



Effect and Mechanism of Marein on Diabetic Retinopathy

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ABSTRACT

This study was aimed at investigating the therapeutic effect of marein on db/db mice and its mechanism of action intervening in diabetic retinopathy. For *in vivo* experiment: 10 healthy db/m mice were used as the control group. Forty healthy db/db mice were randomly divided into the model, positive, ranibizumab and marein groups. There were 10 mice in each group. All mice were adaptively fed for one week. They were given the appropriate drugs by gavage. Tissue material was taken after 12 weeks. Haematoxylin-eosin (H&E) staining was used to detect the pathological changes of retinal tissue. Cell apoptosis was analysed by TUNEL staining. The expression of retinal proteins was detected by tissue immunofluorescence and immunohistochemical staining. The mRNA level of VEGF was detected by RT-qPCR. In the *in vitro* experiments, cell viability was detected by CCK-8 reagent. The expression of related proteins was detected by western blot. We found that marein can effectively reduce triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) and blood glucose concentration (BGC) in db/db mice ($P < 0.05$). The results showed that marein could effectively alleviate diabetic retinopathy, such as oedema, cell body shrinkage, abnormal nuclear morphology, etc., and ultimately enhance retinal function. *In vivo* and *in vitro* experiments showed that after a period of intervention, marein regulates diabetic retinopathy by inhibiting the protein expressions of VEGF, PI3K, fibrin (FN) and spleen tyrosine kinase (SYK), and upregulating the level of E-cadherin, an epithelial marker. Molecular docking results showed that marein could integrate with VEGFA, PI3K and SYK, and the resulting conjugate was more potent than the positive drug metformin. Among the above factors, the combination effect of SYK and marein was the most ideal. To conclude marein can improve glucose and lipid metabolism disorders in db/db mice after a treatment period, so it has positive significance for the prevention and treatment of diabetes. Marein can act on SYK, VEGF and PI3K/AKT factors and change the expression of fibrosis and inflammatory factors in tissues by affecting their pathways. It can integrate with these and many other proteins, and the formed conjugates have a better therapeutic effect.

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Authors' Contribution

SZP, XJL and WXL conceived of the study. ZLPY, ABL, KDRY and ABE participated in its design and coordination. MXM helped to draft the manuscript. All authors read and approved the final manuscript.

Key words

Marein, Diabetic retinopathy, SYK, VEGF, PI3K/AKT

INTRODUCTION

Diabetes mellitus (DM) is the most common type of disease. According to the statistics of relevant departments in 2019, about 463 million adults aged 20–79 years currently have diabetes worldwide (Whiting *et al.*, 2011). Generally, this disease can lead to microangiopathy if not diagnosed in time. Diabetic retinopathy (DR) is a common complication of this disease (Shi *et al.*, 2021), and

its clinical features are mainly reflected in retinal vascular dysfunction, endothelial cell injury, etc. (Antonetti *et al.*, 2012). According to the statistics of relevant departments, more than half the patients with a history of diabetes over 10 years have such diseases (Chisha *et al.*, 2017), and DR will cause irreversible vision loss. Because it has a high incidence and is difficult to cure, it has a serious socioeconomic impact (Simó and Hernández, 2015; Stitt *et al.*, 2016; Liu *et al.*, 2012).

DR is an important contributing factor to blindness, which primarily acts through proinflammatory factors and is considered to be the most common microvascular

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Abbreviations

AKT, protein kinase B; ARPE-19, adult retinal pigment epithelial cell line-19; DMSO, dimethyl sulfoxide; DM, diabetes mellitus; DR, diabetic retinopathy; FN, fibrin; PI3K, phosphatidylinositol kinase; SYK, spleen tyrosine kinase; VEGF, vascular endothelial growth factor.

complication (Vanlandingham *et al.*, 2017; Wang *et al.*, 2019), and retinal pigment epithelial (RPE) cells in tissues are closely related to the formation and development of this disease (Cowley *et al.*, 1989; Idrees *et al.*, 2019; Ryan, 1985). It has been confirmed that the VEGF-A/VEGFR2 signalling pathway plays a critical role in physiological and pathological angiogenesis. At this stage, the use of VEGF antagonists and vitreous injections of ranibizumab is already an effective treatment for DR (Messias *et al.*, 2018). However, after long-term research, many scholars have found that nearly half of the patients treated with anti-VEGF-A have subretinal fibrosis after a long intervention period, but its mechanism of action has not been elucidated (Guo *et al.*, 2020; Zhang *et al.*, 2020). Fibrin (FN) is a macromolecular glycoprotein that can heal injured tissues and organs, as well as improve cellular adhesion growth and the ability of tissues to repair to a greater extent. A significant increase in the amount of this substance in tissues indicates severe organ inflammation. Studies have shown that the expression level of this substance is higher in high glucose environments (Zhang *et al.*, 2017). α -SMA is a marker of myofibroblasts, and when certain organs develop cytopathy due to injury, the degree of fibrosis and organ damage can be obtained by detecting the expression level of this factor. After a long-term study, many scholars have pointed out that when the expression levels of α -SMA, FN and other profibrotic factors decrease, the symptoms of DR can also be effectively relieved, which provides new ideas for the treatment of this disease. Spleen tyrosine kinase (SYK), essentially a member of the non-receptor protein tyrosine kinase, was first extracted from the spleens of pigs (Taniguchi *et al.*, 1991). In recent years, it has been confirmed that this substance plays a crucial role in retinoblastoma, and the intervention of patients using the SYK inhibitor R406 can cause retinoblastoma death (Zhang *et al.*, 2012). In addition, SYK is also involved in the process of the epithelial-mesenchymal transition in retinal epithelial cells (Park and Kim, 2018).

Coreopsis tinctoria is a common medicinal herb that can treat diabetes, of which flavonoids play a major role in its therapeutic effects. Some scholars have pointed out that the dried flowers of this plant have anti-inflammatory and antioxidant properties (Chen *et al.*, 2016; Dias *et al.*, 2010; Wang *et al.*, 2015; Zhang *et al.*, 2013). Marein is extracted from this herb and plays a role in preventing diabetes as well. Flavanomarein (FM), the main active component in *Coreopsis tinctoria*, may have the function of integrating SYK, and its effect will be more significant when it is used in combination with SYK inhibitors (Zhang *et al.*, 2020). Active marein inhibits the massive release of proinflammatory factors and consequently acts to alleviate fibrosis and inflammatory responses (Guo *et al.*, 2020), and additionally increases the high glucose-

induced insulin resistance in HepG2 cells to some extent (Jiang *et al.*, 2016). Based on the above discussion, this study aims to investigate the effect and mechanism of marein in improving DR.

MATERIALS AND METHODS

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Xinjiang Medical University.

Animals and experimental design

The ratio of healthy male SPF (BSK-Leprem2Cd479/Gpt, db/db) mice to (BSK-Leprem2Cd479/Gpt, db/m) mice were selected to be 40:10. The mice were all housed in a specialised experimental centre and provided adequate nutrition during the breeding process. The temperature of the experimental centre was about 25 °C, and the humidity was about 55 °C, with light given alternately during the day. All mice were adaptively fed for one week. The db/m mice were used as the control group, and the other group was divided into the model, positive, ranibizumab and marein groups according to the random sampling principle. In the model group, the feeding method was the same as above. All mice were treated by oral gavage once a day; the positive group was treated with metformin, the ranibizumab group was treated with ranibizumab and the marein group was treated with marein (50 mg/kg). Each group had 10 mice and was fed for 12 weeks.

Reagents

Coreopsis tinctoria was purchased from the Coreopsis Tinctoria Biotechnology Company. Marein is an extract of this herb and a monomeric flavonoid, and its purity of 99.8% was determined by using the areas of peak normalisation method. The high purity marein was dispensed into EP tubes in specific proportions, prepared as a suspension with 0.5% CMC-Na, and transferred at room temperature. A 0.5 g metformin tablet was used to carry out the study. It was pulverised and processed before administration, and a certain concentration of CMC-Na was added proportionally to form a suspension at a dosing concentration of 10 mg/kg. Before the formal study, an appropriate amount of carboxymethylcellulose sodium buffer solution was selected to prepare the 0.5% CMC-Na buffer solution and transferred to a low-temperature environment for preservation. The main reagents and instruments are listed in [Supplementary Data I](#).

Biochemical analysis of animal blood

The mice in each group were fed for 12 weeks, and only water was supplied 12 h before treatment.

The eyeballs were taken for blood sampling in strict accordance with the procedure. When the blood flowed out, the mouse was executed by the dislocation method. The blood was centrifuged and dispensed with the serum, and the obtained samples were tested to analyse the values of fasting blood glucose (FBG), triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). The ocular tissue of the left eye was transferred to an eyeball fixative solution of a certain concentration (Servicebio, G1109-100ML) and stored in the dark at 4°C for subsequent embedding. After the above steps were completed, the right eye was treated with saline and then transferred to a cryogenic environment with an appropriate amount of liquid nitrogen.

Histopathological analysis

The ocular tissue of the left eye was fixed in 4% paraformaldehyde, then dehydrated, embedded and cut into samples of approximately 4 µm thickness. After removing the paraffin, haematoxylin-eosin (H and E) staining was performed separately. The haematoxylin staining solution was selected to stain the nucleus, with the duration of this operation at least 5 min. An ethanol hydrochloride differentiation solution was used to treat for 1 min. The sections were transferred to water at a higher temperature for a period of time, after which they were stained with eosin staining solution for 2 min. Different ethanol concentrations were invaded separately in sequence, and xylene was used for transparency. After the above steps were completed, they were blocked with neutral resin and placed under a light microscope to observe the staining results.

Immunohistochemical staining

Immunohistochemical staining was performed using the standard methods. Paraffin-embedded tissues were sliced into 5–7 µm sections. Following hydration, a citric acid buffer was used at 95°C for 10 min for antigen retrieval. After selecting a certain amount of endogenous peroxidase for blocking for a period of time, the blocking solution was decanted, and diluted primary antibody was added (rabbit polyclonal anti-VEGF-A [ab1316] and anti-SYK [ab190176] antibodies were used as the primary antibodies and were applied at the concentrations of 1:250 and 1:350, respectively). They were placed at room temperature, and the prepared primary antibody was added and incubated overnight at 4°C, followed by several washes in PBS (three times for 5 min each). Biotin-labelled secondary antibodies were added proportionally to the resulting samples, incubated and washed repeatedly. DAB was used for colour development; observation was performed and the time of colour development was set as needed. An

appropriate amount of haematoxylin was selected to stain the nuclei, routinely washed and then differentiated using a certain concentration of hydrochloric acid alcohol and returned to blue. The film was further processed and then sealed with a neutral resin.

Tissue immunofluorescence

Paraffin sections of 2 µm were routinely deparaffinised in water and washed repeatedly. The sections were placed in citrate buffer solution, heated at a high temperature to achieve antigen retrieval and washed several times for later use. Sheep serum was used to block at room temperature, and the sections were transferred to a wet box and left in a low-temperature environment for a period of time. Following primary antibody incubation, sections were rinsed with PBS, and the rinsed sections were incubated with fluorescent dye coupled with secondary antibodies. The samples were transferred to room temperature for a period of time, after which they were washed several times. The nuclei were stained with DAPI, and the resulting samples were observed and photographed under a microscope.

TUNEL assay

DNA fragmentation in retinal apoptosis was detected by a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) kit (FL fluorescein; Roche) according to the manufacturer's standard procedures, and cell nuclei were stained with DAPI (Sigma) for 2 min. The tissue sections were mounted with Mowiol 4-88 (Sigma) before observation and analysis with the Axioskop 40 fluorescence microscope (Zeiss). TUNEL-positive cells in the outer nuclear layer were counted in a masked fashion, and photoreceptor cell apoptosis was expressed as the percentage of total cells in the TUNEL-positive cells.

Quantitative RT-PCR

Total RNA was isolated from the ocular tissue by the RNeasy total RNA purification kit (Qiagen, Valencia, CA). qRT-PCR for evaluating the crossing points for each reaction of each gene was carried out with the primers listed in [Table I](#) using an ABI Prism 7500 sequence detector.

Cell culture and morphological observation

The ARPE-19 cells were incubated in DMEM containing 1% antibiotics (penicillin and streptomycin) and 10% foetal bovine serum (FBS) at 37°C in 5% CO₂. After the cells reached maximum fusion, they were passaged and used for subsequent *in vitro* experiments.

The cell source was the Culture Collection Centre of Wuhan University. The cells were pre-treated after obtaining. The procedure was to treat the cells with 30, 60

and 90 mm glucose for three days, followed by microscopic observation of the morphological changes of the cells.

Table I. Sequences of primers for real-time fluorescence quantitative PCR.

Protein	Forward primer (5'-3')
VEGF	F: TGTGAGCCTTGTTTCAGAGCGG R: ACTCAAGCTGCCTCGCCTTGC
β -Actin	F: CCTGGCACCCAGCACAAT R: GGGCCGGACTCGTCATAC

Cell proliferation and viability test

Cell viability was determined using the cell counting kit-8 (CCK-8; MedChemExpress). In brief, 5,000 ARPE-19 cells were seeded into 96-well plates. Each group was treated with the corresponding drugs. After reaching the predetermined time, 10 μ l of CCK-8 solution was added to each well, and the cells were incubated for 1.5 h. A microplate reader was used to measure the absorbance at 450 nm (TECAN).

Western blot analysis

Cells were processed to obtain proteins for 10% SDS-PAGE and proteins were transferred onto pre-activated polyvinylidene difluoride (PVDF) membranes. Protein-containing membranes were sealed with 5% skimmed milk powder and incubated overnight at 4°C with specific primary antibodies. The membranes were washed with TBST and incubated with the corresponding horseradish peroxidase-coupled secondary antibody at 37°C for 1 h. ECL solution was used for colour development and photographed using a Bio-Rad gel imaging system. The housekeeping protein tubulin was used as a reference in this study.

Statistical analysis

All data obtained in this study were processed using the GraphPad Prism 8, SPSS 26.0 and PS. If the obtained sample means had equal variance, the LSD method was adopted; otherwise, the T3 test was introduced. A one-way analysis of variance was used for comparison. The immunohistochemical staining results recorded in the study were subjected to protein integrated densitometric analysis. $P < 0.05$ indicated a significant difference.

RESULTS

Effect of marein on serum TG, TC, LDL, HDL and BGC in db/db Mice

Through kit detection, it was found that TG ($P < 0.01$), TC ($P < 0.05$), LDL ($P < 0.05$) and BGC ($P <$

0.01) of db/db mice were significantly higher than the control group. This is consistent with the biochemical features of diabetes. It could be seen that after 12 weeks of intervention, FBG was effectively controlled in all mice ($P < 0.05$). After marein had been applied by gavage for 12 weeks, the related factors of mice were controlled, and the comparison showed a significant difference ($P < 0.05$) (Fig. 1).

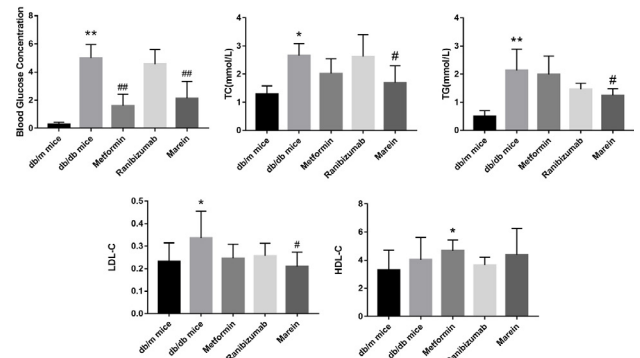


Fig. 1. Effect of marein on serum TG, TC, LDL, HDL and BGC in db/db mice.

Note: The values were expressed as $\bar{x} \pm \text{SEM}$, $n \geq 6$, * $P < 0.05$, ** $P < 0.01$ indicated statistical significance compared with the control group. # $P < 0.05$, ## $P < 0.01$ indicated statistical significance compared with the model group.

Marein can improve the structure and function of retinal tissue in db/db mice

According to the results of H and E staining (Fig. 2A), the db/m mice did not show any abnormalities. The db/db mice exhibited various degrees of retinal oedema and cellular swelling. After the intervention of the positive drug, the symptoms of the lesions were significantly relieved. In addition, the intervention of marein has a good therapeutic effect on retinal oedema and cell swelling in db/db mice.

Effect of marein on the proliferation and apoptosis of retinal tissue in db/db mice

Apoptosis was determined using TUNEL analysis, and the expression of the proliferation marker Ki67 in retinal sections was detected by immunofluorescence. As shown in Figure 2B, the results showed less apoptosis in the db/m mice group, while there was an abnormal increase in apoptosis in the db/db mice group. After metformin intervention, the apoptosis was significantly reduced compared with the model group. The ranibizumab intervention group showed significant apoptosis compared with the normal group. The marein intervention group showed significant differential apoptosis reduction

compared with the model group. It is speculated that marein may affect DR by inhibiting the apoptotic pathway.

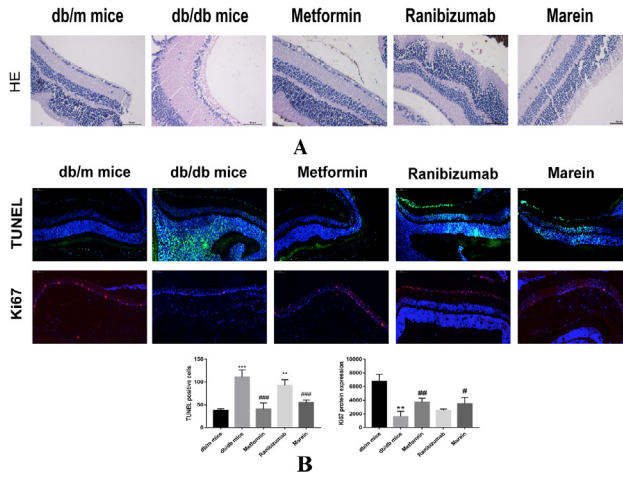


Fig. 2. A: HE staining of retina tissue in experimental mice (400×); B: Effect of marein on proliferation and apoptosis of retinal tissue in db/db mice. Note: The values were expressed as $\bar{x} \pm SEM$, $n \geq 6$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicated statistical significance compared with the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ indicated statistical significance compared with the model group.

The immunofluorescence results showed that in contrast to the db/m mouse group, the expression of the proliferation marker Ki67 in the db/db mouse group was significantly inhibited. This indicated that cell proliferation in retinal tissue of the db/db mouse group was impaired. Compared with the db/db model group, the expression of Ki67 was significantly recovered after metformin treatment ($P < 0.01$). However, the Ki67 expression did not change significantly after ranibizumab treatment. Ranibizumab treatment had little effect on cell proliferation. In addition, marein treatment significantly upregulated the expression of Ki67 in db/db mice. Overall, marein inhibits apoptosis and promotes cell proliferation, thereby controlling diabetic retinopathy.

Marein can affect VEGF-A and SYK protein expression levels in retinal tissue of db/db mice

The immunohistochemical staining results shown in Figure 3A show that VEGF-A expression was significantly downregulated in db/m mice in the normal group. At the same time, the expression of VEGF-A was significantly upregulated in the db/db model mice compared with the normal group, and the statistical results showed $P < 0.01$. The expression of this factor was inhibited to a greater extent in the ranibizumab intervention group compared

with the db/db model group ($P < 0.01$). In addition, the statistical results showed a smaller difference in VEGF-A expression between the metformin and db/db model group. Remarkably, M intervention significantly inhibited the A protein expression in the db/db mice ($P < 0.01$). Interestingly, the mRNA of VEGF-A also showed a trend consistent with the protein level (Fig. 3B). It is implied that the regulation of VEGF-A expression by marein is transcription-dependent and transcription-independent.

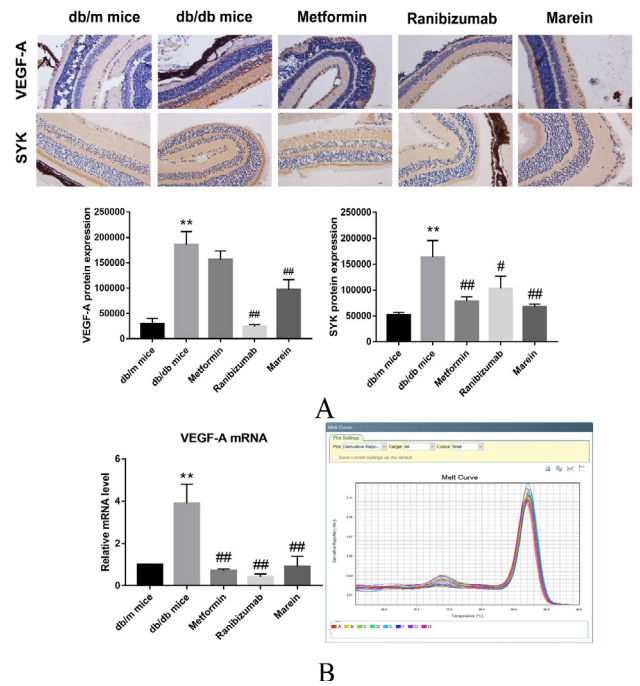


Fig. 3. A: Immunohistochemical staining of VEGF-A and SYK in experimental mice retinal tissue (200×). Note: The values were expressed as $\bar{x} \pm SEM$, $n \geq 6$, * $P < 0.05$, ** $P < 0.01$ indicated statistical significance compared with the control group. # $P < 0.05$, ## $P < 0.01$ indicated statistical significance compared with the model group; B: Effect of Marein on mRNA levels of retinopathy related proteins in retinal tissue of db/db mice. Note: The values were expressed as $\bar{x} \pm SEM$, $n \geq 6$, * $P < 0.05$, ** $P < 0.01$ indicated statistical significance compared with the control group. # $P < 0.05$, ## $P < 0.01$ indicated statistical significance compared with the model group.

After a period of observation, it could be seen that the expression of SYK was moderate in normal db/m mice. After further comparison, it could be seen that the expression of SYK was significantly upregulated in the db/db model group compared with the normal group. The statistical results showed $P < 0.01$. After a period of intervention, SYK was significantly inhibited in the db/db model and metformin intervention groups, and

the statistical results showed $P < 0.01$. The ranibizumab intervention group expressed the same effect compared with the db/db model group. However, after a period of intervention in mice using marein, the inhibition of SYK was more significant compared with mice in the db/db model group, with a significant difference ($P < 0.01$) (Fig. 3A).

Effects of high glucose and marein on cell viability

After the ARPE-19 cells were treated with high glucose for two days, the morphology of the ARPE-19 cells also changed when the concentration of high glucose intervened in these cells was changed, and the ARPE-19 cells changed from oval to fibre-like long shuttle shapes. The proliferation of the ARPE-19 cells would be inhibited when the high glucose concentration increased, as shown in Figure 4A.

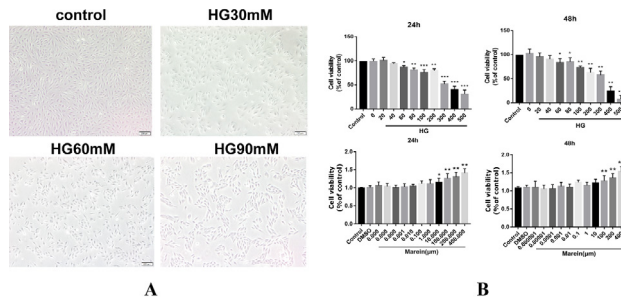


Fig. 4. A: Effect of high glucose on cell morphology and quantity. B: Effects of high glucose and marein on cell viability. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The cell viability decreased when the concentration of high glucose in the ARPE-19 cells and treatment time increased. Compared with the control group after one day of high glucose induction, the result showed that the viability of the ARPE-19 cells was significantly decreased when the high glucose concentration was 60 mM, and the difference was statistically significant. The viability of the ARPE-19 cells was further decreased when the high glucose concentration was 80 mM, and the difference was more significant ($P < 0.01$). The viability of the ARPE-19 cells was significantly decreased after two days of high glucose treatment, and their viability was further decreased when the high glucose concentration was 100 mM, and the difference was more significant ($P < 0.01$), as shown in Figure 4B.

When marein was added, the viability of the ARPE-19 cells increased with the increase of treatment time when the concentration of marein became higher. After treatment of the ARPE-19 cells for one day, their viability increased significantly when the concentration of marein was 10

mM, and the difference was significant ($P < 0.05$). At 100 mM, the viability of the ARPE-19 cells further increased, and the difference was more significant ($P < 0.01$). After two days of treatment, compared with the control group, the viability of the ARPE-19 cells increased significantly when the high glucose concentration was 100 mM, as shown in Figure 4B.

Effect of high glucose on protein expression and fibrosis-related parameters in ARPE-19 cells

When the added high glucose concentration was different, the viability of the ARPE-19 cells was different, and their cellular protein expression was also different. In order to further investigate the effect of high glucose on cellular protein expression and fibrosis indicators, glucose at concentrations of 30, 60, 90, 120 and 150 mM was used for intervention, respectively, for two days. According to the results, when high glucose treatment was performed, intracellular vascular endothelial growth factor (VEGF), actin α -2, intracellular phosphatidylinositol kinase (PI3K), transforming growth factor (TGF- β), SYK and protein kinase B (AKT) were increased, and specifically, when the high glucose concentration rose, the expression also increased. At the same time, fibrosis indicators also increased during the rise of high glucose concentrations, and there was a significant difference in relative indicators between the two groups. In addition, treatment with high glucose would inhibit the expression of the ARPE-19 cell markers. It could be seen that the ARPE-19 cells would develop towards fibrosis after high glucose treatment. In addition, there were significant differences in each index of fibrosis between the two groups when the high glucose concentration was 60 mM, so this was used as the ARPE-19 cytopathic modelling concentration (Fig. 5).

Effect of marein on protein expression in ARPE-19 cells treated with high glucose and fibrosis-related parameters

The results (Fig. 6) show that high glucose could significantly increase the expression of VEGF-A protein in the ARPE-19 cells ($P < 0.01$), and metformin, ranibizumab and marein could downregulate the expression of VEGF-A protein to different extents after the intervention of which the expression of VEGF-A protein downregulated by metformin was the most significant ($P < 0.01$). Compared with the high glucose group, metformin, ranibizumab and marein also decreased the abnormal expression of SYK, of which marein was the most significant. Metformin and marein inhibited the abnormal expression of PI3K, but ranibizumab did not significantly change the expression of PI3K protein compared with the model group. Metformin, ranibizumab and marein interventions all reduced the abnormal expression of TGF- β in the high glucose group.

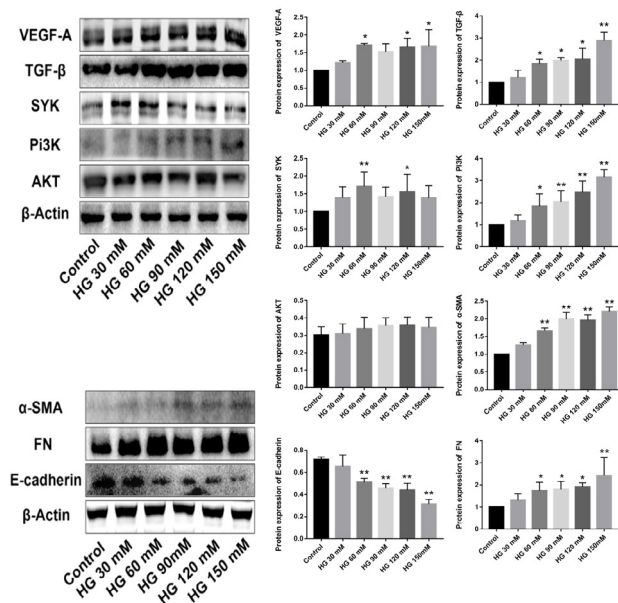


Fig. 5. Effect of high glucose on protein expression and fibrosis-related parameters in ARPE-19 cells. *P < 0.05, **P < 0.01.

For the fibrosis indicators α -SMA and FN, only metformin and marein had an inhibitory effect on them, while ranibizumab monotherapy did not play a role in reducing fibrosis indicators. For the expression of the epithelial marker E-cadherin, high glucose could promote the occurrence of epithelial-mesenchymal transition, thereby reducing its expression, and only marein could rebound its expression.

Molecular docking predicts the binding of M to VEGF-A, PI3K and SYK proteins

The spatial structures of ligands and receptors were downloaded from the ZINC small molecule and PDB protein structure databases. Conventional operations such as hydrogenation and charging of receptors and ligands were performed with the Discovery Studio 4.5 software, and parameters such as the docking centre and pocket size were obtained. Docking was performed with the Auto Dock Vina tool, and finally, the results were processed with DS software. Metformin, a common clinical diabetic drug, was used as a control, and an affinity <0 indicated that the receptor was able to spontaneously bind to the ligand, and the affinity -5.0 kcal/mol was used as a threshold to evaluate the ability of marein to target VEGF-A, PI3K and SYK. The results show that marein had good binding to VEGF-A, PI3K and SYK and was better than metformin. Among them, the best binding of marein to SYK was -7.8 kcal/mol, and its force was mainly formed by H-bond interactions

with amino acid residues SER379, ASN499 and ASP512 of SYK, van der Waals forces with GLY454 and non-covalent bond interactions such as amide- π stacking with LEU453. The docking results showed that marein had good binding to these three proteins, which was used as a reference for later experimental validation (Table II).

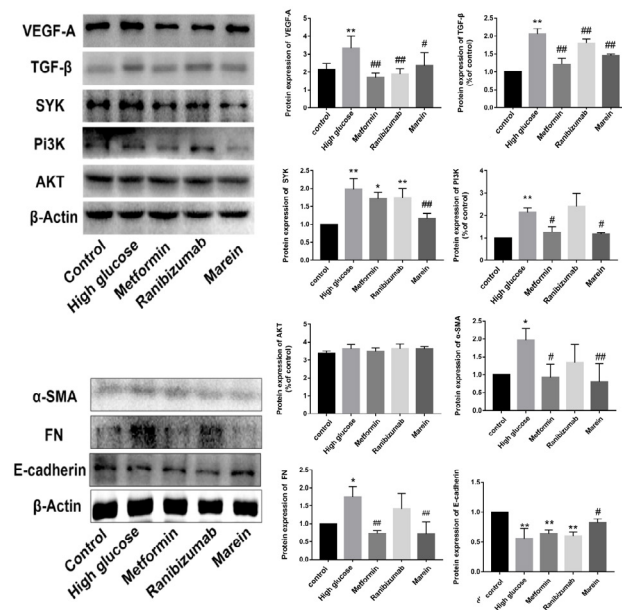


Fig. 6. Effect of marein on protein expression in ARPE-19 cells treated with high glucose. Note: *P < 0.05, **P < 0.01 indicated statistical significance compared with the control group. #P < 0.05, ###P < 0.01 indicated statistical significance compared with the model group.

Table II. Docking results of marein and positive drug metformin with VEGFA, PI3K and SYK molecules, respectively.

Classification	Drug name	Gene name	PDB ID	Affinity / kcal·mol ⁻¹
Positive drug	Metformin	VEGFA	1VPP	-4.5
		PI3K	1H9O	-3.9
		SYK	3SRV	-4.1
Sample	Marein	VEGFA	1VPP	-7.2
		PI3K	1H9O	-6.1
		SYK	3SRV	-7.8

DISCUSSION

Type 2 diabetes mellitus (T2DM) is now a worldwide epidemic (Al-Aubaidy *et al.*, 2021; Feng *et al.*, 2021). It is a syndrome that comprises disorders of sugar, fat and

protein metabolism and is caused by insulin deficiency and/or a reduction in insulin sensitivity of the target tissues (Mohamed *et al.*, 2021). T2DM represents a serious threat to human health, but a number of conventional medications have been developed that can effectively control T2DM and its complications (Hu *et al.*, 2021). However, the long-term use of such drugs can be associated with adverse reactions. Therefore, with the increase in the number of patients with T2DM, research efforts have focused on the production of safer and more effective drugs (Wang *et al.*, 2020). The db/db mouse is a useful model of T2DM because it manifests many of the pathological features of the human disease, including obesity and insulin resistance (IR). Patients with T2DM also demonstrate disorders of lipid metabolism. High TG and FFA concentrations increase beta-cell apoptosis, which reduces insulin secretion at an early stage of the pathogenesis of the disease, and is referred to as lipotoxicity. In this study, after marein was intervened in the mice, it was shown that FBG in serum was effectively controlled, indicating that coreopsis extract has the effect of controlling blood sugar, which provides a new idea for research and treatment of diabetes. In addition, marein inhibited the expression of TG and cholesterol, while metformin did not. The above description shows that the drug has great clinical application value.

The distribution and generation of new blood vessels was undoubtedly the primary factor directly leading to DR, which would develop into severe retinopathy or even blindness if it continued (Wong and Sabanayagam, 2019). However, marein, metformin and ranibizumab could alleviate the above conditions, indicating that ranibizumab, which is specifically used for vitreous injection in the eyeball, is effective in DR and lowering blood glucose, but metformin can also play a protective role in the retina (Li *et al.*, 2018). Under *in vitro* culture conditions, we found that marein had no significant inhibitory effect on cells, but high concentrations of marein could directly enhance the cell viability, and, in a short period of time, an increase in marein concentration could cause an increase in the viability of the retinal epithelial cells ARPE-19. It has a certain protective effect on the early progression of DR and provides a valuable experimental basis as well as research direction for the subsequent study of marein.

Marein could act on db/db mice retinal cells and be involved in the apoptosis and proliferation of such cells. In the process of analysing its mechanism of action, we used TUNEL to analyse apoptosis (Li *et al.*, 2018) and introduced related techniques to detect changes in the expression levels of the proliferation marker Ki67 (Li *et al.*, 2018) to better understand the mechanism of cell proliferation. The results showed little apoptosis without lesions, while the db/db diabetic mouse model was the

opposite. We could speculate that db/db mouse retinas showed high blood glucose due to genetic defects, during which cells were rapidly apoptotic. The comparison between the groups showed that ranibizumab intervention in mice could inhibit the production of new blood vessels (Joachim *et al.*, 2017). However, for apoptosis, ranibizumab did not have any effect, while the other two drugs could have a therapeutic effect.

In terms of cell proliferation, the expression of Ki67 was upregulated, and cells divided rapidly in the db/m normal mouse group, in contrast with the lower expression level of Ki67 in the db/db diabetic group (Takahashi *et al.*, 2018). Therefore, it was speculated that there was a close link between the proliferation of cells and high glucose, which would cause impaired cell proliferation in the case of high blood lipids in tissues. However, the expression of Ki67 was significantly upregulated after intervention with marein, which could also regulate blood glucose and thereby enhance the divisibility of cells, thus playing a role in the treatment of diseases. There was little difference between the db/db model and the ranibizumab intervention groups. This was because ranibizumab did not regulate blood glucose and therefore had no effect on cellular activity, and the condition could not be relieved after intervention. On the other hand, the other two drugs improved cell proliferation and were involved in apoptosis.

In *in vitro* experiments, to observe the inhibitory effect of high glucose on the PI3K-AKT signalling pathway and its effect on endothelial cell proliferation, after treating human retinal epithelial cells with different concentrations of D-glucose, we found that high glucose significantly inhibited the proliferation of epithelial cells (Baj *et al.*, 2020) and the expression of PI3K and AKT. We suspected that high glucose might inhibit epithelial cell proliferation through the PI3K-AKT signalling pathway and participate in the development of diabetic macrovascular and microvascular complications (Song *et al.*, 2019). Therefore, we believe the PI3K-AKT signalling pathway is very important. We also found that both the metformin and marein groups, as well as our targeted VEGF inhibitor ranibizumab group, inhibited abnormally high expression of VEGF at the mRNA level in the retinal tissue of db/db diabetic mice, and the effects were statistically significant. The detection of the expression of VEGF at the mRNA level in the retina of mice can effectively improve the judgement of high vascular endothelial cell division ability because such cytokines are involved in vascular endothelial cell migration, proliferation and also have an important promoting effect on angiogenesis (Han *et al.*, 2019). We believe that VEGF plays a role in promoting vascular permeability and maintaining the normal function of blood vessels. We

observed the expression of VEGF at the mRNA level in the retina of the db/db diabetic mice by the PCR technique. Interestingly, we found that when the expression level of VEGF was dramatically increased in the db/db diabetic mice, highly expressed VEGF could lead to the formation of neovascularization in the macular area of the eyeball, which was also accompanied by the occurrence of high leakage (Sodhi *et al.*, 2019). Neovascularization is fragile, prone to oedema and bleeding; if not treated, it may cause blindness and embolism in the retinal veins. However, the three groups of administration in our study had a better effect on it at the mRNA level, and all of them inhibited the expression of VEGF-A. Thus, in previous experiments, we were more certain about the effects of marein, metformin and VEGF-A monoclonal antibodies, which provided valuable experience for subsequent studies.

The western blot results showed that high glucose (60 mM) could significantly increase the expression of VEGF-A protein in the ARPE-19 cells, and metformin, ranibizumab and marein could downregulate the expression of VEGF-A protein to different extents, with metformin downregulating the expression of VEGF-A protein most significantly. Therefore, we speculated that metformin at the cellular level could reduce the expression of the pathogenic factor VEGF in the cytoplasm in a way that the other two drugs do not. Compared with the high glucose group, metformin, ranibizumab and marein also decreased the abnormal expression of SYK, of which the marein group was the most obvious, which was consistent with the *in vivo* experiment. Therefore, for the SYK pathway, we would pay more attention to the effect of marein on its upstream and downstream, strive to directly find the target and confirm the mechanism. Metformin and marein inhibited the abnormal expression of PI3K, but ranibizumab did not significantly change the PI3K protein compared with the model group. Metformin, ranibizumab and marein administration interventions could reduce the abnormal expression of TGF- β in the high glucose group, which indicated that metformin, ranibizumab and marein could all have an effect on cells, thereby leading to the original abnormally high expression of TGF- β in the cytoplasm from the protein level, thereby preventing the occurrence of fibrosis in the disguised form (Hu *et al.*, 2018).

For fibrosis indicators α -SMA and FN, only metformin and marein had an inhibitory effect on them, which is curious, indicating that anti-VEGF alone is insufficient to improve fibrosis indicators. On the contrary, previous studies have demonstrated that the greatest adverse reaction of ranibizumab was that patients with an injection time of more than two years had different degrees of retinal fibrosis lesions. Therefore, although

anti-VEGF therapy alone could fundamentally inhibit the production of VEGF, fibrosis was something we did not want to see; thus, we needed to pay more attention to its adverse reactions along with anti-VEGF therapy to provide countermeasures. Ranibizumab alone did not play a role in reducing fibrosis parameters. Finally, for the expression of the epithelial marker E-cadherin, high glucose could promote the occurrence of the epithelial-mesenchymal transition, thereby reducing its expression. Interestingly, only marein could rebound the expression of epithelial marker E-cadherin, which had a deeper significance for us to study the coreopsis tinctoria extract marein against diabetes as well as anti-retinopathy and anti-fibrosis processes, which also had a far-reaching effect for us to explore marein on DR. It provides a valuable theoretical basis for subsequent studies.

The spatial structures of different protein ligands and receptors were downloaded based on the database, and the two were simulated using software for hydrogenation and charging. Finally, parameters such as pocket size were obtained, based on AutoDock Vina (Quiroga and Villarreal, 2016), to complete docking, and DS software was used to analyse and process the results. In this process, metformin administration was used as the control group. When the affinity was less than the threshold value 0, it indicated that the two could bind under the interaction of the drug. The effect of marein targeting VEGF-A, PI3K and SYK was evaluated at an affinity threshold of $-5.0 \text{ kcal/mol} -1$. Based on the results of the rating case, it could be seen that this drug had a good affinity to the expression substance, and the effect was better than metformin administration. Marein had the best affinity to SYK. As shown in the Table II, the specific mechanism is that hydrogen bonds can be formed between marein and SYK amino acid residues, and the intermolecular force becomes larger. The analysis results show that marein has a good affinity for these three proteins, and the software analysis results provide the necessary reference for later experiments.

CONCLUSION

Marein can improve glucose and lipid metabolism disorders in db/db mice after a period of treatment, which has positive significance for the prevention and treatment of diabetes. Marein can alleviate the degree of DR by inhibiting retinal tissue cell apoptosis and promoting cell proliferation. Marein can act on SYK, VEGF and PI3K/AKT factors and change the expression of fibrotic and inflammatory factors in tissues by affecting their pathways. This study has reference significance for the development of drugs to treat diabetes and its complications.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Supplementary material

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Statement of conflict of interest

The authors have declared no conflict of interest.

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