

## Original Article

# The role of $\omega$ -3 PUFAs in affecting retinal neovascularization by regulating microglia phenotype

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**Abstract:** Oxygen-induced retinal neovascular disease is a common blinding eye disease in ophthalmology. It was demonstrated that using  $\omega$ -3 polyunsaturated fatty acids ( $\omega$ -3 PUFAs) might be a new strategy for the treatment of this disease. However, the role and mechanism of  $\omega$ -3 PUFAs in regulating retinal neovascularization remains poorly understood. Seven-day-old C57BL/6J mice were randomly divided into control group, oxygen-induced retinopathy (OIR) group, and  $\omega$ -3 PUFAs group in which OIR model mice were fed with  $\omega$ -3 PUFAs diet. Retinal microglia (MG) were detected by immunohistochemistry. The retinal angiogenesis was analyzed by hematoxylin-eosin (HE) staining and retinal smear. Real-time PCR was performed to detect PPAR- $\gamma$  expression. Western blot was used to study iNOS and Arg-1 expression. Enzyme-linked immunosorbent assay (ELISA) was adopted to detect the secretion of interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-12 (IL-12), interleukin-10 (IL-10), and tumor growth factor- $\beta$  (TGF- $\beta$ ). Compared with the control group, MG expression was elevated, retinal angiogenesis was obvious, leakage was enhanced, no perfusion area was increased, PPAR- $\gamma$  expression was decreased, iNOS expression was significantly upregulated, Arg-1 expression was reduced, IL-1 $\beta$  and IL-12 were strengthened, and IL-10 and TGF- $\beta$  were downregulated in OIR group ( $P < 0.05$ ). However,  $\omega$ -3 PUFAs administration significantly inhibited retinal angiogenesis, reduced leakage, declined no perfusion area, upregulated PPAR- $\gamma$  expression, decreased iNOS expression, elevated Arg-1 expression, downregulated IL-1 $\beta$  secretion, and enhanced IL-10 and TGF- $\beta$  expressions compared with the OIR group ( $P < 0.05$ ).  $\omega$ -3 PUFAs can promote macrophage pro-inflammatory/anti-inflammatory phenotype transition possibly through upregulating PPAR- $\gamma$  expression to effectively inhibit the formation of retinal neovascularization. Using  $\omega$ -3 PUFAs is therefore one of the treatment options for vascular proliferative retinopathy.

**Keywords:**  $\omega$ -3 PUFAs, PPAR- $\gamma$ , microglia, phenotype, retinal neovascularization

## Introduction

As a proliferative retinopathy, retinal neovascular disease is a common pathological change of ischemic and hypoxic fundus diseases, including retinopathy of prematurity, retinal vein occlusion, diabetic retinopathy, and age-related macular degeneration [1, 2]. In recent years, with the changes of living standards and lifestyles, and the aging society, the incidence of diabetes keeps increasing. Therefore, the population of patients with diabetic retinopathy and age-related macular degeneration are increasing rapidly. Retinal neovascular disease brings great mental and economic burden to patients, thus has become one of the serious threats to global health [3, 4]. Pathological changes of retinal neovascularization include leakage, hemorrhage, mechanization, which

finally lead to scar formation of the retina. Massive proliferation of retinal fibrous tissue results in retinal detachment, which causes irreversible damage to vision and even blindness [5, 6]. Oxygen-induced retinopathy (OIR) is one of the common complications of blinding retinopathy. Retinal neovascularization is the main pathogenic mechanism [7]. However, the pathogenesis of oxygen-induced retinal neovascular disease is not fully understood [8]. With the development of gene therapy technology, the clinical strategy of intravitreal injection of anti-vascular endothelial growth factor (VEGF) drugs obtained a certain effect [9, 10]. However, the maintenance of visual acuity requires continuous injection, resulting in high cost and a potential risk of infection. At the same time, it was found that inhibition of VEGF may affect normal retinal vascular development

## $\omega$ -3 PUFAs prevent retinal neovascularization

and induce neuronal dysfunction, which seriously limits the clinical value of anti-VEGF therapy [11, 12]. In-depth investigation of the molecular mechanism of retinal neovascularization is a hot topic in ophthalmology research.

$\omega$ -3 polyunsaturated fatty acids ( $\omega$ -3 PUFAs) are essential fatty acids that are mainly derived from fish oil and are also derived from  $\alpha$ -linolenic acid in vegetable oils [13, 14]. It was found that  $\omega$ -3 PUFAs have a wide range of physiological effects, such as regulating immune response, anti-inflammation, and regulating cardiovascular function [15]. It was shown that supplementation of  $\omega$ -3 PUFAs can effectively compensate for the current clinical anti-VEGF treatment deficiency, and is expected to become a new strategy for the treatment [16]. However, the exact role and molecular mechanism of  $\omega$ -3 PUFAs in inhibiting oxygen-induced retinal neovascularization have not been fully elucidated. Therefore, this study aims to analyze the role of  $\omega$ -3 PUFAs in hypoxia-induced retinal neovascularization.

### Materials and methods

#### *Experimental animals*

60 7-day-old healthy and SPF grade C57BL/6J mice were purchased from the experimental animal center. The mice were divided into several cages and placed in a self-made sealed oxygen chamber with 2 lactating females in each cage. Feeding conditions include constant temperature at  $21 \pm 1^\circ\text{C}$ , relative humidity at 50-70%, 12 h day/night cycle, and free diet and water. All experimental procedures were conducted in strict accordance with the experimental design and performed by experienced technicians to minimize animal suffering. This study was approved by the Ethics Committee of our hospital.

#### *Main reagents and instruments*

Sodium pentobarbital was purchased from Shanghai Zhaohui Pharmaceutical Co., Ltd. Western blot related chemical reagents were from Beyotime. ECL reagents were from Amersham Biosciences. Rabbit anti-mouse iNOS monoclonal antibody, rabbit anti-mouse Arg-1 monoclonal antibody, rabbit anti-mouse CD40, and goat anti-rabbit Horseradish peroxidase (HRP)-labeled IgG secondary antibody were purchased from Cell Signaling. IL-1 $\beta$ , IL-12, IL-10, and TGF- $\beta$  ELISA kits were from R&D. The

RNA extraction kit and the reverse transcription kit were purchased from Axygen. The immunohistochemistry kit was purchased from Boster. The ABI 7700 fast quantitative PCR reactor was from ABI. The Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad Corporation. Ophthalmic microsurgical instruments were purchased from Shanghai Medical Instrument Factory. The VD-650 clean workbench was purchased from Suzhou Purification Equipment Factory. The MDG29 microscope was purchased from Leica. OLYMPUS CH-30-313 Biological Microscope and OLYMPUS BX51 Fluorescence Microscope were purchased from Olympus. Image-Pro Plus 6.0 software was purchased from Olympus. The CY-12C oxygen meter was purchased from Shanghai Medical Instrument Factory.

#### *Animal modeling and grouping*

Sixty healthy C57BL/6J newborn mice were equally and randomly divided into three groups, including control group fed with normal diet, OIR group that was established as oxygen-induced retinopathy (OIR) model, and  $\omega$ -3 PUFAs group that the OIR model mice were given a diet of  $\omega$ -3 PUFAs. In the control group, normal newborn rats and lactating mothers were raised under normal air conditions. In the OIR group, mice and lactating mothers were divided into several cages and placed in a home-made sealed oxygen box with two lactating mothers in each cage. The oxygen flow rate in the oxygen tank was controlled to 0.5~1.0 L/min, and the temperature was maintained at  $25^\circ\text{C}$ . The oxygen concentration in the oxygen chamber was detected 4~6 times per day with CY-12C oxygen meter to ensure the oxygen concentration maintained at  $75 \pm 2\%$ . The growth of the mice and the lactating mothers were observed daily. The lactating mothers were alternately placed in the normal oxygen environment for 6-8 h before being placed back in the oxygen chamber. After 5 days of feeding in high oxygen environment, mice were placed in a normal oxygen environment for 5 days. In the  $\omega$ -3 PUFAs group, OIR model mice were given a diet of  $\omega$ -3 PUFAs. After treatment for 15 days, the sample was extracted and stored in liquid nitrogen.

#### *Fluorescein angiography retinal patch*

After anesthetization, mice were fixed and the heart chamber was opened. Next, the hearts

## $\omega$ -3 PUFAs prevent retinal neovascularization

**Table 1.** Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
PPAR- $\gamma$	TCCAACAGAAGGTC GA	ACATCCCATGCAGAG CTC

55°C for 1 min, 35 cycles of 92°C for 30 s, 58-60°C for 45 s, and 72°C for 35 s. GAPDH was used as an internal control. The relative mRNA expression was calculated using  $2^{-\Delta Ct}$  method.

were perfused with FITC-dextran solution and fixed in 4% paraformaldehyde neutral buffer for 3 h under dark. The corneal limb was cut and the lens and vitreous were removed. The retina was carefully peeled off and placed on a glass slide. Then the retina was radially cut along four quadrants centered on the optic disc. The slide was sealed with anti-fluorescent attenuating reagent followed by observation under a fluorescence microscope. The relative area of the retina without reperfusion and neovascularization was measured by Image-Pro Plus 6.0 software.

### *Immunohistochemistry*

Paraffin sections of mouse retinal tissue received antigen repair after dewaxing. Next, the section was washed with PBS, blocked with goat serum, and incubation at room temperature for 20 min. Then the section was incubated with primary anti-mouse CD40 antibody (dilution 1:1000) at 4°C overnight. After washing with PBS, the section was incubated in biotinylated secondary antibody at 37°C for 30 min and horseradish-labeled streptavidin at 37°C for 30 min. After developed with DAB, terminated with tap water, stained with hematoxylin, dehydrated, and sealed, the section was observed under the microscope.

### *ELISA*

IL-1 $\beta$ , IL-12, IL-10, and TGF- $\beta$  level in serum was measured by ELISA. 50  $\mu$ l diluted standard substance was used to establish standard curve. 50  $\mu$ l sample was added to the plate, washed for five times and incubated with 50  $\mu$ l conjugate reagent at 37°C for 30 min. At last, the OD value was measured at 450 nm. Linear regression equation was established using the OD value of standard substance.

### *Real-time PCR*

Total RNA was isolated using Trizol reagent and reversely transcribed into cDNA. The primers were designed by Primer 6.0 software (**Table 1**). Real-time PCR reaction conditions were:

### *Western blot*

Total protein was isolated using RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, 2  $\mu$ g/ml Aprotinin, 2  $\mu$ g/ml Leupeptin, 1 mM PMSF, 1.5 mM EDTA, 1 mM NaVanadate) and quantified by BCA assay. The proteins were separated on 10% SDS-PAGE, transferred to PVDF membrane, blocked for 1 h, and incubated with iNOS and Arg-1 (1:2000 and 1:1000, respectively) primary antibodies at 4°C overnight. After washing, HRP-conjugated secondary antibody (1:2000) was added and incubated for 30 min under dark, followed by being imaged after addition of chemiluminescence reagent for 1 min. Data were analyzed by image processing system software and Quantity one software. The experiment was repeated for four times.

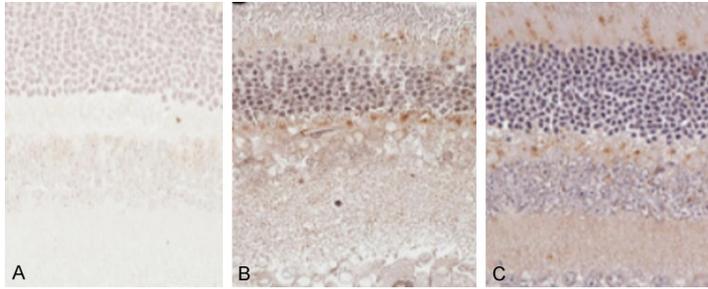
### *HE staining*

The mice were sacrificed by cervical dislocation. The eyeballs were removed and placed in Bouin's solution for 4 h. Next, it was placed in 70% ethanol for fixation. After conventional gradient alcohol dehydration and paraffin-embedding, the sample was cut in sagittal at 4  $\mu$ m thickness with 5 sections per eyeball. The sections with optic nerve sections were not counted. The section was dewaxed in xylene for 10 min  $\times$  2 times, rehydrated in gradient alcohol, and stained in hematoxylin for 15 min. Next, the section was differentiated with 30% hydrochloric acid alcohol for 30 s and bluish with 1% ammonia water. After stained with 1% eosin for 3 min, gradient alcohol dehydration, xylene hyalinization, and neutral gum sealing, observation under light microscope was followed.

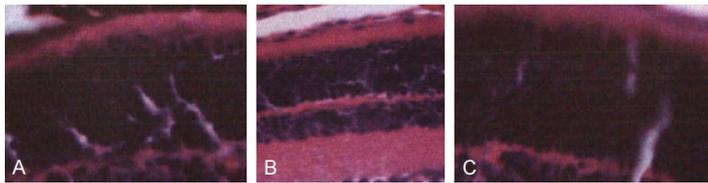
### *Statistical analysis*

Data were analyzed by SPSS 16.0 software. The enumeration data were displayed as percent and compared by chi-square test. The measurement data were shown as mean  $\pm$  standard deviation and compared by one-

## $\omega$ -3 PUFAs prevent retinal neovascularization



**Figure 1.** MG expression in each group ( $\times 10$ ). A. Control; B. OIR group; C.  $\omega$ -3 PUFAs group.



**Figure 2.** Retinal vascular expression in each group ( $\times 100$ ). A. Control; B. OIR group; C.  $\omega$ -3 PUFAs group.

way ANOVA.  $P < 0.05$  indicated a statistical difference.

### Results

#### *$\omega$ -3 PUFAs increases MG expression in retina*

The expression of MG in the retina of each group was analyzed by immunohistochemistry. It was shown that the expression of MG in OIR mice and  $\omega$ -3 PUFAs group was significantly elevated compared with the control group (**Figure 1**), suggesting that MG expression is increased and activated in oxygen-induced retinal neovascular disease. However,  $\omega$ -3 PUFAs administration did not inhibit the expression of MG in oxygen-induced retinal neovascular disease.

#### *$\omega$ -3 PUFAs improves the neovascularization*

HE staining revealed that in the control group, the inner retinal membrane was smooth. There were few endothelial nuclei with only a few retinoic nucleus of the retinal inner retinal membrane. In the OIR group, the vascular endothelial cell nucleus of the intraretinal membrane was observed with proliferated neovascularization.  $\omega$ -3 PUFAs significantly improved the proliferation of neovascularization and reduced the vascular endothelial cell

nucleus in the inner retinal membrane in the OIR group (**Figure 2**).

#### *$\omega$ -3 PUFAs reduce the non-perfused area*

In the control group, retinal blood vessels were derived from the optic disc and distributed radially around, and the blood vessels of the whole retina showed a reticular structure. In the OIR group, the retinal angiogenesis was obvious with significantly enhanced leakage and increased non-perfusion area ( $P < 0.05$ ).  $\omega$ -3 PUFAs treatment significantly reduced the non-perfused area compared with the OIR group ( $P < 0.05$ ) (**Figure 3**).

#### *$\omega$ -3 PUFAs upregulate PPAR- $\gamma$ expression*

Compared with the control group, the expression of PPAR- $\gamma$  in the retina of OIR group was significantly reduced ( $P < 0.05$ ). PPAR- $\gamma$  expression in the  $\omega$ -3 PUFAs group was significantly upregulated compared with the OIR group ( $P < 0.05$ ) (**Figure 4**).

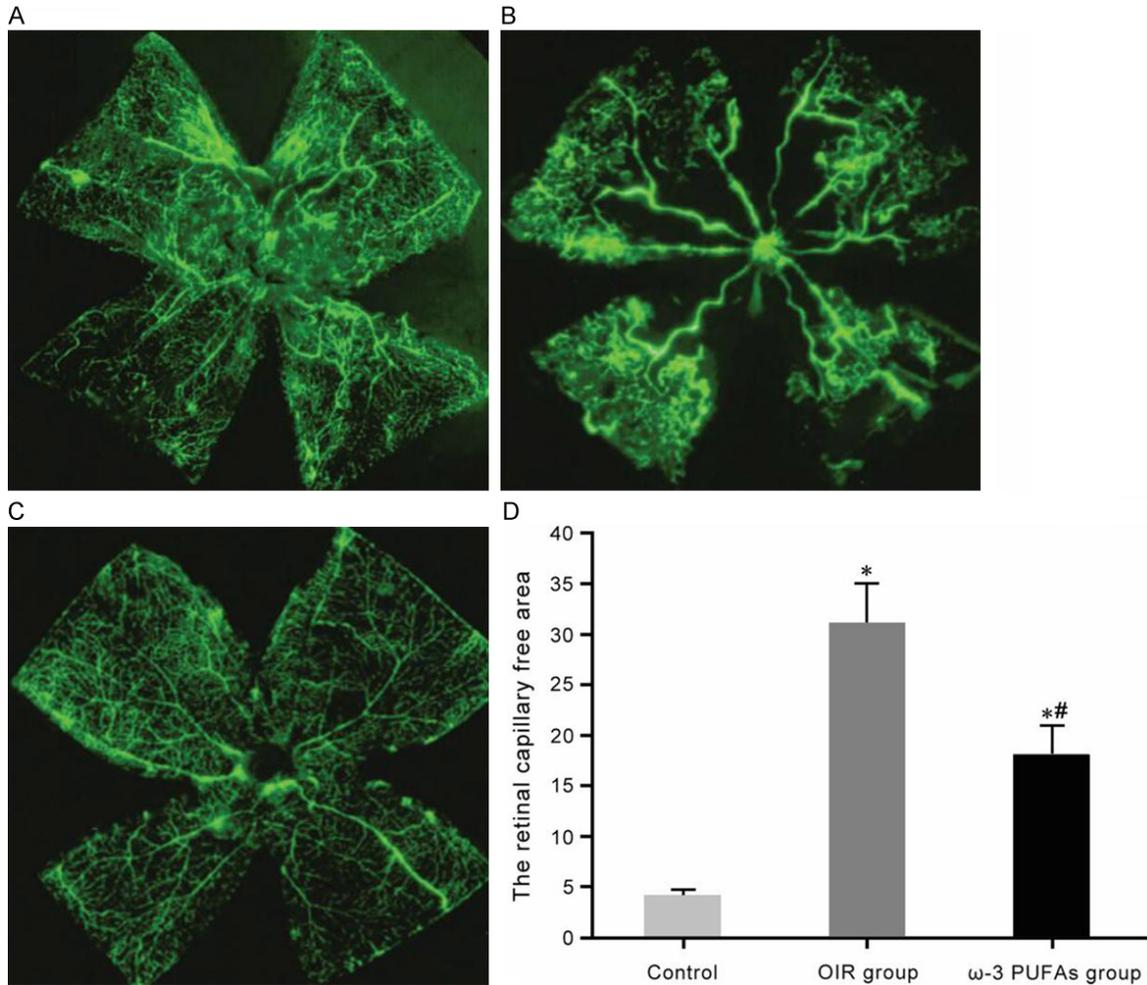
#### *$\omega$ -3 PUFAs reduce iNOS expression and up-regulate Arg-1 expression*

Western blot was adopted to determine proinflammatory phenotype marker iNOS and anti-inflammatory phenotype marker Arg-1 expression in MG cells. It was revealed that compared with the control group, pro-inflammatory phenotype marker iNOS expression was significantly increased in OIR mice, while anti-inflammatory phenotype marker Arg-1 level was significantly declined ( $P < 0.05$ ).  $\omega$ -3 PUFAs administration significantly reduced iNOS expression and upregulated Arg-1 expression compared with OIR group ( $P < 0.05$ ) (**Figure 5**).

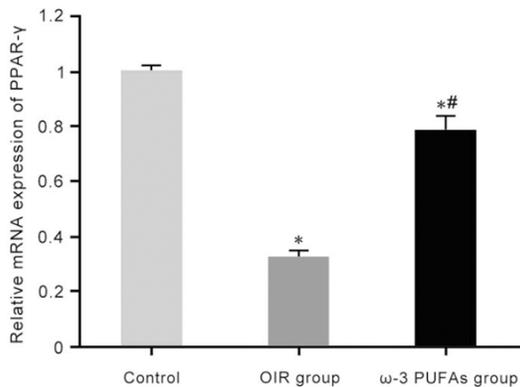
#### *$\omega$ -3 PUFAs decrease IL-1 $\beta$ , IL-12, and increase IL-10, and TGF- $\beta$ secretion*

ELISA analysis demonstrated that inflammatory factors IL-1 $\beta$  and IL-12 increased, whereas anti-inflammatory factors IL-10 and TGF- $\beta$  declined in serum of OIR mice compared with the control group ( $P < 0.05$ ).  $\omega$ -3 PUFAs administration significantly decreased IL-1 $\beta$  and IL-12

## $\omega$ -3 PUFAs prevent retinal neovascularization



**Figure 3.** Fluorescein angiography retinal patch for the observation of retinal blood vessels ( $\times 50$ ). A. Control; B. OIR group; C.  $\omega$ -3 PUFAs group. D. Quantification of the retinal capillary free area. \* $P < 0.05$ , compared with control; \*# $P < 0.05$ , compared with OIR group.



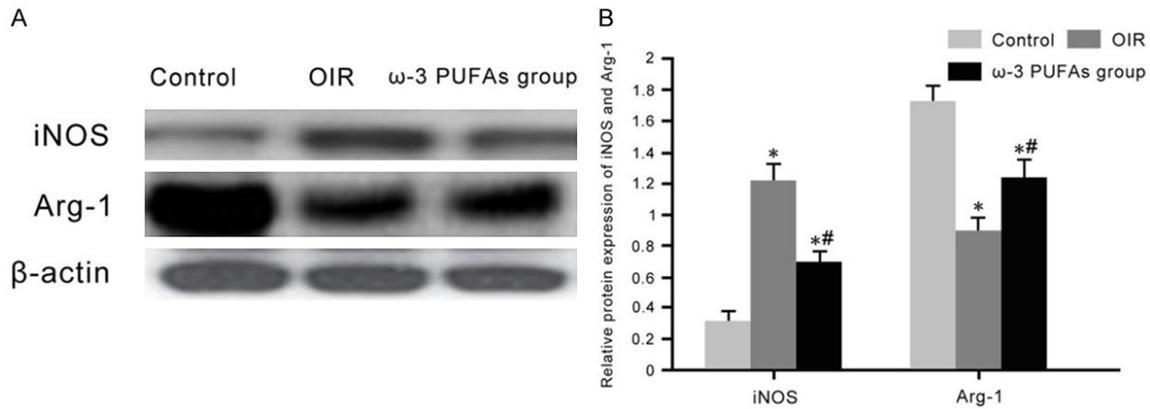
**Figure 4.** PPAR- $\gamma$  expression in each group. \* $P < 0.05$ , compared with control; \*# $P < 0.05$ , compared with OIR group.

secretions, while upregulated IL-10 and TGF- $\beta$  secretions ( $P < 0.05$ ) (Figure 6).

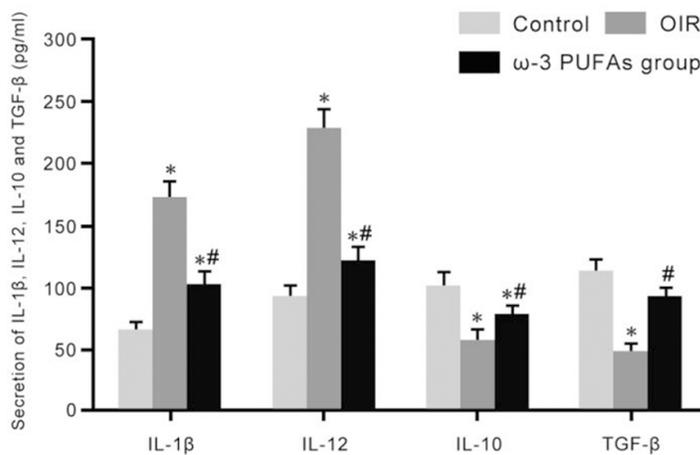
### Discussion

Supplementation of  $\omega$ -3 PUFAs regulates retinal neovascularization, but the specific effects and mechanisms have not been fully elucidated. MG is an immunoreactive cell widely distributed in the central nervous system and plays a key role in mediating the inflammatory response of the central nervous system. It was found that MG is widely distributed in the retina, mainly in the nerve fiber layer, ganglion cell layer, inner plexiform layer, outer plexiform layer, and outer nuclear layer [17].

According to different cell functions, MG can be divided into two types: classical activated type (M1 type) and selective activated type (M2 type) [18, 19]. M1 type MG is pro-inflammatory, which is induced by Th1 type cytokines such as IFN- $\gamma$ , TNF- $\alpha$  and lipopolysaccharide (LPS) or



**Figure 5.** Phenotype marker iNOS and Arg-1 expressions in each group. A. Western blot detection of iNOS and Arg-1 expressions; B. iNOS and Arg-1 expression analysis. \*P < 0.05, compared with control; \*\*P < 0.05, compared with OIR group.



**Figure 6.** IL-1 $\beta$ , IL-12, IL-10, and TGF- $\beta$  secretion analysis. \*P < 0.05, compared with control; \*\*P < 0.05, compared with OIR group.

activated by recognition of pathogen-associated molecular patterns. It triggers an inflammatory response through increasing the secretions of proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-12 [20]. M2 type MG is an anti-inflammatory type, which is activated by Th2 type cytokines such as IL-4, IL-10 and IL-13. It inhibits inflammatory reaction through decreasing the secretions of anti-inflammatory factors TGF- $\beta$ 1 and IL-10. It was suggested that the secretion of TNF- $\alpha$  and the expression and activity of iNOS can be used to identify M1 type MG, while the expression and activity of CD206 and Arg-1 can identify M2 type MG [21]. Activated MG promotes retinal vascular regeneration by exerting anti-inflammatory and neuroprotective effects. Activated MG can facilitate retinal neovascularization and pro-

mote retinal vascular regeneration [22]. Consistent with the role of MG in retinal vascular regeneration, this study confirmed that MG expression was elevated, retinal angiogenesis was obvious, leakage was enhanced, no perfusion area was increased, PPAR- $\gamma$  expression was decreased, iNOS expression was upregulated, Arg-1 expression was reduced, IL-1 $\beta$  and IL-12 were strengthened, and IL-10 and TGF- $\beta$  were downregulated in OIR group. However,  $\omega$ -3 PUFAs administration significantly inhibited retinal angiogenesis, reduced leakage, declined no perfusion area, upregulated PPAR- $\gamma$  expression,

decreased iNOS expression, elevated Arg-1 expression, downregulated IL-1 $\beta$  secretion, and enhanced IL-10 and TGF- $\beta$  expressions compared with the OIR group. It indicated that  $\omega$ -3 PUFAs regulate the phenotype transition of activated MG to inhibit retinal neovascularization.

PPAR- $\gamma$ , belonging to the nuclear hormone receptor superfamily, is a class of ligand-dependent transcriptional regulators. The ligands of PPAR- $\gamma$  are classified into natural ligands and synthetic ligands.  $\omega$ -3 PUFAs can activate PPAR- $\gamma$  expression as the natural ligand. It was reported that PPAR- $\gamma$  has several biological functions, such as regulating fat metabolism, affecting cell differentiation, and inhibiting inflammatory response, while its role in suppressing neovascularization is controver-

sial [23]. It was demonstrated that PPAR- $\gamma$  was involved in the regulation of hypoxia-induced retinal neovascularization [24, 25]. In addition, it was proved that  $\omega$ -3 PUFAs can inhibit the inflammatory response induced by pro-inflammatory phenotype MG through activating PPAR- $\gamma$  [25]. In the present study, we demonstrated that PPAR- $\gamma$  expression is reduced in oxygen-induced retinal neovascularization, whereas administration of  $\omega$ -3 PUFAs activates PPAR- $\gamma$  expression, suggesting that  $\omega$ -3 PUFAs can promote proinflammatory phenotype MG transition into anti-inflammatory phenotype MG possibly through regulating PPAR- $\gamma$  expression, thereby inhibiting hypoxia-induced retinal neovascularization. However, the exact mechanism by which  $\omega$ -3 PUFAs is involved in the regulation of retinal neovascularization requires further investigations. In addition, we plan to explore the role and related mechanisms of  $\omega$ -3 PUFAs in clinical patients, so as to provide a theoretical basis for clinical application.

In conclusion,  $\omega$ -3 PUFAs can promote MG pro-inflammatory/anti-inflammatory phenotype transition possibly through upregulating PPAR- $\gamma$  expression to effectively inhibit the formation of retinal neovascularization, indicating that  $\omega$ -3 PUFAs might be used as one of the treatment options for vascular proliferative retinopathy.

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### Disclosure of conflict of interest

None.

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## $\omega$ -3 PUFAs prevent retinal neovascularization

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