Therapeutic effect of folic acid combined with decitabine on diabetic mice

Gang Du[1], Yong Yan[2], Jun–Feng Gao[3], Chun–Yan Guo[4], Xiao Shen[1], Xun–Wen Lei[1]

[1]The First Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China

[2]Huining Second People's Hospital, Baiyin 730700, Gansu Province, China

[3]The Fourth People's Hospital of Tianshui, Tianshui 741000, Gansu Province, China

[4]Dingxi People's Hospital, Dingxi 743000, Gansu Province, China

Co-first authors: Gang Du and Yong Yan

Correspondence to: Xun-Wen Lei. The First Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China. leixw82@163.com

Abstract

● **AIM:** To evaluate the therapeutic effect of folic acid combined with decitabine on diabetic mice.

● **METHODS:** The diabetic model of *db/db* mice were randomly divided into model group, folic acid group, decitabine group, folic acid combined with decitabine group, and C57 mice as normal control group. The density of retinal blood vessels and retinal thickness were detected by fundus photography and optical coherence tomography, respectively. Pathological changes of retina were observed by hematoxylin-eosin (HE) staining. The homocysteine (Hcy) in serum was detected by enzyme linked immunosorbent assay (ELISA). TdT-mediated dUTP nick-end labeling (TUNEL) was used to detect apoptosis in retinal tissue. Evans blue dye was used to detect the permeability of retinal blood vessels. The platelet endothelial cell adhesion molecule-1 (CD31) and vascular endothelial growth factor receptor (VEGFR) protein were detected by Western blot. The 3-nitrotyrosine (3-NT) and 4-hydroxynonanine (4-HNE) were detected by immunohistochemistry.

● **RESULTS:** The density of retinal blood vessels, retinal thickness, retinal vascular permeability and the proportion of apoptotic cells of retinal tissue in the model group increased significantly than control group ($P<0.05$). The Hcy in serum and the levels of CD31, VEGFR, 3-NT, and 4-HNE in retinal tissue increased significantly in the model group ($P<0.01$). Folic acid and decitabine both reversed these changes significantly, and the combination of the folic acid and decitabine worked best.

● **CONCLUSION:** The combination of folic acid and decitabine has a more significant protective effect on the retina in diabetic mice.

● **KEYWORDS:** diabetic model folic acid; decitabine; apoptosis; mouse

INTRODUCTION

Diabetic retinopathy (DR) is one of the most serious microangiopathic complications of diabetes mellitus (DM). Long-term chronic hyperglycemia injury leads to morphological and functional abnormalities in retinal tissues, damages microvascular endothelial cells, and results in abnormal angiogenesis[1-2]. Currently, many research have found that multiple drugs can exhibit certain therapeutic effects on DR by targeting angiogenesis[3-4].

It has been demonstrated that hyperglycemia-induced oxidative stress can lead to apoptotic damage of retinal endothelial cells, changes in vascular permeability, disorder of neovascularization and changes in metabolism of glycolipid-protein in retinal tissues[5-7]. Herein, homocysteine (Hcy) is the main cause of oxidative stress in DM and is an important independent factor in DR[8]. The free sulfhydryl groups on Hcy are prone to auto-oxidation, producing hydrogen peroxide and other oxygen radicals, leading to lipid peroxidation. Hcy can also inhibit the activity of various antioxidant enzymes such as glutathione peroxidase, which can weaken the antioxidant function of the body and cause oxidative stress reactions[9]. Meanwhile, Hcy can induce the expression of various adhesion factors in vascular endothelial cells, promote adhesion of inflammatory cell, cause endothelial coagulation, anti-coagulation disorder and promote thrombosis[10-12].

Hcy is an intermediate of the methionine cycle, which directly affects the Hcy content in blood and tissues. In this cycle, folic acid acts as a one-carbon carrier, and is catalyzed by

5-methyltetrahydrofolic acid Hcy methyltransferase, which provides a methyl group to Hcy, methylating Hcy to afford methionine (Met). Therefore, folic acid supplementation can promote the conversion of Hcy to Met and reduce the Hcy content in blood. It has been demonstrated that supplementation of folic acid can protect retinal vascular endothelial cells from damage in the hyperglycemia state[13], reduce the local oxidative stress state[14], reduce Hcy serum levels, and alleviate retina thinning in early DR models[15].

The ratio of S-adenosine Met (SAM) and S-adenosine homocysteine (SAH) in the methionine cycle (SAM/SAH ratio) plays an important role in DNA methylation. In the process of DNA methylation, methyltransferase can indirectly promote the conversion of SAM to SAH by accelerating methyl metabolism, resulting in the decrease of SAM and the increase of SAH, the precursor of Hcy, thus manifesting as a high Hcy state, the high Hcy and SAH levels will further aggravate the oxidative stress state and affect the development of DR[16]. As a specific inhibitor of methyltransferase, decitabine can protect retina by inhibiting methyl metabolism, reducing the conversion of SAM to SAH, thereby reducing Hcy levels, improving DR progression, and preventing mtDNA damage[17]. Currently, the natural product quercetin has shown potential therapeutic effects in DR by inhibiting NLRP3 inflammasome and autophagy signaling pathways, which are involved in the dysregulation of neovascularization[18]. However, there is currently no research exploring new treatment strategies for DR based on the methionine cycle. In summary, folic acid reduces Hcy levels by promoting the remethylation of Hcy to generate Met, whereas decitabine reduces Hcy levels by indirectly inhibiting SAM demethylation and increasing the SAM/SAH ratio to inhibit Hcy production. Both alone may alleviate DR by reducing the level of Hcy and inhibiting the resulting local oxidative stress[15]. However, the effect of the combination of the two on DR has not been reported. Therefore, in this experiment, folic acid was combined with decitabine to intervene in DM model mice to explore the therapeutic effect on DR.

MATERIALS AND METHODS

Ethical Approval This experimental study was carried out in the laboratory at the First Hospital of Lanzhou University and Gansu University of Chinese Medicine between March 2021 and August 2022. The Ethics Committee approval for this study was obtained from the First Hospital of Lanzhou University, Lanzhou, China (No.LDYYLL2021-135).

Experimental Animals Forty male *db/db* mice (16-week-old, weighing 57±5 g), and 10 male C57BL/Ksj mice (16-week-old, weighing 22±2 g) were purchased from Changzhou Cavens experimental animal Co., Ltd. with license No. SCXK (SU) 2016-0010. All the mice were raised in the specific

pathogen-free (SPF) barrier environment of Gansu University of Chinese Medicine which simulated natural day and night conditions, with daytime relative temperature of 21°C±2°C, relative humidity of 40%-45%, daily sunshine time of 12h, and free feeding and water intake.

Drugs and Reagents All materials were as follows: folic acid (HPLC purity≥97%; F7876, Sigma-Aldrich, USA), blood glucose meter and blood glucose test paper (BD, USA), inverted fluorescence microscope (BX53, Olympus, Japan), Evans blue dye (E2129, Sigma-Aldrich, USA), mouse Hcy enzyme linked immunosorbent assay (ELISA) kit (ml037451, Shanghai mlbio, China), TdT-mediated dUTP nick-end labeling (TUNEL) apoptosis detection kit (Alexa fluor 488; 40307es50, Yeasen Biotechnology, Shanghai, Co., Ltd.), mouse anti-3-nitrotyrosine (3-NT) monoclonal antibody (ab61392, Abcam, UK), rabbit anti-4-hydroxynonanine (4-HNE) polyclonal antibody (ab46545, Abcam, UK), HRP-labeled Goat anti-rabbit polyclonal IgG(H+L) (ab6721, Abcam, UK), HRP-labeled goat anti-mouse IgG(H+L) (ab6789, Abcam, UK), Alexa Fluor® 488-labeled goat anti-rabbit IgG(H+L) (ab150077, Abcam, UK), BeyoECL Plus (P0018, Beyotime Biotechnology, Shanghai, Co., Ltd.), radio immunoprecipitation assay lysis buffer (P0013C, Beyotime Biotechnology, Shanghai, Co., Ltd.), SDS-PAGE protein sample loading buffer (P0286, Beyotime Biotechnology, Shanghai, Co., Ltd.).

Groups and Treatment Ten C57BL/Ksj mice used as the normal control group were fed with common diet, 40 *db/db* mice with fasting blood glucose ≥11.1 mmol/L were randomly divided into 4 groups: model group (*n*=10), folic acid group (*n*=10), decitabine group (*n*=10) and folic acid+decitabine group (*n*=10). Among them, folic acid group was fed by gavage at 70 µg/kg·d with folic acid once a day. The decitabine group was injected intraperitoneally with decitabine at the dose of 0.25 mg/kg once every 5d, the folic acid+decitabine group was intervened with the combination of 70 µg/kg·d folic acid and 0.25 mg/kg decitabine[19]. The normal group was given the same dosage of normal saline by gavage and injection, respectively. The above groups were intervened for 30d.

Angiogenesis in Retinal Tissue Detected by Fundus Photography After the intervention, the eye of the mice anesthetized by intraperitoneal injection of 10% chloral hydrate with a dose of 30 mg/kg were fixed, the pupils of both eyes were dilated, and the 20 D indirect mirror was fixed on the fundus camera (nonmyd) in front of the lens of α-D III), fix the mouse eyelids, make the mouse's eyeballs directly in front of the indirect lens through adjustment, and adjust the mouse's position until a clear fundus image is observed and photographed through the fundus camera.

Retina Thickness Detected by Optical Coherence Tomography After the intervention, the mice were anesthetized with 30 mg/kg intraperitoneal injection of 10% chloral hydrate, fixed the rat eyes, mydriasis in both eyes, fixed the rat eyes, measured the retinal thickness at the two disc diameters above, below, nasal and temporal, and took the average value. The high reflection layer from the inner limiting membrane of the retina to the retinal pigment epithelium was measured manually using the built-in software of the detection system, which was defined as the thickness of the retina.

Cells Apoptosis in Retinal Tissue Detected by TUNEL Test The eyeball slices fixed with 4% paraformaldehyde were dewaxed in water. Protease K (20 μg/mL) incubated the slices at room temperature for 30min. Then slices were washed with phosphate buffered saline (PBS) for 3 times, added 50 μL Alexa Fluor 488-12-dUTP labeling mix, and incubated at 37°C in dark for 60min. Washed with PBS for 3 times and dropwise added of 50 μL PI solution (1 μg/mL) at room temperature for 5min, slices were rinsed with deionized water, and observed and photographed under microscope.

Hcy in Serum Detected by ELISA After the intervention, 1% pentobarbital sodium was injected intraperitoneally to anesthetize the mice, the blood was taken by extracting the eyeballs. The blood was naturally coagulated at room temperature for 20min, centrifuged at 3000 rpm for 10min, and the serum was taken. The content of Hcy in serum was detected using the ELISA kit according to manufacturer's protocol.

Expression of 3-NT and 4-HNE Protein in Retina Detected by Immunohistochemistry After hydrated and repaired of antigen, the paraffin sections of retinal tissue were washed with PBS, added 3% H_2O_2, and incubated at room temperature for 10min to block endogenous peroxidase. After washed with PBS, the paraffin sections were incubated with 10% goat serum at room temperature for 30min, with 3-NT and 4-HNE antibody overnight at 4°C, and with biotin labeled goat anti-rabbit secondary antibody at room temperature for 1h. After washed with PBS, the sections were stained with HRP-labeled Streptomyces ovalbumin, incubated at room temperature for 15min, and added 3,3'-diaminobenzidine, then stained with hematoxylin, dehydrated with gradient alcohol, added xylene, and observed under the microscope.

Expression of CD31 and VEGFR Detected by Western Blot The total protein in the retina were extracted using radio immunoprecipitation assay lysis buffer. Briefly, the retina was lysed in radio immunoprecipitation assay lysis buffer for 30min at 4°C, The lysates were centrifuged at 13 000 rpm for 10min, the supernatants were separated and added 5× loading buffer, boiled for 5min for the denatures. Equal amounts of protein (30 μg) were subsequently loaded in SDS-PAGE gel for electrophoresis. Following concentration, separation and membrane transfer, the membranes were blocked overnight with 10% skim milk in 0.5% Tween 20 in PBS (PBST) for 1h at room temperature and then incubated with rabbit antiserum specific for CD31 and VEGFR (diluted 1:2000) at 4°C overnight, and subsequently washed with PBS. HRP-conjugated goat anti-rabbit IgG (diluted 1:5000) in PBS was incubated as the secondary antibody reacted for 2h at room temperature. Antibody binding was visualized by ECL, exposed and developed with automatic exposure instrument, and take photos for analysis. The gray value of each band was analyzed by imagePro plus software.

Statistical Analysis SPSS 21.0 data statistics software package was used for data statistics. The measurement data are expressed as mean±standard deviation (SD). One way ANOVA is used for multi group comparison, LSD-t test is used for pairwise comparison between groups, and the difference is statistically significant at $P<0.05$.

RESULTS

Angiogenesis and Retinal Thickness Changes The neovascularization and retinal thickness increased significantly in the model group compared with normal group and decreased significantly in folic acid, decitabine, and decitabine+folic acid groups compared with model group. There were no significant changes between folic acid group and decitabine group. Compared with folic acid group and decitabine group, the neovascularization and retinal thickness in decitabine+folic acid group were significantly reduced (Figure 1).
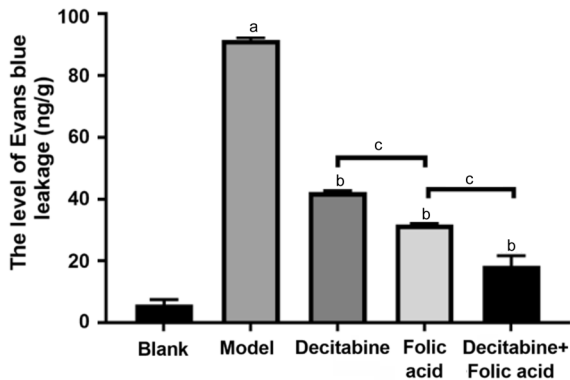
Changes of Retinal Vascular Permeability The Evans blue leakage value in the retina of the model group increased significantly compared with the normal group ($P<0.01$) and decreased significantly in folic acid, decitabine, and folic acid+decitabine groups compared with the model group ($P<0.01$). Evans blue leakage in folic acid group was less than decitabine group ($P<0.01$) and the folic acid+decitabine group was the least ($P<0.01$; Figure 2).

Changes of Hcy Level in Serum Serum Hcy level in the model group increased significantly compared with the normal group ($P<0.01$) and decreased significantly in folic acid, decitabine, and folic acid+decitabine groups compared with the model group ($P<0.01$). The serum Hcy level in folic acid+decitabine group was the most significant decrease among the three groups ($P<0.01$; Figure 3).

Changes of Retinal Cell Apoptosis After one month of intervention, the proportion of apoptotic cells in the retina of the model group increased significantly compared with the normal group ($P<0.01$) and decreased significantly in the folic acid, decitabine, and folic acid+decitabine groups compared with the model group ($P<0.01$). It was the most significant decrease in folic acid+decitabine group ($P<0.01$; Figure 4).
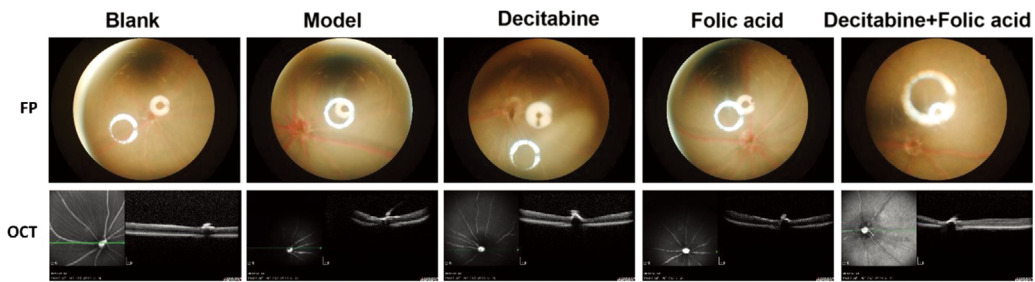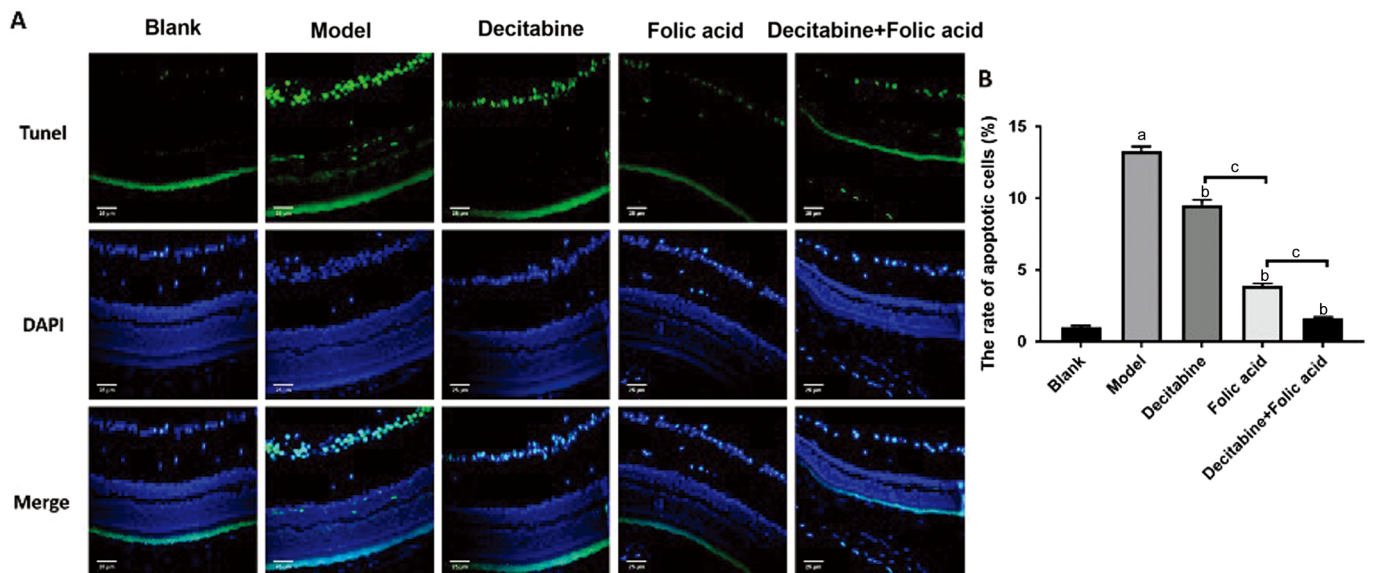
Figure 1 The effect of folic acid combined with decitabine on the angiogenesis and thickness of retina in diabetic mice The retinal vascularization was observed by fundus photography (FP), and the retinal thickness changes were observed by optical coherence tomography (OCT).

Figure 2 Effect of folic acid combined with decitabine on vascular permeability in retina of diabetic mice, and retinal permeability changes were detected by Evans blue staining [a]$P<0.01$ vs blank group, [b]$P<0.01$ vs model group, [c]$P<0.01$.
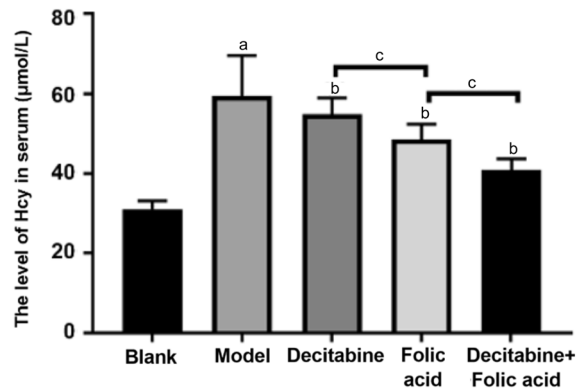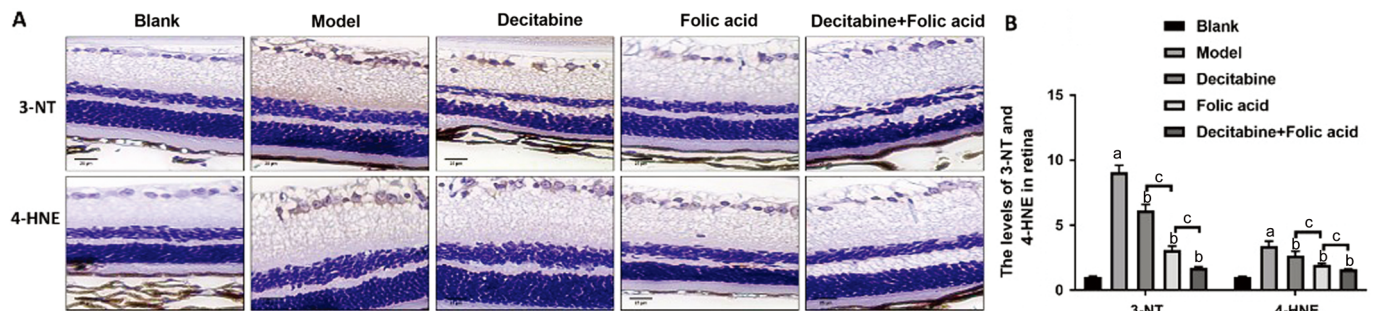
Figure 3 The effect of folic acid combined with decitabine on serum Hcy level in diabetic mice ELISA assay was used to detect the level of Hcy in serum of mice. [a]$P<0.01$ vs blank group, [b]$P<0.01$ vs model group, [c]$P<0.01$.
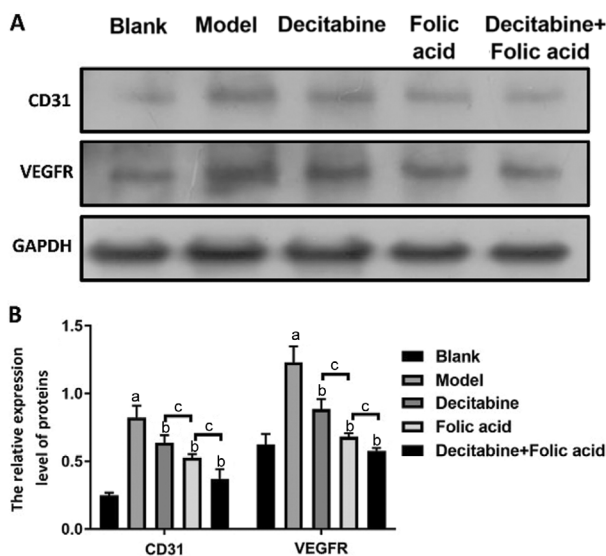

Figure 4 The effect of folic acid combined with decitabine on the cell apoptosis of retina in diabetic mice The cell apoptosis of retinal tissue was detected by Tunel staining (A, ×200), and the rate of apoptotic cells was analyzed using Image pro plus software (B). [a]$P<0.01$ vs blank group, [b]$P<0.01$ vs model group, [c]$P<0.01$.

Changes of 3-NT and 4-HNE in Retina The 3-NT and 4-HNE proteins increased significantly in the model group compared with normal group ($P<0.01$) and decreased significantly in folic acid, decitabine, and decitabine+folic acid groups compared with model group ($P<0.01$), especially in decitabine+folic acid group ($P<0.01$; Figure 5).

Expression Changes of CD31 and VEGFR in Retina The CD31 and VEGFR proteins increased significantly in the model group compared with normal group ($P<0.01$) and decreased significantly in folic acid, decitabine, and decitabine+folic acid groups compared with model group ($P<0.01$), especially in decitabine+folic acid group ($P<0.01$; Figure 6).

Figure 5 The effect of folic acid combined with decitabine on the expression of 3-NT and 4-HNE in retina of diabetic mice Immunohistochemistry was used to detect the changes of 3-NT and 4-HNE in the retina of mice (A, ×200), and the optical density of the region stained by 3,3'-diaminobenzidine dye was analyzed using Image pro plus software (B). 3-NT: 3-nitrotyrosine; 4-HNE: 4-hydroxynonenal. [a]$P<0.01$ vs blank group, [b]$P<0.01$ vs model group, [c]$P<0.01$.

Figure 6 The effect of folic acid combined with decitabine on the expression of CD31 and VEGFR in retina of diabetic mice The expression of CD31 and VEGFR protein in the retina of mice was detected by Western blot, and the optical density of the bands was analyzed using Image pro plus software (B). CD31: Platelet endothelial cell adhesion molecule-1; VEGFR: Vascular endothelial growth factor receptor. [a]$P<0.01$ vs blank group, [b]$P<0.01$ vs model group, [c]$P<0.01$.

DISCUSSION

The results of this study revealed that folic acid, decitabine and its combination could significantly inhibit the damage of retinal tissues in mice induced by DM, increase the thickness of retinal tissues, decrease the permeability of retinal vascular, reduce vascular damage and generation as well as apoptosis of retinal tissues. At the molecular level, folic acid, decitabine and the combination of both could significantly reduce the levels of Hcy in serum and the expression of angiogenesis-related factors CD31 and VEGFR in retinal tissues, and decrease the levels of oxidative stress-related indicators 3-NT and 4-HNE in mice affected by DM. Nevertheless, the therapeutic and protective effects of folic acid combination with decitabine on DM retinal tissues were significantly better than those of the two alone.

DR is caused by long-term damage of hyperglycemia to the micro-vessels in retinal tissues. It has been demonstrated that both folic acid and decitabine can protect DM retinal tissues[13,15]. It is of great significance to improve and alleviate DR[20]. Likewise, the results of this study revealed that both folic acid and decitabine significantly inhibited DM-induced structural damage to the retinal tissues of mice, decreased retinal vascular permeability, reduced vascular damage, generation and apoptosis of retinal tissues, and the effect of folic acid was better than that of decitabine. The protective effect of folic acid combined with decitabine on DM retinal tissues was found to be significantly better than the effect of both alone. It has been demonstrated that folic acid can reduce Hcy levels by promoting Hcy methylation[15,21-22], while decitabine can reduce Hcy levels by indirectly inhibiting SAM demethylation, increasing the SAM/SAH ratio and inhibiting Hcy production[17,23]. High levels of Hcy in blood can cause elevated active oxygen in retinal tissues[17], tissue and vascular endothelial cell damage[24], and increased inflammation[25]. High level of active oxygen in retinal tissues caused by high levels of Hcy is an important factor in the occurrence and development of DR. Therefore, the better alleviating effect of folic acid combined with decitabine on DR may be related to the fact that the combination of folic acid and decitabine inhibited the production of Hcy and promoted the metabolism of Hcy. Therefore, in this study, the levels of Hcy in mice serum were further explored and the results showed that the effect of folic acid combined with decitabine on the reduction of Hcy levels in the serum of DM mice was significantly stronger than the effect of the two alone.

It has been demonstrated that abnormally elevated plasma Hcy levels caused by hyperglycemia disrupt the balance of free radical production and scavenging, leading to elevated active oxygen in retinal tissues, producing oxidative stress, damage to retinal tissues and blood vessels, leading to DR[16,26], which is an important independent factor for the occurrence and development of DR[27]. In terms of molecular

mechanisms, sustained high active oxygen can lead to changes in mitochondrial membrane permeability in retinal tissues cells and their vascular endothelial cells, elevated Bax, decreased Bcl-2, and cytochrome C entry into the cytoplasm, causing activation of the cellular caspase protein cascade, which in turn induces apoptosis, causing inflammation and tissue damage[28]. In this study, oxidation levels in retinal tissues were further investigated, and the combination of folic acid with decitabine was found to significantly reduce the content of 4-HNE, 3-NT in DM retinal tissues compared to folic acid or decitabine alone. 4-HNE and 3-NT are important indicators of cellular active oxygen levels, of which 4-HNE is an important indicator of unsaturated fatty acid lipid oxidative damage[29] and 3-NT is an important indicator of protein oxidative damage[30]. It is evident that the better protective effect of folic acid combined with decitabine on DM retinal tissues is based on the combined effect of the two in reducing the Hcy levels in serum of DM mice, which further reduces the active oxygen levels in the retinal tissues of *db/db* mice more effectively.

In conclusion, compared with folic acid or decitabine alone, the protective effect of folic acid combined with decitabine on DM retinal tissues is more significant, which can further reduce the level of Hcy in the blood of DM mice, inhibit oxidative stress in DM retinal tissues, prevent the development of apoptosis and inflammation in retinal cells, and thus inhibiting the development of DR. It provides a theoretical basis for the clinical application of folic acid combined with decitabine in the treatment of DR.

ACKNOWLEDGEMENTS

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