• Basic Research •

Effects of autophagy inhibitor 3-methyladenine on a diabetic mice model

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Abstract

● **AIM:** To investigate the role of autophagy inhibitor 3-methyladenine (3-MA) on a diabetic mice model (DM) and the potential mechanism.

● **METHODS:** Male C57BL/6J mice were randomly divided into a normal control group (NC group) and an DM group. DM were induced by multiple low-dose intraperitoneal injection of streptozotocin (STZ) 60 mg/kg•d for 5 consecutive days. DM mice were randomly subdivided into untreated group (DM group), 3-MA (10 mg/kg•d by gavage) treated group (DM+3-MA group) and chloroquine (CQ; 50 mg/kg by intraperitoneal injection) treated group (DM+CQ group). The fasting blood glucose (FBG) levels were recorded every week. At the end of experiment, retinal samples were collected. The expression levels of pro-apoptotic proteins cleaved caspase-3, cleaved poly ADP-ribose polymerase 1 (PARP1) and Bax, anti-apoptotic protein Bcl-2, fibrosis-associated proteins Fibronectin and type 1 collagen α1 chain (COL1A1), vascular endothelial growth factor (VEGF), inflammatory factors interleukin (IL)-1β and tumor necrosis factor (TNF)-α, as well as autophagy related proteins LC3, Beclin-1 and P62 were determined by Western blotting. The oxidative stress indicators 8-hydroxydeoxyguanosine (8-OHdG) and malondialdehyde (MDA) were detected by commercial kits.

● **RESULTS:** Both 3-MA and CQ had short-term hypoglycemic effect on FBG and reduced the expression of VEGF and inflammatory factors IL-1β and TNF-α in DM mice. 3-MA also significantly alleviated oxidative stress indicators 8-OHdG and MDA, decreased the expression of fibrosis-related proteins Fibronectin and COL1A1, pro-apoptotic proteins cleaved caspase-3, cleaved PARP1, as well as the ratio of Bax/Bcl-2. CQ had no significant impact on the oxidative stress indicators, fibrosis, and apoptosis related proteins. The results of Western blotting for autophagy related proteins showed that the ratio of LC3 II/LC3 I and the expression of Beclin-1 in the retina of DM mice were decreased by 3-MA treatment, and the expression of P62 was further increased by CQ treatment.

● **CONCLUSION:** 3-MA has anti-apoptotic and anti-fibrotic effects on the retina of DM mice, and can attenuate retinal oxidative stress, VEGF expression and the production of inflammatory factors in the retina of DM mice. The underlying mechanism of the above effects of 3-MA may be related to its inhibition of early autophagy and hypoglycemic effect.

● **KEYWORDS:** diabetic mellitus; 3-methyladenine; autophagy; fibrosis; apoptosis; mice
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INTRODUCTION

The number of patients with diabetes and diabetic retinopathy (DR) is increasing dramatically worldwide[1-2]. According to the report of Khan *et al*[2] in 2020, diabetes has become the ninth cause of human death and is expected to affect 693 million adults in 2045, which means that one in ten people worldwide are living with diabetes.

According to recent reports, about one in five people with diabetes will develop DR[3].

DR is the main cause of blindness in middle-aged and elderly people, and is one of the most hidden diabetic microvascular lesions. Most patients have severe pathological changes when they are discovered[4]. The pathological changes of DR include retinal capillary aneurysms, hemorrhagic spots, rigid exudates, cotton wool spots, beading veins, macular edema, abnormal formation of retinal microvessels, and fibrosis[5]. Chronic hyperglycemia can lead to increased production of advanced glycation end products (AGEs) and vascular endothelial growth factor (VEGF), and activation of polyols, hexosamine and protein kinase C pathways, leading to retinal oxidative stress, inflammation, neovascularization, and pericyte apoptosis[6]. There are two types of DR, proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR)[7]. PDR is more harmful to vision and can lead to severe vision loss or even complete blindness compared with NPDR[7], and the current clinical strategy for the treatment of PDR is still anti-VEGF[8]. However, a recent long-term follow-up study found that anti-VEGF therapy can lead to retinal fibrosis[9-10]. Therefore, it is necessary to explore a therapeutic strategy that is both anti-VEGF and anti-fibrosis.

Autophagy is an evolutionarily highly conserved cellular process and a subcellular membrane rearrangement process. When cells suffer from hypoxia, lack of nutrition (such as in starvation) and are in a harmful environment (such as misfolded proteins, damaged organelle or microbial invasion), certain components are encapsulated by the double-membrane autophagic vesicles and transported to the lysosome for fusion, which are then degraded by lysosomal hydrolase and released to cytoplasm for recycling[11]. Apoptosis of retinal Müller cells (rMCs) and pericytes is another important pathological feature of DR[12]. Recently, it has been reported that autophagy is closely related to the apoptosis of rMCs and pericytes in DR, under severe stress of high glucose, autophagy up-regulation promotes the death of retinal rMCs and pericytes[13-14].

3-Methyladenine (3-MA) is a common autophagy inhibitor, which inhibits autophagy by inhibiting class III phosphatidylinositol 3-kinase (PI3K)[15]. Recently, studies from Bo *et al*[15] showed that 3-MA inhibits the polarization of macrophages toward M2 phenotype by targeting the PI3K/Akt signaling pathway, thereby exerting an anti-fibrotic effect on the experimental subretinal fibrosis. Moreover, Wang and Wu[16] reported that 3-MA could inhibit retinal apoptosis in the rat model of ischemic-reperfusion injury. However, it's unclear whether 3-MA has anti-fibrotic and anti-apoptotic effects on diabetic retina. Therefore, this study intends to explore the effect of 3-MA on retinal fibrosis and apoptosis as well as oxidative stress, VEGF expression and inflammatory factors in diabetic mice. In addition, we also used autophagy inhibitor chloroquine (CQ) as a reference drug to investigate the mechanism of 3-MA on the retina of diabetic mice.

MATERIALS AND METHODS

Ethical Approval The experiment was carried out according to the protocols approved by the Institutional Animal Ethics Committee (IAEC). The study was approved by the Committee on the Ethics of Animal Experiments of the Medical College of Yangtze University (No.CJYXBEC2019-083) and was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antibodies and Reagents 3-MA was purchased from Abmole Bioscience (Houston, TX, USA). CQ was purchased from Meilunbio (Dalian, Liaoning Province, China). Bicinchoninic acid (BCA) protein assay kit and malondialdehyde (MDA) assay kit were purchased from Beyotime (Shanghai, China). Glycated serum protein (GSP) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China). The enzyme-linked immunosorbent assay (ELISA) kits for 8-hydroxydeoxyguanosine (8-OHdG) was purchased from Elabscience (Wuhan, Hubei Province, China). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Bioss (Beijing, China). Anti-VEGF antibody was purchased from ABclonal Technology (Wuhan, Hubei Province, China). Antibodies to COL1A1, cleaved caspase-3 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies to Beclin-1, Fibronectin were purchased from Proteintech Group, Inc. (Rosemont, IL, USA). Antibodies to LC3, SQSTM1/P62, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, cleaved PARP1, Bcl-2, Bax were purchased from Abcam (Cambridge, MA, UK). Secondary antibodies for Western blotting, and streptozotocin (STZ) were purchased from Sigma (St. Louis, MO, USA).

Experimental Animals Male C57BL/6J mice weighing 18–22 g were obtained from the Experimental Animal Center of Three Gorges University (Yichang, Hubei Province, China) and housed at 22°C, 50% humidity with a 12h-light/12h-dark cycle with unlimited access to water and food. The 24h water intake of the mice were recorded weekly.

Induction of Diabetes Diabetic mouse model was established using our previous method[17], which was slightly improved. In brief, the type 1 diabetic mouse model was induced by multiple low-dose intraperitoneal injection of STZ solution (prepared in pre-chilled citrate buffer pH 4.5 at a dose of 60 mg/kg•d for 5 consecutive days). The mice in normal control group (NC group) were injected with equal amount of citrate buffer. Blood glucose was measured two weeks after the first intraperitoneal injection of STZ solution. Mice with blood glucose higher than 16.7 mmol/L (250 mg/dL) were considered diabetic and used for further studies.

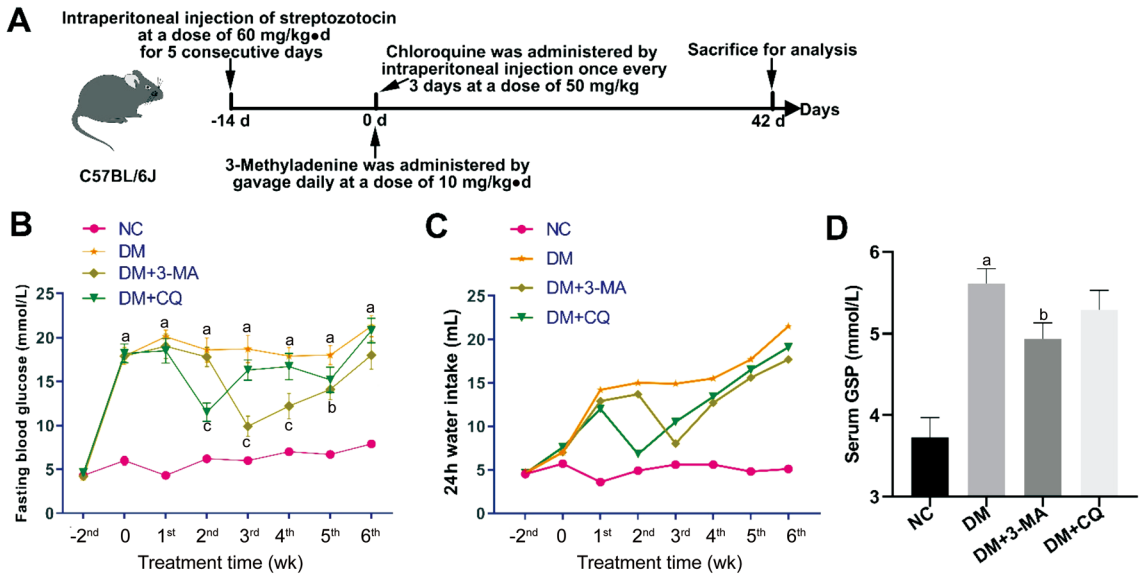

Figure 1 3-MA had a short-term hypoglycemic effect on FBG in STZ-induced diabetic mice A: Schematic diagram of animal experimental design; B: Trend graph of weekly FBG; C: Trend chart of 24h water intake per week; D: Serum GSP detected by a commercial biochemical assay kit. All data are represented as mean±SEM, $^{a}P<0.001$ *vs* the NC group; $^{b}P<0.05$, $^{c}P<0.001$ *vs* the DM group. FBG: Fasting blood glucose; 3-MA: 3-methyladenine; STZ: Streptozotocin; DM: Diabetes mice model; NC: Normal control; CQ: Chloroquine; SEM: Standard error of mean; GSP: Glycated serum protein.

Treatment of Animals Diabetic mice were randomly divided into diabetes mice model untreated group (DM group), 3-MA treated group (DM+3-MA group) and DM+CQ group, and were raised under the same conditions as the non-diabetic mice in NC group. The DM+3-MA group were given 3-MA aqueous solution (10 mg/kg•d) by gavage; the DM+CQ group were given CQ aqueous solution (50 mg/kg) by intraperitoneal injection every 3d; and the NC and DM groups were given equal amount of normal saline (Figure 1A).

Fasting Blood Glucose The fasting blood glucose (FBG) levels were recorded every week. Fasting was started from 21:00 the day before testing FBG, and the FBG levels were measured after fasting for 10h using Accu-chek Performa (Roche, Germany). Approximately 3 μL of blood was collected in conscious mice *via* tail vein puncture.

Serum Glycated Serum Protein Assay After 6wk of treatment with 3-MA or CQ, blood samples were collected into the tubes without anticoagulants *via* enucleation of the eye, and centrifuged at 3000 rpm/min for 10min to obtain the serum. Serum glycosylated serum protein (GSP) was determined using commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Assessment of Oxidative Stress Index The retinal samples were ground and homogenized. The concentrations of 8-OHdG and MDA in retinal tissues were detected by commercial kits according to the manufacturer's instructions, and the final levels of 8-OHdG and MDA were normalized to the protein concentration of retinal tissue homogenate.

Western Blotting The retinal samples were homogenized in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing 1% protease inhibitors for 10min. The homogenate was centrifuged at 12 000 rpm/min for 30min at 4°C, and the protein concentration in supernatant was quantified using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein lysate (40 μg/well) were separated by electrophoresis in 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V and transferred to polyvinylidene fluoride (PVDF) membrane (Sigma, St. Louis, MO, USA) at 200 mA for 120min. Membranes were blocked in 5% nonfat milk for 2h at room temperature, and then incubated with primary antibody overnight at 4°C. Then, the membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Kerui Biotechnology, Wuhan, Hubei Province, China) at room temperature for 1–2h and imaged using a Bio-Rad ChemiDoc MP chemiluminescence imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA) with enhanced chemiluminescence (ECL) developer, and the image data was then imported into Image J for additional analysis. The expression level of each protein was normalized to GAPDH on the same blot.

Statistical Analysis Data were represented as mean±standard error of mean (mean±SEM). IBM SPSS statistics 25 (Version X; IBM, Armonk, NY, USA) was used for statistical analysis. Multiple group comparisons were conducted using one-way ANOVA, and comparisons between two groups were conducted using Student's *t*-test. $P<0.05$ was considered statistically significant.

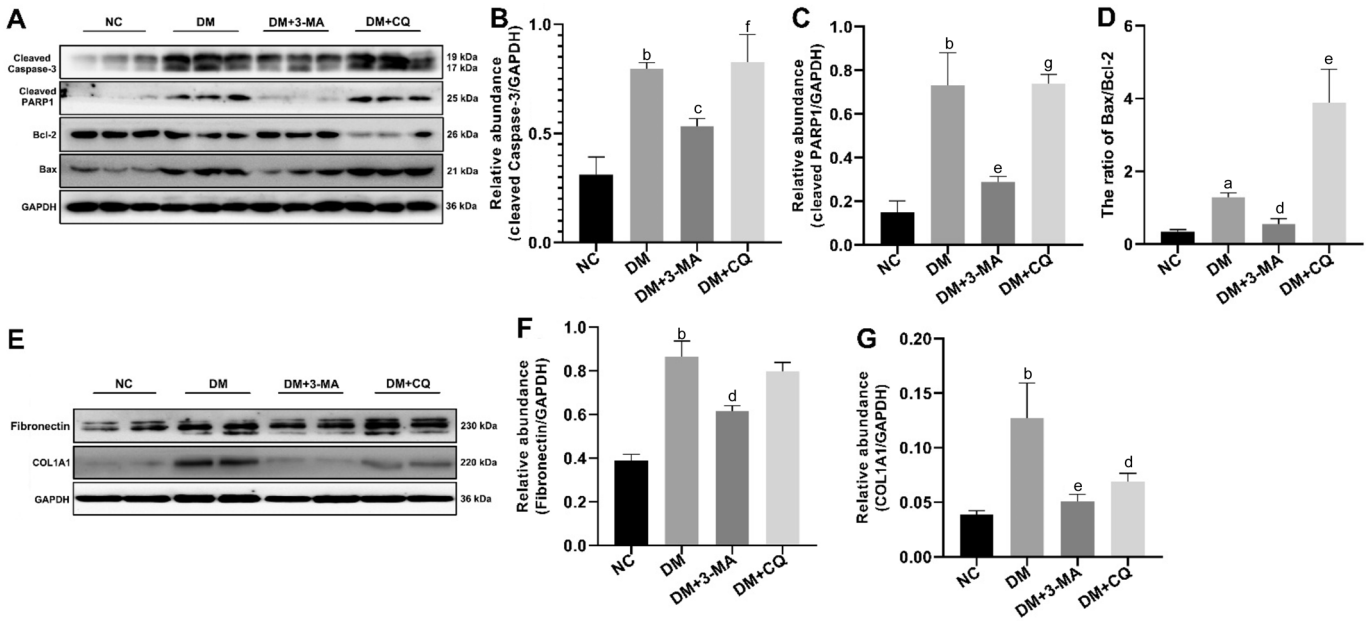

Figure 2 3-MA reduced the expression of apoptosis-related proteins and fibrosis-associated proteins in the retina of STZ-induced diabetic mice A: Retinal tissue lysates were subjected to immunoblot analysis with antibodies to cleaved caspase-3, cleaved PARP1, Bcl-2, Bax, and GAPDH; B: Expression level of cleaved caspase-3 was quantitated by densitometry and normalized with GAPDH; C: Expression level of cleaved PARP1 was quantitated by densitometry and normalized with GAPDH; D: Expression levels of Bcl-2 and Bax were calculated by densitometry and the ratio of Bax/Bcl-2 was determined; E: Retinal tissue lysates were subjected to immunoblot analysis with antibodies to Fibronectin, COL1A1, and GAPDH; F: Expression level of Fibronectin was quantitated by densitometry and normalized with GAPDH; G: Expression level of COL1A1 was quantitated by densitometry and normalized with GAPDH. All data were represented as mean±SEM. $^{a}P<0.01$ and $^{b}P<0.001$ *vs* the NC group; $^{c}P<0.05$, $^{d}P<0.01$, $^{e}P<0.001$ *vs* the DM group; $^{f}P<0.05$, $^{g}P<0.001$ *vs* the DM+3-MA group. PARP1: Poly ADP-ribose polymerase 1; COL1A1: Type 1 collagen α1 chain; 3-MA: 3-methyladenine; STZ: Streptozotocin; DM: Diabetes mice model; NC: Normal control; CQ: Chloroquine; SEM: Standard error of mean; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

RESULTS

Short-term Hypoglycemic Effect of 3-MA on Fasting Blood Glucose in STZ-induced Diabetic Mice It is well known that hyperglycemia is the cause of the pathogenesis of DR[8], therefore, we examined the impact of 3-MA and CQ on blood glucose in STZ-induced diabetic mice. As shown in Figure 1B, 3-MA significantly reduced FBG levels in STZ-induced diabetic mice at the third week of treatment. CQ reduced FBG levels in STZ-induced diabetic mice at the second week of treatment. However, the FBG levels were then gradually increased in both 3-MA and CQ treated groups. The results of water intake showed that the change trend of 24h water intake was basically consistent with that of blood glucose level (Figure 1C). Serum GSP is a biochemical marker for routine monitoring in DR patients, which can indirectly reflect blood glucose levels in the past 2–3wk[18-19]. As shown in Figure 1D, 3-MA reduced serum GSP levels in STZ-induced diabetic mice. CQ showed a trend of reducing serum GSP, but the difference was not statistically significant. These results suggest that 3-MA had a short-term hypoglycemic effect on FBG in STZ-induced diabetic mice.

3-MA Reduced the Expression of Apoptosis-Related Proteins and Fibrosis-associated Proteins in the Retina of STZ-induced Diabetic Mice Apoptosis of rMCs and pericyte is an important pathological feature of DR[12]. Therefore, the expression levels of apoptosis-related proteins cleaved caspase-3, cleaved PARP1, Bcl-2 and Bax were evaluated. As shown in Figure 2A–2D, the ratio of Bax/Bcl-2 and the expression levels of cleaved Caspase-3 and cleaved PARP1 were significantly up-regulated in the retina of STZ-induced diabetic mice compared with NC mice. 3-MA significantly reduced the ratio of Bax/Bcl-2 and the expression levels of cleaved caspase-3 and cleaved PARP1 in the retina of STZ-induced diabetic mice, while CQ had no significant effect on the expression of cleaved caspase-3 and cleaved PARP1, and further increased the ratio of Bax/Bcl-2 in the retina of STZ-induced diabetic mice.

It has been documented that epithelial-mesenchymal transition (EMT), extracellular matrix (ECM) deposition and fibrosis are present in the retina of DR patients[20-22]. Fibronectin and collagen I are the main ECMs that cause retinal fibrosis, and COL1A1 is a peptide chain that constitutes collagen I[23-24]. As shown in Figure 2E–2G, the expression levels of Fibronectin and COL1A1 were significantly up-regulated in the retina of diabetic mice compared with NC mice. 3-MA significantly decreased the expression levels of

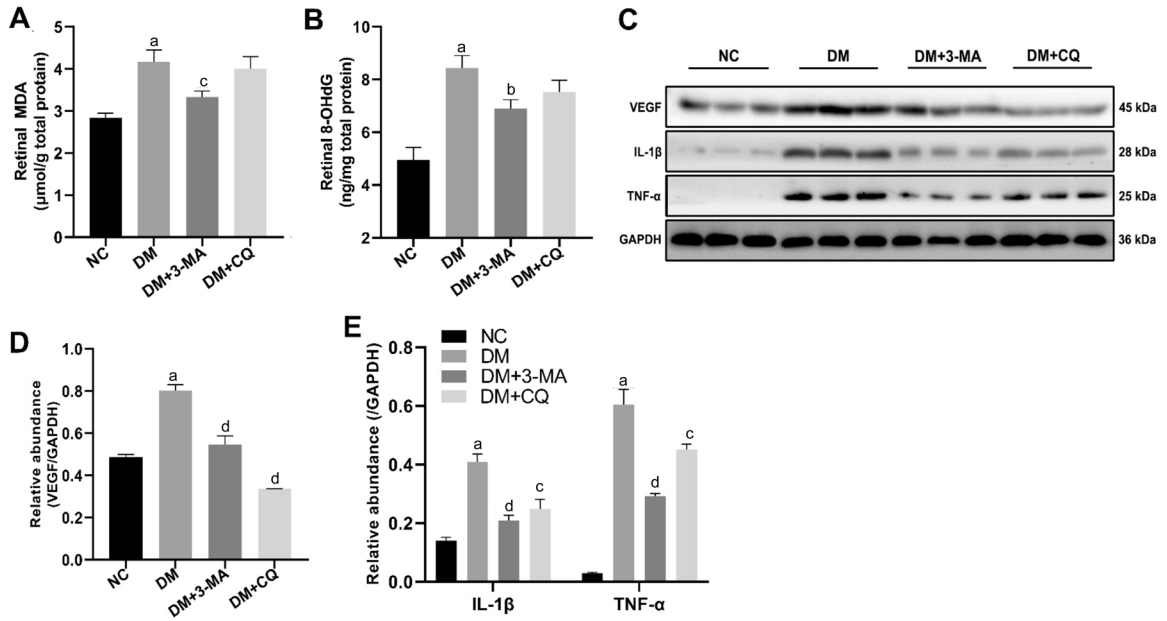

Figure 3 3-MA alleviated oxidative stress, expression of VEGF, and production of inflammatory factors in the retina of STZ-induced diabetic mice A: Retinal MDA detected by a commercial biochemical assay kit; B: Retinal 8-OHdG detected by a commercial ELISA kit; C: Retina tissue lysates were subjected to immunoblot analysis with antibodies to VEGF, IL-1β, TNF-α, and GAPDH; D: Expression level of VEGF was quantitated by densitometry and normalized with GAPDH; E: Expression levels of IL-1β and TNF-α were quantitated by densitometry and normalized with GAPDH. All data are represented as mean±SEM. $^{a}P<0.001$ *vs* the NC group; $^{b}P<0.05$, $^{c}P<0.01$, $^{d}P<0.001$ *vs* the DM group. MDA: Malondialdehyde; 8-OHdG: 8-hydroxydeoxyguanosine; VEGF: Vascular endothelial growth factor; IL-1β: Interleukin-1β; TNF-α: Tumor necrosis factor-α; 3-MA: 3-methyladenine; STZ: Streptozotocin; DM: Diabetes mice model; NC: Normal control; CQ: Chloroquine; ELISA: Enzyme-linked immunosorbent assay; SEM: Standard error of mean; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Fibronectin and COL1A1 in the retina of diabetic mice. CQ only down-regulated the expression level of COL1A1 in the retina of diabetic mice.

3-MA Alleviated Oxidative Stress, Expression of VEGF, and Production of Inflammatory Factors in the Retina of STZ-induced Diabetic Mice It has been documented that MDA and 8-OHdG are well-known biochemical markers of oxidative stress in DR[25-26]. MDA indicates lipid peroxidation, and 8-OHdG means DNA oxidative damage[25,27]. Hyperglycemia causes oxidative stress, which acts as an autophagy inducer[28-29]. To understand whether 3-MA can suppress retinal oxidative stress in STZ-induced diabetic mice, we examined the expression levels of MDA and 8-OHdG in mice retina. As shown in Figure 3A and 3B, the expression levels of MDA and 8-OHdG were markedly increased in the retina of diabetic mice compared with NC mice. 3-MA decreased the expression levels of MDA and 8-OHdG in the retina of diabetic mice, and CQ had no significant impact on MDA and 8-OHdG, suggesting that 3-MA reduced retinal oxidative stress in STZ-induced diabetic mice.

The persistent accumulation of free radicals and inflammatory mediators in the retina of patients with chronic diabetes promotes pathological angiogenesis[30-31]. IL-1β and TNF-α are classic inflammatory mediators. VEGF is the culprit that directly promotes retinal pathological angiogenesis[32]. Consequently, IL-1β, TNF-α, and VEGF were assessed in this study. As shown in Figure 3C-3E, the expression levels of VEGF, IL-1β, and TNF-α in the retina of diabetic mice were significantly increased compared with NC mice. Both 3-MA and CQ remarkably down-regulated the expression levels of VEGF, IL-1β, and TNF-α in the retina of diabetic mice.

3-MA Inhibited the Expression of Early Autophagy Related Proteins in the Retina of STZ-Induced Diabetic Mice To investigated whether the effect of 3-MA on the retina of diabetic mice is related to the regulation of autophagy, the expression levels of early autophagy related proteins LC3 and Beclin-1, as well as late autophagy protein P62 were detected. As shown in Figure 4, the ratio of LC3 II/LC3 I and the expression levels of Beclin-1 and P62 were significantly up-regulated in the retina of diabetic mice compared with NC mice. 3-MA significantly down-regulated the ratio of LC3 II/LC3 I and the expression level of Beclin-1 (Figure 4), suggesting that 3-MA inhibited early autophagy in the retina of diabetic mice. CQ further up-regulated the expression of P62 in the retina of diabetic mice (Figure 4), implying that CQ inhibited late autophagy in the retina of diabetic mice.

DISCUSSION

DR is one of the most common microvascular complications of diabetes[4]. According to the latest epidemiological survey in China, the incidence of DR in diabetes in four years is

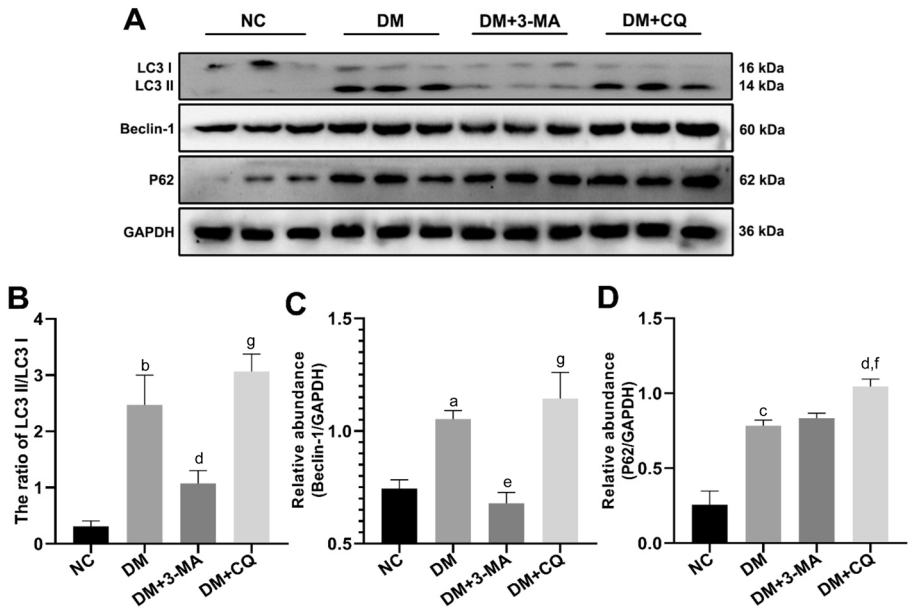

Figure 4 3-MA inhibited the expression of early autophagy related proteins in the retina of STZ-induced diabetic mice A: Retinal tissue lysates were subjected to immunoblot analysis with antibodies to LC3, Beclin-1, P62, and GAPDH; B: Expression levels of LC3 I and LC3 II were calculated by densitometry and the ratio of LC3 II/LC3 I was determined; C: Expression level of Beclin-1 was quantitated by densitometry and normalized with GAPDH; D: Expression level of P62 was quantitated by densitometry and normalized with GAPDH. All data are represented as mean±SEM. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ *vs* the NC group; $^{d}P<0.05$, $^{e}P<0.01$ *vs* the DM group; $^{f}P<0.05$, $^{g}P<0.01$ *vs* the DM+3-MA group. LC3: Microtubule-associated protein light chain 3; P62: Sequestosome 1; MDA: Malondialdehyde; 8-OHdG: 8-Hydroxydeoxyguanosine; VEGF: Vascular endothelial growth factor; IL-1β: Interleukin-1β; TNF-α: Tumor necrosis factor-α; 3-MA: 3-methyladenine; STZ: Streptozotocin; DM: Diabetes mice model; NC: Normal control; CQ: Chloroquine; SEM: Standard error of mean; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

10.70%[33]. Although current treatment methods can effectively prevent visual impairment in some patients, some patients may still experience partial or complete visual impairment after receiving treatment. A recent study showed that 3-MA alleviates ischemia reperfusion induced retinal injury in rats[16], but whether 3-MA can alleviate the retinal injury induced by diabetes remains unclear. In this study, we found that 3-MA lowered the blood glucose levels, reduced the expression of apoptosis-related proteins and fibrosis-associated proteins, alleviated oxidative stress, VEGF expression and the production of inflammatory factors in the retina of STZ-induced diabetic mice, and inhibited the expression of early autophagy-related proteins.

Apoptosis of retinal cells plays a crucial role in DR[12]. The retina is sensitive to high glucose environment, and hyperglycemia up-regulates autophagy and activates polyol and hexosamine pathways, causing apoptosis of retinal cells (*e.g.*, rMCs, pericytes, and neurons)[6,13], which in turn causes fundus exudation, leakage, and vision loss[34-35]. Recently, some studies have reported that 3-MA inhibits the apoptosis of retinal cells caused by ischemia-reperfusion[16]. Our study found that 3-MA significantly decreased the expression of apoptosis related proteins in the retina of diabetic mice, indicating that 3-MA has an anti-apoptotic effect on retinal cells in diabetic mice.

ECM is a complex network composed of proteins and polysaccharides, which can provide a scaffold for the attachment and movement of histiocyte, and is an important regulator of cell behavior. Disordered assembly and accumulation of ECM proteins can lead to fibrosis in many diseases[36]. Fibronectin is one of the main components of ECM proteins and an important component of fibrotic deposits. In DR, it can provide environmental support for vascular VEGF to promote retinal neovascularization, leading to the progression of non-proliferative DR to proliferative DR[37]. In diabetes, the expression of fibronectin and other ECM proteins in retinal blood vessels significantly increased[38-39], and this change occurred before the clinical manifestations of vascular dysfunction in DR[40]. It has been reported that 3-MA alleviates fibrosis in animal models of various diseases and inhibits the expression of signaling molecules related to fibrosis[41-42], as well as alleviates experimental subretinal fibrosis through the PI3K/Akt signaling pathway[15]. Consistent with the above reports on 3-MA inhibiting fibrosis, the present study found that 3-MA inhibited the increase of fibronectin and COL1A1 (part of ECM) in the retina of diabetic mice. The above results suggested that 3-MA may have an anti-fibrotic effect on the retina of diabetic mice.

VEGF is one of the most important mediators mediating the progression of DR. Under hyperglycemia, retinal ischemia and hypoxia can lead to an increase in VEGF and promote retinal neovascularization[12,43]. Currently, it is unanimously believed that anti-VEGF therapy is the primary choice for DR treatment[44]. In addition, sustained high-circulating glucose increases oxidative stress in the retina and also activates the inflammatory cascade[45]. The persistent accumulation of free radicals such as reactive oxygen species (ROS) and inflammatory mediators such as IL-1β in the retina of patients with chronic diabetes also promotes pathological angiogenesis[30-31]. To investigate the effect of 3-MA on retinal angiogenesis in diabetic mice, the expression levels of retinal MDA, 8-OHdG, VEGF and inflammatory factors IL-1β, TNF-α were assessed. The results showed that the expression levels of MDA, 8-OHdG, VEGF, and inflammatory factors IL-1β, TNF-α were significantly up-regulated in the retina of STZ-induced diabetic mice. 3-MA significantly down-regulated the expression levels of these molecules, suggesting that 3-MA alleviated oxidative stress, VEGF expression, and the production of inflammatory factors in the retina of STZ-induced diabetic mice. 3-MA may have a preventive effect on DR angiogenesis.

It has been reported that hyperglycemia induces autophagy but leads to lysosomal dysfunction[13]. In order to explore whether the inhibition of 3-MA on retinal apoptosis, fibrosis, VEGF, oxidative stress and inflammation in diabetic mice is related to its regulation of autophagy, the early stage autophagy related proteins LC3, which marks the existence of autophagosomes, and Beclin-1[46-47], which marks the induction of autophagosome formation, as well as the late stage autophagy related protein P62[46], a cargo protein degraded by an autophagy-lysosome system were examined. Moreover, the effects of CQ, another autophagy inhibitor which blocks the final stage of autophagy by increasing the pH value of the lysosome and inhibiting the fusion of autophagosome with lysosome[48] on apoptosis-related proteins, fibrosis-related proteins, VEGF, oxidative stress related molecules and inflammatory factors were tested. The data showed that autophagy was induced in the retina of diabetic mice, which is in line with the results reported in the literature that high glucose induces autophagy in human retinal endothelial cells[49]. However, autophagic flux was blocked, indicating that hyperglycemia caused retinal autophagy defect, which is consistent with the study by Lopes de Faria *et al*[13]. 3-MA significantly down-regulated the ratio of LC3 II/LC3 I and the expression level of Beclin-1, and CQ obviously up-regulated the expression level of P62 in the retina of diabetic mice, suggesting that 3-MA and CQ inhibited the early and late autophagy in the retina of diabetic mice, respectively. In the present study, although CQ reduced the levels of VEGF and inflammatory factors, it did not reduce the levels of oxidative stress related molecules and fibrosis related proteins in the retina of diabetic mice, and even further increased the ratio of pro-apoptotic protein Bax to anti-apoptotic protein Bcl-2, which may be related to its further aggravation of lysosome dysfunction and inhibition of final autophagy. In addition, it was reported that rapamycin, an autophagy inducer, aggravates retinal cell apoptosis in retinal ischemia-reperfusion injury model[50], suggesting that inducing autophagy with functional defects may exacerbate retinal cell damage. Our results indicated that the anti-apoptotic and anti-fibrotic effects of 3-MA, as well as its ability to alleviate oxidative stress and reduce the production of VEGF and inflammatory factors, are at least partially related to its inhibition of autophagy with functional defects by inhibiting early autophagy.

Hyperglycemia is the main risk factor for DR. The present study found that both 3-MA and CQ have a short-term hypoglycemic effect on diabetic mice. Consistent with our findings, some studies have also reported that CQ has a hypoglycemic effect[51-52], which may be the reason why CQ inhibits oxidative stress, VEGF expression and the production of inflammatory factors in our study. However, the hypoglycemic effect of 3-MA has not been reported in the past. It was reported that 3-MA enhances glucose induced insulin secretion and synthesis by inhibiting phosphodiesterase and elevating cyclic adenosine monophosphate level in rat pancreatic islets[53], which may be the reason of hypoglycemic effect of 3-MA observed in this study. Therefore, the above effects of 3-MA on the retina of diabetic mice may also be partially associated with its hypoglycemic effect.

In conclusion, our study demonstrates that 3-MA has anti-apoptotic and anti-fibrotic effects on the retina of diabetic mice, and can attenuate retinal oxidative stress, VEGF expression and the production of inflammatory factors in the retina of diabetic mice. The underlying mechanism of the above effects of 3-MA on the retina of diabetic mice may be related to its inhibition of early autophagy and hypoglycemic effect. Therefore, 3-MA may have a protective effect on DR. The results of this study may provide a new therapeutic strategy for the treatment of DR.

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Conflicts of Interest: Ren HW, None; **Yu W,** None; **Wang YN,** None; **Zhang XY,** None; **Song SQ,** None; **Gong SY,** None; **Meng LY,** None; **Gan C,** None; **Liu BJ,** None; **Gong Q,** None.

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