Effect of Interleukin 6 on Scleral Fibroblast Proliferation, Differentiation, and Apoptosis Involved in Myopic Scleral Remodeling

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Abstract
Introduction: Scleral hypoxia (HO) is present in myopic eyes, and interleukin (IL)-6 is increased in the aqueous humor of patients with high myopia. The aim of this study was to investigate the effects of IL-6 on scleral fibroblast proliferation, differentiation, and apoptosis under conditions of HO and the possible role of IL-6 in myopic scleral remodeling. Methods: Primary human scleral fibroblasts (HSFs) were cultured using a tissue mass adherent method. First, cells were cultured under conditions of HO (2% O₂) or normoxia (NO, 20% O₂) for different times. A quantitative real-time polymerase chain reaction (qRT-PCR) and immunofluorescence were used to detect the expression of IL-6 in HSFs. Next, cells were divided into five groups: NO, HO, HO plus IL-6, HO plus IL-6 receptor inhibitor (IL6RI), and HO plus IL-6 and IL6RI. The groups were treated separately for 72 h. Cell counting kit-8 assay and flow cytometry were used to detect cell proliferation and apoptosis, respectively. Western blotting and qRT-PCR were used to detect the expression of various genes in the transforming growth factor-β1/Smad2/matrix metalloproteinase-2 pathway; these methods and immunofluorescence were also used to detect transdifferentiation of HSFs. Results: HO resulted in upregulation of IL-6 expression in HSFs. Compared with NO, HO resulted in diminished cell proliferation and increased apoptosis and differentiation in HSFs; the above trend was further enhanced by the addition of IL6RI. Compared with the HO group, the addition of IL-6 led to a decrease in cell proliferation and an increase in apoptosis and differentiation of HSFs; the above trends showed opposite changes after the addition of both IL-6 and IL6RI. Additionally, IL-6 and IL6RI exerted opposite regulatory effects on the transforming growth factor-β1/Smad2/matrix metalloproteinase-2 pathway under conditions of HO. Conclusion: HO caused HSFs to overexpress IL-6. IL-6 has a role in scleral remodeling in myopic eyes through affecting the proliferation, differentiation, and apoptosis of HSFs.

Introduction
In the past half-century, the prevalence of myopia has been increasing annually. According to recent epidemiological surveys, the prevalence of myopia in the global population will be approximately 49.8% by 2050, and the...
prevalence of high myopia (HM) will be approximately 9.8% [1]. HM is often associated with prolongation of the eye axis and fundus changes and is often complicated by retinal detachment and macular neovascularization. HM is one of the main causes of visual impairment and can lead to blindness [2, 3]. HM develops from myopia, which is associated with excessive lengthening of the ocular axis, leading to scleral thinning, a finding that has been confirmed in previous studies in animal and human models of myopia [4, 5].

The concept of scleral remodeling has been developed as a result of research into the mechanisms of scleral thinning during the development of myopia, and it is believed that scleral remodeling associated with myopia is the synthesis and degradation of the scleral extracellular matrix (ECM), of which type I collagen secreted by scleral fibroblasts (SFs) is the main component. The reduced synthesis or increased degradation of type I collagen owing to various causes can lead to HM [6]. In recent years, it has been proposed that visual signals associated with myopia lead to reduced choroidal capillary permeability and blood flow [7]. The choroid nourishes the adjacent inner sclera, and reduced choroidal capillary permeability and blood flow lead to reduced levels of oxygen and nutrient supply to the sclera, especially the inner sclera, and ultimately to scleral hypoxia (HO). This in turn promotes myofibroblast transdifferentiation and a reduction in collagen synthesis. These changes lead to scleral thinning and weakening, and ultimately to axial hyperextension [8]. In highly myopic eyes, the eyeball is further dilated, the choroid is further thinned, scleral HO is more severe, and scleral remodeling is more intense [9].

A study on inflammatory factors in highly myopic eyes showed higher levels of interleukin 6 (IL-6) and matrix metalloproteinase-2 (MMP-2) in the aqueous humor [10–12]. IL-6 is a cytokine that can be produced by many types of cells, including fibroblasts [13]. IL-6 is involved in inflammatory and immune processes [14], and its synthesis and secretion can be induced under inflammatory conditions [15]. Additionally, MMP-2 is mainly secreted by SFs and is involved in the degradation of various collagen proteins in the sclera, including type I collagen [16]. A study of myocardial HO also demonstrated that HO drives cardiac fibroblasts to overexpress IL-6, and that overexpressed IL-6 plays a role in hypoxic myocardial remodeling through the transforming growth factor (TGF)-β1/Smad2/MMP-2 signaling pathway [17], which has not been addressed in myopia studies.

SFs are the main target cells in the sclera. Maintenance of the stability of the scleral ECM and scleral thickness relies on the secretion of SFs and the production of various substances as well as their own proliferation, transdifferentiation, and apoptosis. According to the above, we hypothesize that HO induces inflammatory changes in SFs that result in overexpression of IL-6, which in turn affects the proliferation, differentiation, and apoptosis of SFs to further influence scleral ECM changes and thereby participate in scleral remodeling. Because IL-6 can be derived from both fibroblasts and other pathways, we examined both autocrine IL-6 and exogenous recombinant IL-6 in human SFs (HSFs) to investigate whether IL-6 plays a role in scleral remodeling by affecting the proliferation, differentiation, and apoptosis of HSFs under conditions of HO. Our findings may provide a reliable basis for the prevention, control, and treatment of myopia, especially HM.

Materials and Methods

Cell Culture

Human scleral tissue with no specific eye disease was obtained from discarded sclera after traumatic eye removal surgery at Yongchuan Hospital, Chongqing Medical University. Human primary SFs were extracted using the tissue block apposition method with in 24 h of sclera isolation. Cells were crawled out in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) containing 20% fetal bovine serum (FBS; Biological Industries, Kibbutz Be’er Haemek, Israel) and 1% penicillin-streptomycin. The medium was changed every 3 days until SFs had grown from the tissue blocks. The medium was then changed to 10% FBS and culture and passing were continued. After identification with wave proteins and keratin, fibroblasts from the fourth to eighth passages were used in this experiment.

Cell Treatments and Groups

HSF cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, and cell passage was performed when cell density reached 80%–90%. First, based on previous studies related to cellular HO [18–20], cells were divided into a normoxia (NO) group with 20% O2 and a HO group with 2% O2. The medium was replenished on the day following passaging. Cells were then placed into a conventional CO2 incubator with pre-adjusted parameters of a hypoxic tri-gas incubator (Heal Force, Shanghai, China) for 24 h, 48 h, and 72 h to determine the optimal hypoxic effect time of IL-6 secretion by HSFs. Next, recombinant IL-6 (1 ng/mL; R&D Systems, Minneapolis, MN, USA) and the IL-6 receptor inhibitor (IL6Ri) tocilizumab (10 ng/mL; Selleck Chemicals, Houston, TX, USA) were either added or not added to the different groups of media according to the grouping. Cells were grouped into NO, HO, HO plus IL-6 (HO+IL-6), HO plus IL6Ri (HO+IL6Ri), and HO plus IL-6 and IL6Ri (HO+IL-6+IL6Ri). Each group was placed in the corresponding incubator for 72 h. Other target genes were then tested.
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Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from HSFs using Trizol reagent, according to the manufacturer’s instructions. Reverse transcription was performed with the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Tokyo, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with TB Green® Premix Ex Taq™ II (Takara). The primer sequences used in this study are shown in Table 1. Target gene expression was normalized using the 2-ΔΔCt method from the Ct values of the respective mRNAs relative to the housekeeping gene β-actin.

Western Blotting

Proteins of HSFs were extracted in RIPA lysis buffer containing phosphatase inhibitors and protease inhibitors. In total, 30 μg of proteins were separated on SDS-PAGE gels and then transferred to polyvinylidene fluoride membranes. After blocking with tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% skim milk powder for 1 h at room temperature, the membranes were incubated with diluted primary antibodies (TGF-β1, Smad2, p-Smad2, α-smooth muscle actin (SMA), MMP-2; Abcam, Cambridge, MA, USA) and α-tubulin (Beyotime, Shanghai, China) overnight at 4°C. After rinsing three times with TBST, the membranes were incubated with Goat Anti-Rabbit IgG H&L (HRP; Abcam) for 1 h at room temperature. After rinsing three times with TBST, Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA) was used for chemiluminescence detection. The immunoreactive bands were analyzed in triplicate using Image Pro Plus software, Version X (Media Cybernetics, Silver Spring, MD, USA).

Immunofluorescence

Cells were inoculated into 24-well plates, fixed for 10 min, and then permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Cells were incubated with diluted primary antibodies (vimentin, keratin, IL-6, and α-SMA; Abcam, Cambridge, MA, USA) and α-tubulin (Beiyotime, Shanghai, China) overnight at 4°C. After rinsing three times with TBST, the membranes were incubated with Goat Anti-Rabbit IgG H&L (HRP; Abcam) for 1 h at room temperature. After rinsing three times with TBST, Immunoblot Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA) was used for chemiluminescence detection. The immunoreactive bands were analyzed in triplicate using Image Pro Plus software (Version X; Media Cybernetics, Silver Spring, MD, USA).

Flow Cytometry

An Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) was used for apoptotic cell determination with a CytoFLEX flow cytometer (Beckman Coulter, Miami, FL, USA), according to the manufacturer’s instructions. The percentage of apoptotic cells was defined as the sum of the percentages of early and late apoptotic cells.

Cell Counting Kit-8 Assay

After resuspension, approximately 3 × 10^4/100 μL/well cells were incubated in 96-well plates for 0 h, 24 h, 48 h, and 72 h. Cell counting kit-8 regent (Dojindo, Kumamoto, Japan) was used for cell viability assays, following the manufacturer’s protocol. The optical density was read at 450 nm with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

The Student’s t test or one-way analysis of variance was performed to compare differences among groups. p values <0.05 were considered statistically significant. Continuous variables are presented as mean ± standard error of the mean. GraphPad Prism software was used for the statistical analysis in this study (GraphPad Software Inc, San Diego, CA, USA).

Results

Isolation and Identification of Primary HSFs

Primary HSFs were cultivated following a tissue block culture protocol (Fig. 1a). All the HSFs were derived from the scleral tissue of the same eye. Spindle-shaped cells were typically fibroblasts. Primary HSFs could grow to confluence in approximately 3–4 weeks after crawling out of the tissue mass. To identify SFs, immunofluorescence imaging was used. As shown in Figure 1b, vimentin (+) keratin (−) cells were considered HSFs.

HSFs Express IL-6 and HO Increases IL-6, TGF-β1, Smad2, and MMP-2 Expression

We first confirmed that HSFs expressed IL-6 itself, and then evaluated the trend of HSFs’ own IL-6 expression under HO conditions. HSFs were cultured under HO conditions for 24 h, 48 h, and 72 h. To normalize the expression levels of IL-6 under different HO time treatments, levels in the NO culture were used as controls. qRT-PCR (Fig. 2a) and immunofluorescence (Fig. 2b) showed that HSFs expressed IL-6 by themselves, and the expression of IL-6 was upregulated after different HO time treatments, with the greatest upregulation of IL-6 at 72 h of HO. We also found that HO treatment upregulated TGF-β1, Smad2, and MMP-2 simultaneously, and the upregulation trend was consistent with that of IL-6.

Table 1. Oligonucleotide primers used in qRT-PCR

| Human genes | Sequences |
|-------------|-----------|
| IL-6        | Forward:(5′–3′) CCTGAACCTTCCAAAGATGCC |
| TGF-β1      | Reverse:(5′–3′) TTACACGGCAAGGTCTCCTCA |
| Smad2       | Forward:(5′–3′) GAATTCCTGGGATACCTCAG |
| MMP-2       | Reverse:(5′–3′) GCACAATCGGGTGACATCAA |
| α-SMA       | Forward:(5′–3′) CTCAAGCTCATCTACTGTCG |
| β-Actin     | Reverse:(5′–3′) CATCACGGAACCTGAATCTT |

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Effect of IL-6 on HSF Proliferation under HO Conditions

Previous studies have shown that the IL6RI tocilizumab can specifically bind to the IL-6 receptor (IL6R), thereby affecting the biological effects of IL-6 [21]. To determine whether IL-6 had an effect on the proliferation of HSFs under conditions of HO, we divided the cells into five groups (NO, HO, HO+IL-6, HO+IL6RI, and HO+IL-6+IL6RI) and incubated them for 0 h, 24 h, 48 h, and 72 h. We added the cell counting kit-8 reagent and assessed cell proliferation using optical density readings with an enzyme marker. As shown in Figure 3, the different treat-
Fig. 2. Expression of IL-6 and its downstream genes at different times under HO compared with NO. (a) Expression levels of IL-6, TGF-β1, Smad2, and MMP-2 mRNA. (b) Expression level of IL-6 (1:50) protein. Results are presented as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 versus NO; *p < 0.05, **p < 0.01, ***p < 0.001 versus different hypoxic times. Scale bar, 50 μm. IL, interleukin; TGF, transforming growth factor; MMP, matrix metalloproteinase; SEM, standard error of the mean.
ment groups were read once at each of the same time points using an enzyme marker. The results showed that HO inhibited the proliferation of HSFs as compared with NO. The addition of recombinant IL-6 under HO conditions further inhibited cell proliferation compared with the HO group alone, whereas the simultaneous addition of IL-6 and IL6RI weakened the inhibitory effect produced by the addition of IL-6 alone. Unexpectedly, the proliferation of HSF cells was further inhibited by the addition of IL6RI under HO conditions compared with the HO group alone, suggesting that autocrine IL-6 from HSFs under HO conditions has a protective effect on cell proliferation under conditions of HO.

Effect of IL-6 on Transdifferentiation of HSFs under HO Conditions

To test the effect of IL-6 on cellular transdifferentiation of HSFs under HO conditions, HSFs were divided into five experimental groups, as above. α-SMA is a marker protein of myofibroblasts and was also used in this study as a marker of SF transdifferentiation to myofibroblasts. HSFs were cultured under NO and HO conditions for 72 h, and then the mRNA and protein changes of α-SMA were analyzed using qRT-PCR (Fig. 4a), western blotting (Fig. 4b), and immunofluorescence (Fig. 4c). As shown in Figure 4, the expression of α-SMA was upregulated by HO compared with NO, and the addition of IL-6Ri under HO conditions further upregulated the expression of α-SMA. This indicated that autocrine IL-6 from HSFs under HO inhibits cellular transdifferentiation. Compared with the HO group alone, the addition of recombinant IL-6 under HO conditions further upregulated expression of the above genes, whereas the addition of both IL-6 and IL6RI attenuated the upregulation caused by IL-6 alone. Thus, exogenous IL-6 showed a promoting effect on the transdifferentiation of HSFs.

Effect of IL-6 on HSF Apoptosis under HO Conditions

To determine whether IL-6 affects the apoptosis of HSFs under conditions of HO, each of the above five subgroups was cultured for 72 h; Annexin V/propidium iodide staining and flow cytometry were performed. The results showed (Fig. 5) that HO increased apoptosis in HSFs as compared with NO, and apoptosis was further increased by the addition of IL6RI alone under HO conditions, suggesting that HO prompted the autocrine IL-6 of HSFs to inhibit its own apoptosis. The addition of IL-6 under HO conditions further increased apoptosis compared with the HO group, but the addition of IL-6 and IL6RI attenuated this effect caused by IL-6. Thus, exogenous IL-6 showed a pro-apoptotic effect on apoptosis in HSFs.

Effect of IL-6 on TGF-β1/Smad2/MMP-2 Pathway under HO Conditions

To test whether IL-6 could activate the expression of TGF-β1-related signaling molecules under HO conditions, HSFs were divided into five experimental groups:
Fig. 4. Effect of IL-6 on the transdifferentiation of HSFs under hypoxic conditions. a qRT-PCR analysis of mRNA expression of α-SMA. b Cells subjected to immunoblot analysis using an antibody directed against α-SMA (1:10,000). c Cells analyzed via immunofluorescence staining using an antibody directed against α-SMA (1:300). Results presented as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 versus NO; *p < 0.05, **p < 0.01, ***p < 0.001 versus HO. Scale bar, 50 μm. HSF, human scleral fibroblast; IL, interleukin; qRT-PCR, quantitative real-time polymerase chain reaction; SMA, smooth muscle actin; SEM, standard error of the mean.
NO, HO, HO+IL-6, HO+IL6RI, and HO+IL-6+IL6RI. HSFs were incubated under NO and HO conditions for 72 h and then analyzed using qRT-PCR and western blotting. We examined the mRNA and protein expression of each pathway gene in the HSFs. As shown in Figure 6, HO activated the TGF-β1/Smad2/MMP-2 pathway in comparison with NO; this pathway was further activated by the addition of IL6RI under HO conditions. The addition of exogenous recombinant IL-6 further activated the TGF-β1/Smad2/MMP-2 pathway compared with the HO group, whereas the addition of both IL-6 and IL6RI attenuated this activation induced by the addition of IL-6 alone. This indicated that either HO promoted autocrine IL-6 from HSFs or the addition of exogenous recombinant IL-6 can have an effect on the TGF-β1/Smad2/MMP-2 pathway, with the former showing inhibition and the latter showing promotion.

**Discussion**

Various studies have shown that scleral ECM remodeling plays a key role in the development of HM [22]. Our data showed that HO promoted overexpression of IL-6 by HSFs, suggesting that scleral HO may lead to inflammatory changes in the sclera. We demonstrated that IL-6 plays a role in scleral remodeling in myopic eyes by influencing the proliferation, differentiation, and apoptosis of HSFs in a manner that affects scleral ECM remodeling. To our knowledge, the present study is the first to explore the relationship between inflammatory factors and scleral remodeling in myopic eyes, formally incorporating inflammatory factors into the etiology of myopia.

The results of our study showed that IL-6 has a bidirectional regulatory effect on the proliferation, differen-
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**Fig. 6.** IL-6 induces activation of the TGF-β1/Smad2/MMP-2 signaling pathway under hypoxic conditions. Expression of target gene detected after treatment for 72 h under different grouping conditions. 

a–c qRT-PCR analysis for mRNA expression of TGF-β1, Smad2, and MMP-2. 

Cells underwent immunoblot analysis using antibodies directed against TGF-β1 (1:1,000), Smad2 (1:2,000), pSmad2 (1:1,000), and MMP-2 (1:1,000). Results presented as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 versus NO; #p < 0.05, ##p < 0.01, ###p < 0.001 versus HO. 

HSF, human scleral fibroblast; IL, interleukin; TGF, transforming growth factor; MMP, matrix metalloproteinase; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean.

**tiation, and apoptosis of HSFs under conditions of HO.** SF autocrine IL-6 might have a protective effect on SFs themselves under HO conditions by promoting proliferation and inhibiting apoptosis and transdifferentiation, whereas recombinant IL-6 has a negative effect on cells under HO conditions by promoting apoptosis, promoting transdifferentiation, and inhibiting proliferation. It is well known that during wound healing, myofibroblasts exhibit a pro-fibrotic phenotype with upregulated expression of ECM components, such as fibronectin and collagen [23]. Unlike transient myofibroblasts observed during wound healing, myofibroblasts that transdifferentiate during myopia development express lower levels of Col1a1 and Col1a2, which may be because the sclera does not contain blood vessels [8]. In the sclera, type I collagen is mainly synthesized and secreted by SFs. When IL-6, other than cellular autocrine IL-6, inhibits the proliferation of fibroblasts and promotes their apoptosis and transdifferentiation into myofibroblasts, the result is a decrease in the total number of HSFs that secrete more type I collagen; this will eventually lead to a decrease in the total amount of type I collagen synthesized in the sclera and thinning and remodeling of the sclera.

The two-sided effect of IL-6 that occurs under HO conditions may be related to two pathways of biological signaling by IL-6, namely, classical signaling and trans-signaling [24]. In classical signaling, IL-6 first binds to the membrane-bound non-signaling IL6R on the target cell membrane to form a complex, and then binds to the membrane glycoprotein gp130 subunit to form a signaling complex that transmits signals to target cells [25, 26]. In humans, IL6R is also present in a soluble form (sIL6R), and sIL6R can bind to IL-6 to form a complex that stimulates gp130-expressing cells and initiates subsequent signaling [27]. Thus, even in the absence of membrane-bound non-signaling IL6R, IL-6 signaling can still be transmitted; this process of IL-6 and sIL6R is known as trans-signaling [28]. It is generally believed that classical IL-6 signaling is involved in the anti-inflammatory and regenerative properties of IL-6, but IL-6 trans-signaling...
is thought to be the pro-inflammatory response induced by this cytokine [26, 29]. We speculate that when the sclera is in a state of HO, autocrine IL-6 from fibroblasts and from atrial aqueous or scleral-adjacent tissue cells may play different roles in myopic scleral ECM remodeling through different delivery pathways. Taken together, reducing IL-6 from sources other than SFs could increase type I collagen synthesis and reduce collagen breakdown by MMP-2, thereby controlling the onset and progression of myopia. Current findings on chronic inflammatory factors in highly myopic eyes also show increased levels of IL-6 in the aqueous humor [10, 12]. Elevated IL-6 in the aqueous humor can originate from any cell in the eye that secretes IL-6, such as retinal pigment epithelial cells [30] and microglia [31] in the retina. HSFs like cardiac fibroblasts can also secrete IL-6 [17].

Vogt–Koyanagi–Harada (VKH) disease is a bilateral uveitis disease in which the primary site of inflammation in the eye is the choroid. One study showed that choroidal thinning is present during VKH disease recovery, as in pathological myopia, and VKH disease shows progression of myopia and an increase in the ocular axis during the recovery period [32]. Although it remains unclear whether choroidal thinning in highly myopic eyes is a cause or a consequence of increased ocular axis, it links inflammation, choroidal thinning, and increased ocular axis in HM and indirectly provides strong evidence for our study.

Our results suggested that IL-6 has both promoting and inhibitory effects on scleral remodeling in myopic eyes under HO conditions. HSFs oversecreted IL-6 under HO conditions and showed an indirect protective effect on scleral ECM thinning and scleral remodeling, whereas exogenous recombinant IL-6 exhibited the opposite effect. All these effects were mediated by influencing the value-added differentiation, apoptosis, and secretion of MMP-2 by SFs. These results suggest that drugs that inhibit the IL-6 retransmission signaling pathway, as well as those that improve scleral HO (oxygen administration or anti-hypoxic drugs), may be potential targets for the treatment of scleral remodeling in highly myopic eyes. Additionally, IL-6 family members exert different functions via their respective specific receptors, which may not exist simultaneously or may not be spatially restricted [24]. Therefore, an in-depth study of IL-6 and its family members and their signaling pathways is important for a comprehensive understanding of myopia and the development of more effective drugs.

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Statement of Ethics

This study was approved by the Medical Ethics Committee of Yongchuan Hospital affiliated to Chongqing Medical University Hospital (approval No. 2020103). All procedures followed the tenets of the Declaration of Helsinki, and written consent was obtained from all patients.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All the authors participated in the design, interpretation of the studies and analysis of the data, and review of the manuscript. Ling Liu, Yujie Fan, and Liming Zhang conducted the experiments. Wenjun Zhou, Shichun Liu, and Hua Li provided the key idea for the experiment. Ling Liu analyzed the data and wrote the manuscript. Shengfang Song and Hua Li critically revised the manuscript for intellectual content. All the authors read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed in this study are included in this article. Further inquiries can be directed to the corresponding author.
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