL-1 $\beta$ and TNF- $\alpha$ contents in supernatant of retinal endothelial cells and Muller cells*

The contents of IL-1 $\beta$  and TNF- $\alpha$  in the supernatant of retinal endothelial cells and Muller cells were analyzed by ELISA. The contents of IL-1 $\beta$  and TNF- $\alpha$  in the supernatant of retinal endothelial cells and Muller cells were apparently higher in EIU group than those in control group ( $P < 0.05$ ) (**Figures 7, 8**).

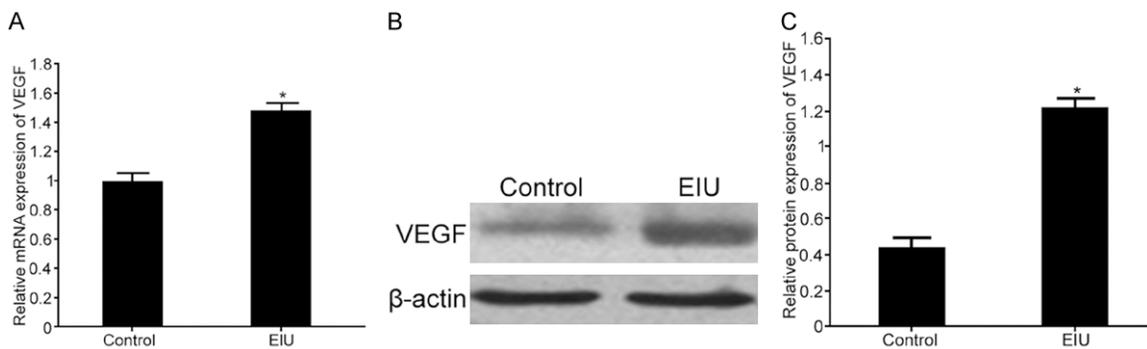
## Discussion

Uveitis is a type of eye disease frequently occurring in young. It has a wide range of types with complicated pathogenesis. Improper treatment can lead to blindness, thus causing widespread concerns around the world. Since its pathogenesis and recurrence mechanism is not entirely clear, the prevention has not been proceeded and the treatment effect is also unsatisfactory [17, 18]. Posterior EIU may cause retinal infiltration, leading to disc damage, retinal tissue damage, and retinal vasculitis [19]. Retinal endothelial cells are one of the important cells that maintain retinal function, and endothelial cell damage and denudation can cause retinal damage [14]. As retinal-specific glial cells that play an important role in maintaining the morphology and physiological function of the retina, Muller cells account for more than 90% of all cells in the retina [20]. The morphological and physiological functions and metabolic changes of Muller cells seriously affect the pathogenesis of the retinopathy [21]. This study confirmed that retinal endothelial cells and Muller cell proliferation was decreased with enhanced Caspase 3 activity in EIU group, suggesting that uveitis damaged retinal endothelial cells and decreased Muller cell expression.

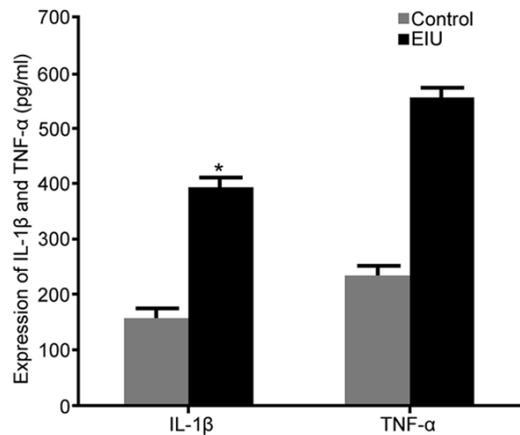
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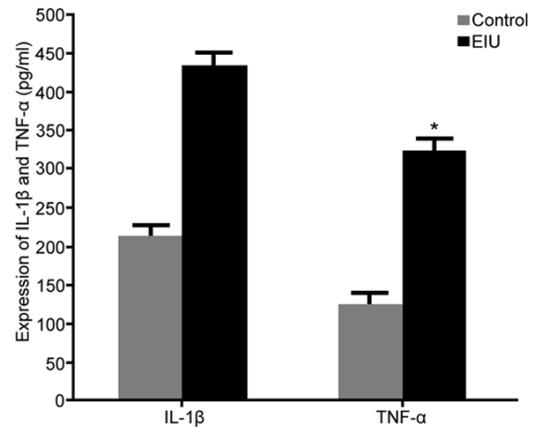
**Figure 5.** The impacts of EIU on VEGF expression in retinal endothelial cells. A. Real-time PCR detection of VEGF mRNA expression; B. Western blot detection of VEGF protein expression; C. VEGF protein expression analysis. \*P < 0.05, compared with control.



**Figure 6.** The impacts of EIU on VEGF expression in Muller cells. A. Real-time PCR detection of VEGF mRNA expression; B. Western blot detection of VEGF protein expression; C. VEGF protein expression analysis. \*P < 0.05, compared with control.



**Figure 7.** The function of EIU on IL-1β and TNF-α contents in supernatant of retinal endothelial cells. \*P < 0.05, compared with control.



**Figure 8.** The function of EIU on IL-1β and TNF-α contents in supernatant of Muller cells. \*P < 0.05, compared with control.

In-depth investigation revealed that VEGF expression was increased, while IL-1β and TNF-α secretion was enhanced in retinal endothelial cells and Muller cells from EIU group. VEGF has multiple isoforms that play a role through bind-

ing to its corresponding receptor (VEGFR). Most VEGFRs are located on the surface of endothelial cells and can alter vascular permeability after ligand binding. Thus, in the formation of EIU, VEGF participates in regulation by altering

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vascular permeability; and VEGF participates in the entire EIU pathology by promoting neovascularization [22]. IL-1 $\beta$  and TNF- $\alpha$  are potent regulators that inhibit the proliferation of retinal endothelial cells and Muller cells. They regulate the interaction between cells and the matrix, leading to the chemotaxis of macrophages and subsequent uveitis-induced retinopathy [23]. However, the specific mechanism of EIU on retinal endothelial cell injury and Muller cells still needs further investigation.

In conclusion, EIU causes retinal endothelial cell damage and Muller cell expression reduction by promoting inflammatory factors secretion, facilitating VEGF expression, and inducing apoptosis.

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### Disclosure of conflict of interest

None.

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