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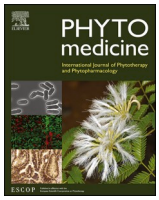

Original Article

Protective effect of alizarin on vascular endothelial dysfunction via inhibiting the type 2 diabetes-induced synthesis of THBS1 and activating the AMPK signaling pathway

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ABSTRACT

Background: In this study, we investigated the protective effects of alizarin (AZ) on endothelial dysfunction (ED). AZ has inhibition of the type 2 diabetes mellitus (T2DM)-induced synthesis of thrombospondin 1 (THBS1). Adenosine 5'-monophosphate- activated protein kinase (AMPK), particularly AMPKα2 isoform, plays a critical role in maintaining cardiac homeostasis.

Purpose: The aim of this study was to investigate the ameliorative effect of AZ on vascular injury caused by T2DM and to reveal the potential mechanism of AZ in high glucose (HG)-stimulated human umbilical vein endothelial cells (HUVECs) and diabetic model rats.

Study design: HUVECs, rats and AMPK$^{-/-}$ transgenic mice were used to investigate the mitigating effects of AZ on vascular endothelial dysfunction caused by T2DM and its *in vitro* and *in vivo* molecular mechanisms.

Methods: In type 2 diabetes mellitus rats and HUVECs, the inhibitory effect of alizarin on THBS1 synthesis was verified by immunohistochemistry (IHC), immunofluorescence (IF) and Western blot (WB) so that increase endothelial nitric oxide synthase (eNOS) content *in vitro* and *in vivo*. In addition, we verified protein interactions with immunoprecipitation (IP). To probe the mechanism, we also performed AMPKα2 transfection. AMPK's pivotal role in AZ-mediated prevention against T2DM-induced vascular endothelial dysfunction was tested using AMPKα2$^{-/-}$ mice.

Results: We first demonstrated that THBS1 and AMPK are targets of AZ. In T2DM, THBS1 was robustly induced by high glucose and inhibited by AZ. Furthermore, AZ activates the AMPK signaling pathway, and recoupled eNOS in stressed endothelial cells which plays a protective role in vascular endothelial dysfunction.

Conclusions: The main finding of this study is that AZ can play a role in different pathways of vascular injury due to T2DM. Mechanistically, alizarin inhibits the increase in THBS1 protein synthesis after high glucose induction and activates AMPKα2, which increases NO release from eNOS, which is essential in the prevention of vascular endothelial dysfunction caused by T2DM.

Abbreviations: Ach, acetylcholine; AMPK, AMP-activated protein kinase; AZ, alizarin; EDR, endothelium-dependent vasorelaxation; eNOS, Endothelial nitric oxide synthase; GHb, Glycated hemoglobin; HUVECs, human umbilical vein endothelial cells; IR, insulin resistance; NO, nitric oxide; SNP, sodium nitroprusside; STZ, streptozotocin; T2DM, type 2 diabetes mellitus; VECs, vascular endothelial cells; THBS1, thrombospondin 1.

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Introduction

Previously known as "noninsulin-dependent diabetes mellitus" or "adult-onset diabetes mellitus," (Henning, 2018), type 2 diabetes mellitus (T2DM) is present in 90–95 % of all adult patients with diabetes (Xu et al., 2018). Approximately 95 % of adults have type 2 diabetes mellitus, which is primarily brought on by insulin resistance (IR) and insufficient insulin production (DeFronzo, 2004). Study has demonstrated that T2DM can cause a number of complications and multiple organs injury, such as nephropathy (Thipsawat, 2021), neuropathy (Agashe and Petak, 2018), retinopathy (Saw et al., 2019), and accelerates the development of cardiovascular disease (Quinn, 2002). Research has demonstrated that endothelial function is compromised in macro- and micro-vascular problems resulting from hyperglycemia in both animal models and human subjects (Meza et al., 2019). However, there is still no effective and clinical treatment to improve endothelial dysfunction caused by T2DM. Therefore, it is important to prob the pathogenesis of vascular function injury caused by T2DM deeply and seek effective therapeutic methods.

Patients with T2DM have a 2-3-fold increased risk of cardiovascular disease compared to non-T2DM patients (Emerging Risk Factors et al., 2010) and cardiovascular disease accounts for approximately 80 % of mortality in T2DM (Emerging Risk Factors et al., 2015). Abnormal metabolism in T2DM including IR, dyslipidemia, and other metabolic milieus act on the vascular wall, leading to vascular endothelial dysfunction (Kaur et al., 2018). Vascular endothelial dysfunction is a potential factor in the pathogenesis of diabetic vascular disease, where an abnormal metabolic environment leads to an imbalance of endothelium-derived dilating and contracting factors (Dhananjayan et al., 2016), resulting in vascular and end-organ damage. The vasodilator factor NO, for example, is produced by endothelial nitric oxide synthase, but under pathological conditions eNOS uncouples and NO production is reduced (Harrison, 1997).

Alizarin is a compound belonging to the anthraquinone group, which is extracted from the root of a traditional Chinese herb called *Rubia cordifolia* L (Xu et al., 2022). *Rubia* has the effects of hemostasis, stasis-dispersing. Numerous biological actions have been described for alizarin (Takahashi et al., 2002), including an antigenotoxic activity (Fotia et al., 2012) and a strong inhibitory effect on tumor cell development that has been confirmed. Because alizarin red staining is a calcium chelator, it was employed for Jie D (Ding et al., 2021) et al. to aid see the bone and demonstrate skeletal mineral content; Alizarin has the ability to lower blood sugar and enhance IR, as shown by Lei X et al. (Xu et al., 2019). The study by LT Xu et al. concluded that AZ significantly reduces fasting blood glucose, postprandial blood glucose in diabetic mice and may be an effective compound for improved IR (Xu et al., 2019). Although these effects of alizarin have been widely known for many years, its mechanism is still unclear in improving type 2 diabetes-induced vascular function injury.

Since it is the primary source of nitric oxide (NO) generation in vascular endothelial cells (VECs), endothelial nitric oxide synthase (eNOS) is a crucial regulator of cardiovascular homeostasis. Because vascular smooth muscle can relax as a result of NO release from VECs, eNOS is essential for blood vessel vasodilation and blood pressure management (Yu et al., 2021). It is believed that hyperglycemia causes vascular damage by altering the bioavailability of NO and causing reactive oxygen species (ROS) and reactive nitrogen species (RNS) to build up, which causes endothelial dysfunction (Kaur et al., 2018). All eukaryotic cells include the highly conserved AMP-activated protein kinase (AMPK) sensor of cellular energy state. Stimuli that raise the cellular AMP/ATP ratio activate AMPK (Shaw et al., 2004). Dysregulation of energy homeostasis is considered to be a major cause behind the alterations in many different human diseases, including T2DM (Carling, 2017). For the purpose of preventing or treating T2DM, activation of AMPK in skeletal muscle, liver, and adipose tissue improves metabolism, insulin sensitivity, and gene expression (Long and Zierath, 2006).

The glycoprotein known as secreted thrombospondin-1 (THBS1) has a molecular mass of 150–180 kDa (Isenberg and Roberts, 2020). The known regulatory functions of THBS1 have expanded from disrupting cell adhesion to modulating inflammation, responses to hypoxic and genotoxic stress, redox signaling, stem cell self-renewal, and autophagy (Roberts and Isenberg, 2021). Research has shown that THBS1 controls endothelial-dependent vasorelaxation and eNOS activation through Cell surface receptor 47 (CD47) (Bauer et al., 2010).

Based on the results of the above studies, we used alizarin in the prevention and treatment of T2DM-related vascular impairment. In this study, we hypothesized that alizarin exerts a protective effect against type 2 diabetes-induced vascular functional impairment by inhibiting high-glucose (HG) and high-fat-induced THBS1 synthesis and activating AMPKα2. In this study, we investigated the mechanisms by which alizarin regulates THBS1 as well as AMPKα2 to ameliorate endothelial dysfunction in T2DM, enriching the study of related mechanisms and providing theoretical support for disease-targeted therapies.

Materials and methods

Materials

Sigma supplied alizarin with a purity level of $>$ 95.0 %. To cause experimental diabetes, streptozotocin (STZ) was obtained from Sigma (Sigma Aldrich, St. Louis, MO, USA). Sigma Aldrich (St. Louis, MO) provided the acetylcholine (ACh, cas. 9000-81-1) and sodium nitroprusside (SNP, cas. 13755-38-9) that were acquired. Proteintech Group (Rosemount, Illinois, USA) provided the antibody against THBS1 (NO: 67241-1-Ig), and Abcam (Cambridge, MA, USA) provided the antibody against AMPKα2 (NO: ab105028) and CD47 (NO: ab314170). The source of the antibody against eNOS (NO: #32027) and IgG (NO: #2729) was Cell Signaling Technology, located in Boston, USA. The source of the GAPDH antibody (NO: #AF7021) was Affinity Bioscience, located in Cincinnati, Ohio, in the United States. The Nanjing Jiancheng Institute of Biological Engineering (Nanjing, Jiangsu, China) provided the nitric oxide assay kit.

Animals

Purchased from Henan Experimental Animal Centre, these male Sprague-Dawley (SD) rats (n = 48) weighed 220 ± 10 g and were 8–10 weeks old. Male mice devoid of the AMPKα2 gene (AMPKα2$^{-/-}$ mice) (n = 24) weighing 20-25 g and aged 6-8 weeks were acquired from the Department of Endocrinology, Metabolism and Cancer, Institute Cochin, Universite Paris Descartes, France. Every animal was raised at the Xin-Xiang Medical University's SPF-level animal housing, fed only conventional feed and pure water, and maintained at a consistent temperature of 21±2 ℃ and 40 %−60 % humidity with a 12-hour light/12-hour dark cycle. Every experiment was conducted under rigorous with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was reviewed and approved by the Ethics Committee of Xinxiang Medical University (No: XXLL-2022S579).

Establishing model and experimental group

In order to create type-2 diabetes mellitus models, the rats and mice in the experimental group were fed a diet high in fat and carbohydrates (which included 10 % lard, 2.5 % cholesterol, 20 % saccharose, 1 % sodium cholate, and 66.5 % basic diets) for the duration of the experiment. A single intraperitoneal injection of streptozotocin (STZ; pH 4.5) in 0.1 mol/L of citrate acid buffer at 150 mg/kg in mice and 60 mg/kg in rats was administered to the diabetic animals four weeks later. Following three days, the animals' caudal veins were used to measure the blood glucose levels. After two consecutive fasting blood glucose values of ≥16.7 mmol/L, the diabetes model was deemed successful and

was incorporated into the subsequent experiment. Glycated hemoglobin (GHb) was detected by double antibody sandwich method.

The current study on animals consisted of two parts; the first animal study employed SD rats. Following a week of adaptive feeding, the rats were split into six groups at random and given the following care: (One) control group ($n = 8$), (two) Alizarin groups (10 mg/kg/day, $n = 8$), (three) T2DM groups ($n = 8$), (four) T2DM + Alizarin groups (10 mg/kg/day, $n = 8$), (five) T2DM + Metformin groups (100 mg/kg/day, $n = 8$), (six) T2DM + Alizarin groups (10 mg/kg/day, $n = 8$) + Metformin groups (100 mg/kg/day, $n = 8$). Oral gavage (po) was used to provide medication to all rats for a duration of four weeks.

The purpose of the second animal investigation is to determine whether or not alizarin is AMPK-dependent in its ability to prevent T2DM-induced vascular function damage.

After a week of adaptive feeding, AMPKα2$^{-/-}$ mice were randomly assigned to four groups and given the following treatments: The study included four groups of mice: (1) AMPKα2$^{-/-}$ ($n = 6$); (2) AMPKα2$^{-/-}$ mice + Alizarin (15 mg/kg/day, $n = 6$); (3) AMPKα2$^{-/-}$ mice + T2DM ($n = 6$); and (4) AMPKα2$^{-/-}$ mice + T2DM + Alizarin (15 mg/kg/day, $n = 6$). Oral gavage (po) was used to provide medications to all AMPKα2$^{-/-}$ mice for a duration of 4 weeks.

Isoflurane inhalation was used to put the mice to sleep, and then the aortas were carefully removed in preparation for the next set of trials.

Pharmacokinetic study

SD rats were administered alizarin (10 mg/kg) by oral gavage and fasted for 12 h prior to the administration of alizarin. Blood samples were collected from the orbital venous sinus by puncture at 0.25, 0.33, 0.5, 0.67, 1, 1.5, 2, 3, 4, 6, 7.7, 18, and 24 h before and after administration, respectively, and placed in heparinised EP tubes, which were immediately centrifuged at 12,000 g for 10 min at 4℃ to separate the plasma. 100 μl of plasma was mixed with 20 μl of 1 % formic acid for further analysis. The blood drug concentration at each collection time point was calculated according to LabSolutions LCMS Ver.5.6 data processing software, and the data were automatically fitted with DAS 2.1 pharmacokinetic software package to calculate the relevant pharmacokinetic parameters, including C_{max}, T_{max}, $t_{1/2}$, $AUC_{0\rightarrow t}$, and so on.

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection Center (ATCC, Manassas, VA, USA). HUVECs were cultivated at 37 ℃ in an incubator with 95 % humidified air and 5 % CO2 and passaged every three days in Endothelial Cell Medium (ECM, ScienCELL, California, CA, USA) supplemented with 10 % fetal bovine serum (ScienCELL, California, CA, USA), 100 μg/mL penicillin, and 100 μg/mL streptomycin.

Six groups of cells were created, and each group received the following treatment: (1) Control group: cells did not receive any drug during the experiment. (2) Alizarin group: HUVECs were incubated with 10 μM alizarin for 72 h. (3) High glucose group: HUVECs were incubated with 30 mmol/L glucose for 72 h. (4) High glucose + Alizarin group: After HUVECs were incubated with 30 mmol/L glucose for 72 h, cells were incubated with 10 μM alizarin for 72 h. (5) High glucose + Low expression of AMPKα2 group: After HUVECs were incubated with 30 mmol/L glucose for 72 h, cultured cells were washed with Serum- and Antibiotic-free Opti-Minimal Essential Medium, seeded in 6-well plates to 70–80 % confluence. Transfection reagent and siRNA are each diluted in serum-free medium, mixed and incubated for 10 min at room temperature to form siRNA/lipid complexes. This complex is then added to each well with siRNA at a final concentration of 0.7 ug/ml per well. Forty-eight hours post-transfection, cells were collected to determine AMPKα2 protein expression levels by Western blot analysis. (6) High glucose + Overexpression of AMPKα2 + Alizarin group: After HUVECs were incubated with 30 mmol/L glucose for 72 h, cells were incubated with 10 μM alizarin for 72 h. Briefly, viral constructs or control vectors are transfected into HEK-293 T cells along with packaging plasmids. Forty-eight hours after transfection, progeny viruses released by HEK-293 T cells were collected and filtered for HUVEC infection.

Network pharmacology and molecular docking

The active components of alizarin were screened using the Bioinformatics Analysis Tool for Molecular Mechanism of Traditional Chinese Medicine (BATMAN-TCM) database and the Swiss Target Prediction database. The Swiss Target Prediction and BATMAN databases were used to obtain targets. DisGeNET and GeneCards databases were searched for proteins associated with T2DM. Using common AZ/T2DM targets from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, a protein-protein interaction (PPI) network was built. Gene Ontology (GO) function analysis was conducted using the Metascape platform, whereas Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted using the Cytoscape plug-in ClueGO. The software programs AutoDock Vina and iGEMDOCK were used for molecular docking. For network mapping, LigPlos and Pymol were utilized.

Histological analysis

Tetrahydrofuran and gradient alcohol were used to dehydrate the tissues after they had been treated with 4 % paraformaldehyde. The specimens were fixed in paraffin and then cut into transverse slices that were 5 μm thick. Cells were stained with hematoxylin-eosin after being fixed in 95 % alcohol. Hematoxylin-eosin (H&E) staining of tissues and cells was then performed in order to examine the morphological alterations in the myocardium. For the histology analysis, a light electron microscope (400-fled, Olympus) was utilized.

Measurement of endothelium-dependent vasorelaxation

Aortic rings from mice or rats were placed in an organ bath filled with Kreb buffer. Phenylephrine (1 μmol/L) administration elicited a contractile response. Endothelium-dependent vasorelaxation (EDR) was induced at the contraction plateau by adding either sodium nitroprusside (SNP) or accumulative acetylcholine (ACh), as previously mentioned. A ratio of phenylephrine-induced vasoconstriction to ACh- or SNP-induced vasodilation was used to calculate relaxation, with a ratio of 1 representing 100 % of relaxation.

Transfection of siRNA against AMPKα2

We bought scrambled siRNA and small interfering RNA (si-RNA) against AMPKα2 from OriGene Biotechnology (SR321410). Using Lipofectamine 2000 (Invitrogen, USA), scrambled or target siRNA were transfected into HUVECs.

Measurement of NO release

A NO-sensitive fluorescent probe called 4,5-diaminofluorescein-2 (DAF-2) was used to measure the amount of NO released. After adding DAF-2 (5 μM) to the HUVECs, they were incubated at 37℃ for one hour. Using a multifunctional detection microplate reader (Tecan, Genios-Pro, Austria) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, the fluorescence levels of the supernatant were determined.

Immunohistochemical assay

Paraffin sections of tissue were cut to 8 μm, deparaffinized and dehydrated in preparation for immunohistochemical staining. Thermal antigen repair was then performed and the sections were blocked with 5

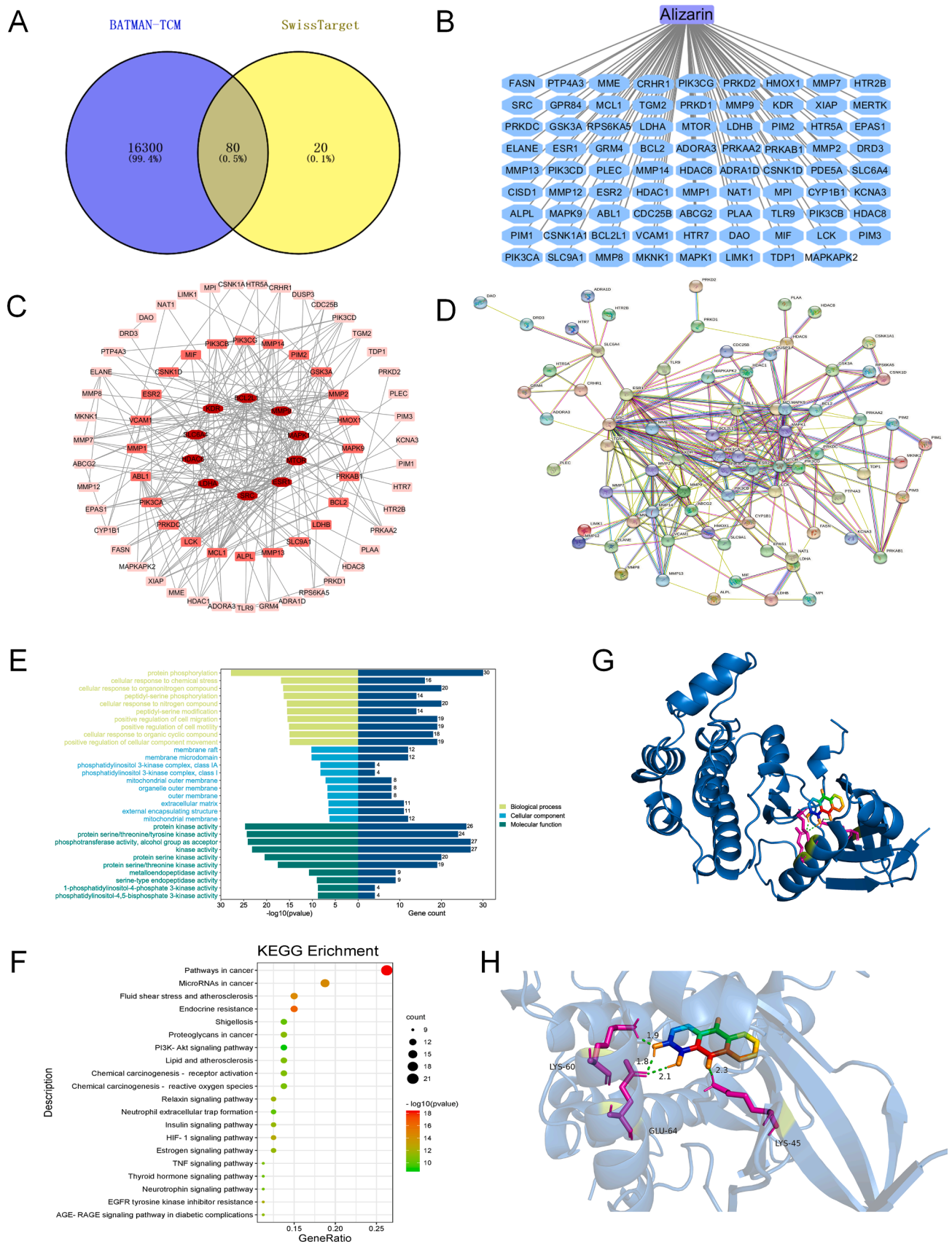

Fig. 1. Construction of the drug-target pharmacology network and molecular docking. (A) The number of potential targets for alizarin by taking an intersection of two databases. (B) The drug-targets interaction pharmacology network. (C) Core targets of drug-targets. The darker the color, the greater degree value which represents. (D) PPI network of potential targets of alizarin. (E) GO enrichment analysis. (F) KEGG pathway analysis of potential targets of alizarin. (G) Molecular models of the binding of alizarin with AMPKα2 shown as 3D diagrams. (H) Detail drawing of the connection between alizarin and AMPKα2.

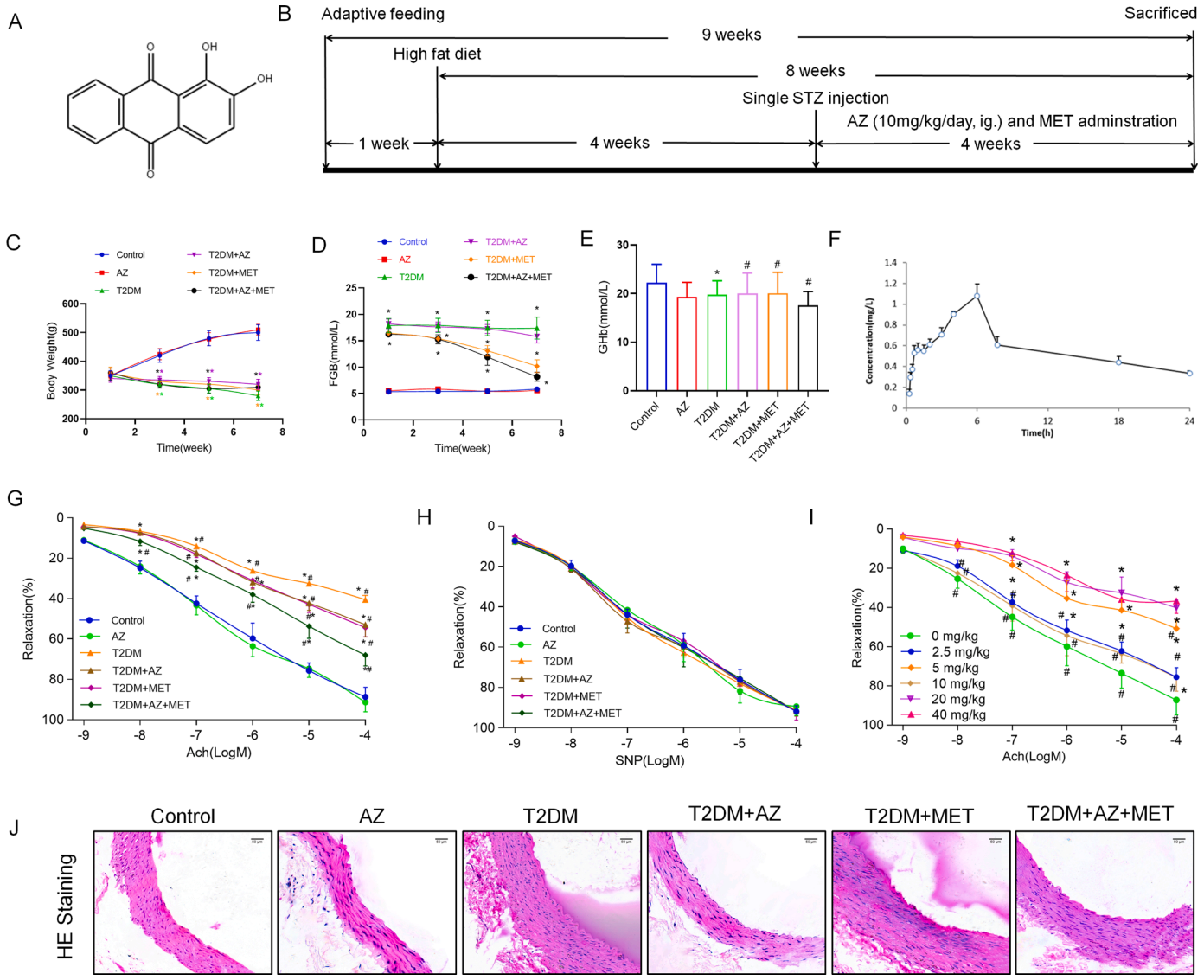

Fig. 2. Effect of AZ on the changes in different indicators of STZ-induced T2DM in rats. (A) Chemical structure of alizarin. (B) The protocol of animal experiment. (C-E) Determination of blood glucose monitoring index, including body weight (C), fasting blood glucose (D), GHb (E). (F) The plasma concentration-time profiles of alizarin after oral administration at 10 mg/kg of alizarin in SD rats (n = 6). (G-H) Endothelium-dependent relaxation induced by acetylcholine (Ach) and endothelium-independent relaxation induced by sodium nitroprusside (SNP) in organ chamber. (I) Endothelium-dependent relaxation induced by acetylcholine (Ach) given different concentrations of alizarin. (J) H&E staining of vascular endothelial sections. All data were expressed as mean ± SD. $^{*}p<0.05$, vs. control; $^{\#}p<0.05$ vs. T2DM group.

% BSA for 30 min. To detect the expression of AMPKα2, eNOS and THBS1, primary antibodies were incubated on sections overnight at 4 ℃. DAB and hematoxylin staining was performed after incubating the secondary antibody for one hour at room temperature.

Immunofluorescence staining

To determine the expression of THBS1, eNOS, and AMPKα2, respectively, primary antibodies were incubated with the sections and cells overnight at 4 ℃ for immunofluorescence labeling. Prior to this, the sections and cells were blocked with 5 % BSA. Following three PBST washes, the slices and cells were evaluated after being mounted with DAPI and treated for two hours at room temperature with a secondary fluorescent antibody in the dark. Every picture was gathered using the digital pathology section scanner.

Immunoprecipitation

Firstly, cell lysates were prepared, cells were collected and then to centrifuge tubes, broken by sonication lysis and supernatants were collected. Protein concentration was determined by BCA. THBS1 IP was performed by spinning 500 μl of lysate with 4 μg of primary antibody or corresponding IgG and incubating overnight at 4 ℃. Next, magnetic beads were added and spun at 4 ℃ for one hour. The beads were then washed 3 times in Wash buffer. Finally, the beads were resuspended in 2X sample buffer and heated at 95 ℃ for 10 min. Immunoblotting is performed as described in the Western Blotting section.

Western blotting

Following the administration of the relevant therapies, total proteins were collected from rats, mice, and HUVECs, and analyses were performed. Equal volumes of the total protein were separated by 12 % SDS-PAGE and transferred to nitrocellulose membranes after the protein

Table 1
The main pharmacokinetic parameters of alizarin after oral administration at 10 mg/kg of alizarin in SD rats (mean ± SD., $n = 6$).

Parameters	Units	Dose(10 mg/kg)
$t_{1/2}$	H	13.524±2.901
T_{max}	H	6.106±0.137
C_{max}	mg/L	1.082±0.102
AUC_{0-96}	mg•h/L	13.52±0.742
$AUC_{0-\infty}$	mg•h/L	20.063±1.421
MRT	H	21.299±3.548
CL	mL/h	1.002±0.077

content was quantitatively determined using a BCA assay kit. The membranes were then washed in blocking solution (5 % skim milk). Anti-rabbit, anti-mouse, or peroxidase (HRP)-conjugated anti-goat secondary antibodies were used to identify proteins, and an ECL substrate kit was used to visualize the results.

Statistical analysis

The means ± standard deviation (SD.) of six separate experiments is used to show the data. Using the Statistics Package for Social Science software, the data were analyzed using ANOVA, and the statistical significance of the differences between the control and treatment groups was determined using an LSD post hoc test. A P value of less than 0.05 was deemed statistically significant.

Results

The results of network pharmacology and molecular docking analyses on alizarin

First, we obtained 100 and 16,300 target genes from the BATMAN-TCM (http://bionet.ncpsb.org/batman-tcm/) and Swiss Target Prediction databases (http://www.swisstargetprediction.ch/), respectively. Then, by taking an intersection of the compound target genes of two databases, we finally obtained the 80 main targets of Alizarin (Fig. 1A). We constructed a protein-protein interaction (PPI) network of the interaction of the Alizarin targets (Fig. 1D). Further, we used CytoScape 3.9.0 software to build a drugs-targets interaction network, as shown in Fig. 1B. We obtained the degrees by using the network analysis cytoNCA plug-in and arranged target genes according to the degree; the greater the node degree, the more biological functions the node has in the network, a visual network was established using Cytoscape v.3.9.0 software (Fig. 1C). We used the Metascape platform (http://metascape.prg/gp/index.html) to conduct Gene Ontology functional analysis (Fig. 1E) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (Fig. 1F) for the 80 main target of alizarin. Finally, Using AutoDock Vina software, the target proteins AMPKα2 in the molecular docking were molecularly docked with the active component alizarin (Fig. 1G, 1H). The docking score is −7.41 kcal·mol^{-1}, which shows AZ could easily enter and bind the active pocket of the AMPKα2 protein.

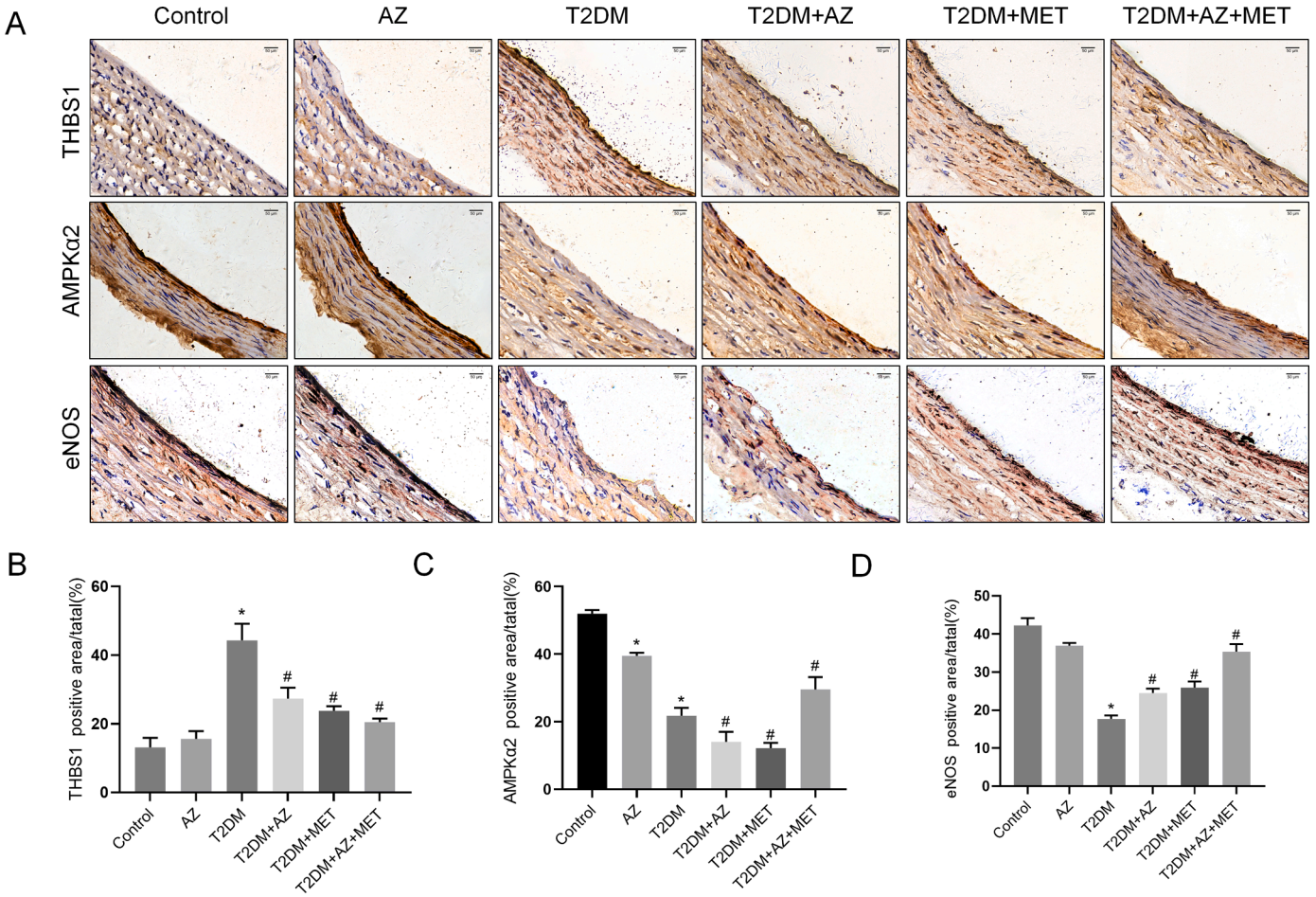

Fig. 3. AZ alleviated the endothelial dysfunction in T2DM rats. (A) The immunohistochemistry detection of THBS1, AMPKα2, eNOS in vascular endothelium. (B) Quantitative analysis of positive intensity of THBS1. (C) Quantitative analysis of positive intensity of AMPKα2. (D) Quantitative analysis of positive intensity of eNOS. All data were expressed as mean ± SD. *$p<0.05$, vs. control;$^{\#}p<0.05$ vs. T2DM group.

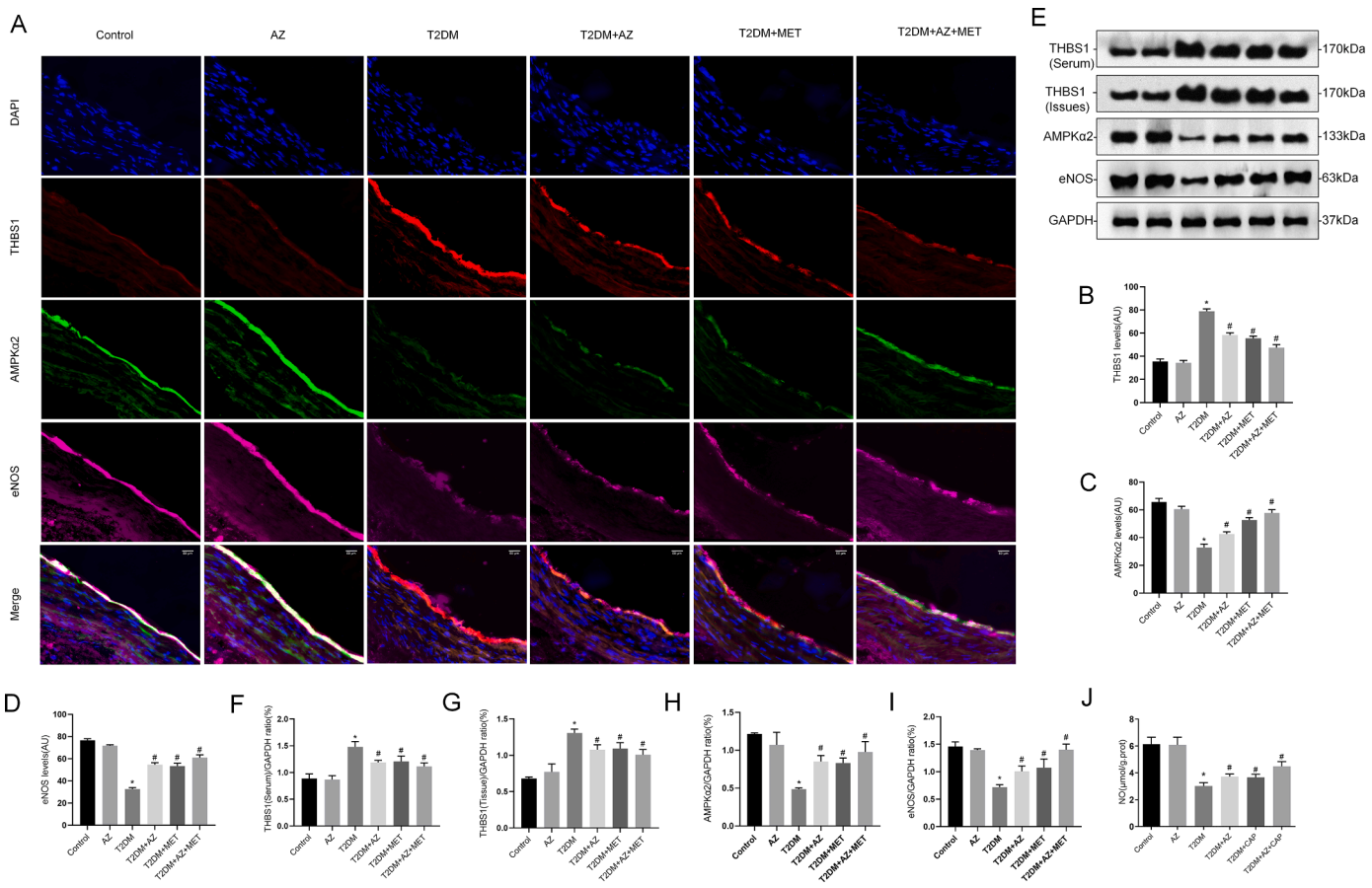

Fig. 4. Alizarin activate eNOS activity by inhibiting the expression of THBS1 protein and activating AMPK pathway in rats. (A), The immunofluorescence detection of rats in vascular endothelium (THBS1, red; AMPKα2, green; eNOS, purple). (B-D), Quantitative analysis of fluorescence intensity of THBS1, AMPKα2, eNOS. (E-I), Western blot analysis of THBS1 in serum and tissue, AMPKα2, eNOS in endothelial cells ($n = 3$). (J), NO level was measured in rats. All data were expressed as mean ± SD. $^{*}p<0.05$, vs. control; $^{\#}p<0.05$ vs. high glucose group. All data were expressed as mean ± SD.

Alizarin improves diabetes detection in STZ-induced T2DM rats

The structural formula of alizarin is shown in Fig 2A. The flow chart of the rat experiment we performed is shown in Fig 2B. The weight levels of the successfully modeled T2DM rats were very low from the third week compared to the control group (Fig.2C). The fasting blood glucose level in T2DM rats was notably greater than that of the control group (Fig.2D). Glycated hemoglobin (Fig. 2E), one of the diagnostic criteria for type 2 diabetes, was reduced. To determine the dose of alizarin administered, we tested endothelium-dependent vasodilation after administration of different concentrations of alizarin, and the results showed that the best aortic diastolic function was achieved at a dose of 10 mg/kg (Fig. 2I). We further performed experiments related to pharmacokinetics and obtained relevant parameters (Fig. 2F, Table 1). Although the therapeutic effect of AZ in T2DM rats was lower than that of metformin, it still had a blood glucose-lowering effect. In addition, the combination of AZ and metformin showed the best results in all aspects.

Alizarin alleviates endothelial dysfunction in T2DM rats

In SD rats, normal controls had clear, normal vascular structure, tightly packed cells, regular morphology and regular distribution. In the model group, the blood vessels were subjected to long-term pressure, the wall was dilated and the cells were disorganized. By hematoxylin-eosin staining analysis, the vascular endothelial cells of T2DM rats showed obvious damage (Fig. 2J). This was also confirmed by cellular experiments with HUVECs performed in vitro (Fig. 5A, B). Further, alizarin treatment improved vascular damage. One characteristic that sets T2DM-induced endothelial dysfunction apart is endothelium-dependent diastolic dysfunction (Li et al., 2016); therefore, we investigated endothelium-dependent vasorelaxation (EDR). In T2DM rats, AZ dramatically enhanced aortic diastolic function as compared to the T2DM group (Fig. 2G), and these data confirm that AZ attenuates ED in T2DM rats. At the same time, AZ did not impact endothelium-independent relaxation induced by SNP (Fig. 2H). It has been observed that decreased NO generation in vascular tissue impairs ACh-induced endothelium-dependent relaxation, which impacts both relaxation and vasoconstriction (Wang et al., 2009). It is further suggested that AZ may affect the EDR by influencing NO content. At present, we do not know what pathway influences NO release.

Alizarin upregulates THBS1 expression and function in diabetic vessels

First, we determined the amount of THBS1, AMPKα2, eNOS in rats' vascular tissue by immunohistochemistry and immunofluorescence (Figs. 3A, 4A). There were no significant changes in THBS1 in the group administered alone compared to the normal group (Figs. 3B, 4B), suggesting that under normal conditions, AZ does not strongly correlate with THBS1. When the T2DM group was compared to the control group, there was a significant increase in THBS1. THBS1 levels in the AZ-treated group were notably lower than those in the T2DM group (Figs. 3A, 4A, F, G). Next, we examined the levels of THBS1 in rat serum and tissues by western blotting, and the results were as expected (Fig. 4E). According to related studies, THBS1 has a role in regulating eNOS, and we further detected the eNOS content. The T2DM group had lower levels of protein expression than the control group (Fig. 3A, D, 4A,

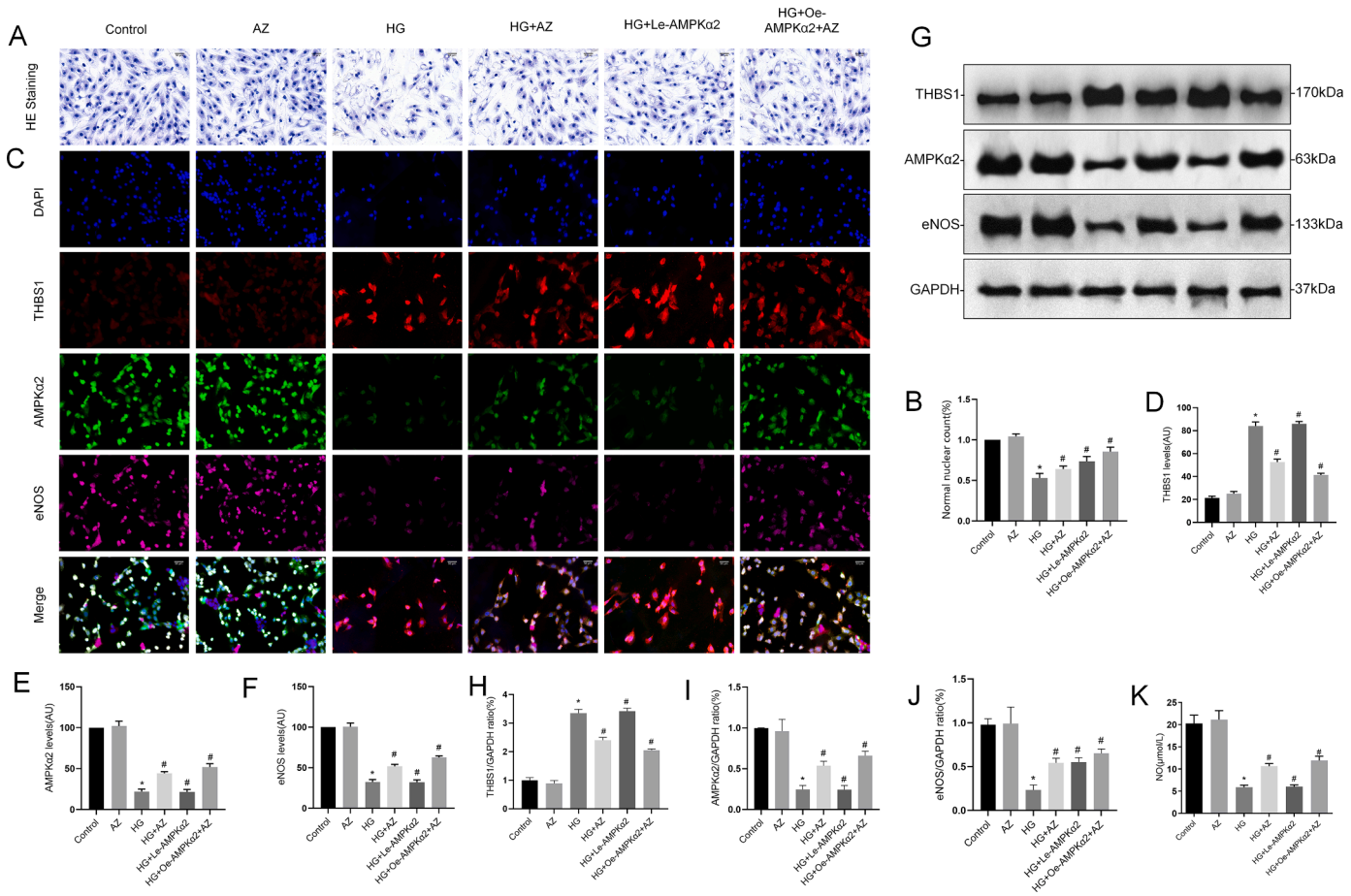

Fig. 5. In HUVECs, AZ decreases the expression of THBS1, increases the expression AMPKα2, eNOS protein, and increases NO release. (A) Representative images of HUVECs stained with hematoxylin and eosin (H&E) in each group. (B) Quantitative results of (A). (C) The immunofluorescence detection of HUVECs (THBS1, red; AMPKα2, green; eNOS, purple). (D-F) Quantitative analysis of fluorescence intensity of THBS1, AMPKα2, eNOS. (G-J) Western blot analysis of THBS1, AMPKα2, eNOS in endothelial cells (n = 6). (K) NO level was measured in HUVECs. All data were expressed as mean ± SD. $^{*}p<0.05$, vs. control; $^{\#}p<0.05$ vs. high glucose group.

E, D, I). Regulation of AMPK has been effective in the treatment of diabetes and cardiovascular problems (Madhavi et al., 2019). Moreover, AMPK is widely expressed in a variety of biological systems' tissues. Therefore, we examined the content of AMPKα2, which was significantly reduced in the T2DM group (Fig. 4C, H). Conversely, AZ treatment increased the expression level of AMPKα2 protein (Figs. 3A, C, 4A, E). Next, we examined the NO content, which presented a consistent trend with eNOS (Fig. 4J). Collectively, these findings imply that by blocking the production of THBS1 protein, AZ may help treat vascular endothelial dysfunction in type II diabetes mellitus.

Alizarin inhibits the upregulation of THBS1 in HUVECs and improves endothelial function by activating AMPKα2

To look into the specific mechanism by which AZ reduces endothelial dysfunction brought on by T2DM, we used a lentiviral-mediated strategy to overexpress AMPKα2 (AMPKα2-OE) in HUVEC prior to AZ treatment, and, at the same time, we transfected si-AMPKα2 to make the protein low-expressed (AMPKα2-LE). In comparison to the normal group, the model group had a significant increase in THBS1, and a significant decrease in AMPKα2 and eNOS (Fig. 5C, G). Compared with the model group, THBS1 was decreased and AMPKα2 and eNOS were significantly increased in the model administration group, indicating that alizarin could reduce the expression of THBS1 and activate the AMPKα2 pathway in response to disease (Fig. 5C, G, E, I). There was no significant change in the fluorescence intensity of THBS1 protein and eNOS protein in the high-glucose and AMPKα2-low expression groups compared to the high-glucose group, indicating that AZ affected the activity of eNOS through AMPKα2 (Fig. 5D, F, H, J). The HG- and AZ-treated AMPKα2 overexpression groups had significantly lower THBS1 protein and lower eNOS content compared with the model administration group, suggesting that AMPKα2 interfered with the eNOS-lowering effect of THBS1. We further examined the level of NO, which was consistent with the expected results (Fig. 5K). In summary, AZ caused a decrease in THBS1 expression; Together with previous results, it was confirmed that AZ affects eNOS activity by activating AMPKα2. Combined with the results, we found that AZ attenuated endothelial dysfunction by lowering THBS1 and activating AMPKα2.

We set up (i) high glucose + AMPKα2 low-expression group; (ii) high glucose + AMPKα2 low-expression + AZ group; (iii) high glucose + AMPKα2 overexpression group; (ii) high glucose + AMPKα2 overexpression + AZ group. Compared with the high glucose + AMPKα2 low-expression group, the high glucose + AMPKα2 low-expression + AZ group showed a decrease in the content of THBS1 and a slight increase in the content of AMPKα2 and eNOS, demonstrating that AZ could inhibit the expression of THBS1 and activate AMPKα2, which led to the elevation of eNOS expression (Fig. 6A-D). Immunofluorescence and WB results were consistent (Fig. 6E-H). Compared with the high glucose + AMPKα2 overexpression group, the high glucose + AMPKα2 overexpression + AZ group similarly showed a decrease in the content of THBS1, which also achieved elevated eNOS expression (Fig. 6A, E).

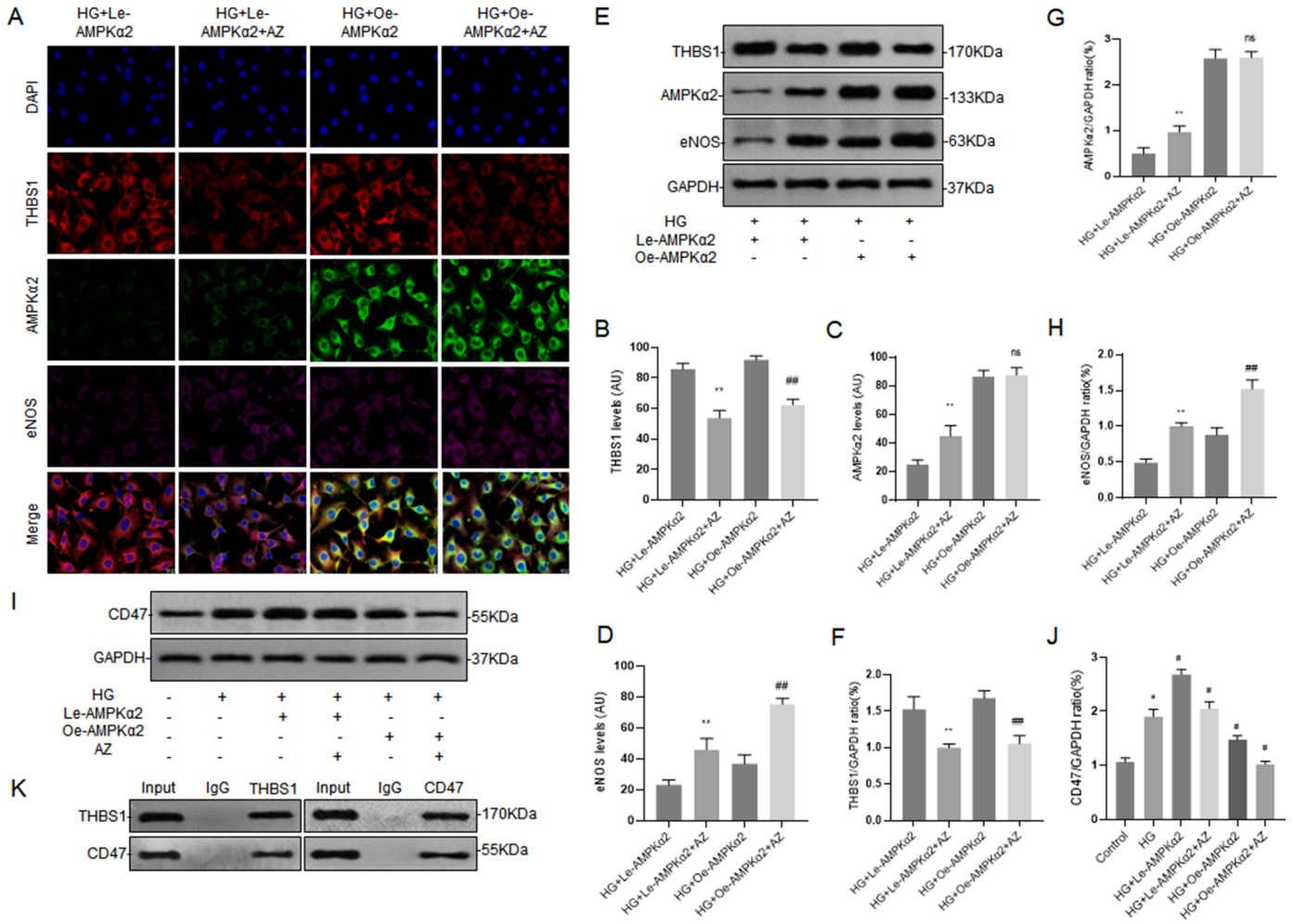

Fig. 6. AZ exerts a blocking effect by activating AMPKα2 and inhibits CD47. (A) The immunofluorescence detection of HUVECs (THBS1, red; AMPKα2, green; eNOS, purple). (B-D) Quantitative analysis of fluorescence intensity of THBS1, AMPKα2, eNOS. (E-H) Western blot analysis of THBS1, AMPKα2, eNOS ($n = 3$). (I-J) Western blot analysis of CD47 ($n = 3$). (K)Western blotting after immunoprecipitation (IP) of AMPKα2 and CD47 in protein extracts of HUVECs. IP with corresponding IgG as a negative control. All data were expressed as mean ± SD. $^{*}p<0.05$, vs. high glucose + Le-AMPKα2; $^{\#}p<0.05$ vs. high glucose + Oe-AMPKα2.

Alizarin blocks eNOS uncoupling by inhibiting CD47 through activation of AMPKα2

In order to explore the mechanism of action of AMPKα2, we first performed immunoprecipitation of AMPKα2 and THBS1, and found that the results were negative, which proved that there was no protein interaction between AMPKα2 and THBS1. We further performed immunoprecipitation of THBS1 and CD47 and found positive results, proving that the interaction between THBS1 and CD47 proteins occurred (Fig. 6K). We further examined the expression of CD47 under conditions of low and overexpression of AMPKα2 (Fig. 6I, J). The expression of CD47 was found to be elevated in the high glucose + AMPKα2 low expression group compared to the high glucose group, which showed a significant decrease after drug administration. Under the condition of overexpression of AMPKα2, the expression of CD47 was reduced and further decreased after administration of the drug.

Alizarin activates eNOS expression through the AMPKα2 signaling pathway

According to the aforementioned research, AMPK might be essential for AZ-mediated protection against T2DM-induced endothelium deterioration. AMPKα2-KO mice were employed to investigate the necessity of AMPKα2 in the AZ-mediated prevention of T2DM. We first assayed the level of AMPKα2 (Fig. 7C, H). Results from western blot and immunofluorescence analysis demonstrated that the model group's THBS1 expression was noticeably higher than that of the control group (Fig. 7A, B, E, F, G). The administered group's eNOS content did not significantly differ from that of the model group (Fig. 7D, I). Combined with previous findings, this suggests that alizarin activates eNOS expression through the AMPKα2 signaling pathway. The expression of eNOS, an enzyme closely related to NO release, was closely correlated with NO content (Fig. 7J). We further examined the NO content in the vasculature and found it to be as expected.

Discussion

A variety of biological activities, including decreasing cholesterol levels (Gao et al., 2010) and preventing atherosclerosis (Wang et al., 2012), are attributed to the anthraquinone type to which AZ belongs. However, its improvement of vascular endothelial damage is seldom reported. As a result, we concentrated on how alizarin protects endothelium cells.

In this study, we explored the AZ on T2DM-induced vascular endothelial damage. We have come to the conclusion that: (1) AZ reduces the binding of THBS1 to CD47 by inhibiting the increase in THBS1 synthesis induced by high glucose and high lipids, thereby weakening the inhibitory effect on eNOS activity; (2) AZ reduces CD47 expression by activating the AMPK signaling pathway and inhibits the blockade of eNOS activity by the THBS1-CD47 complex (Fig. 8). It has been demonstrated

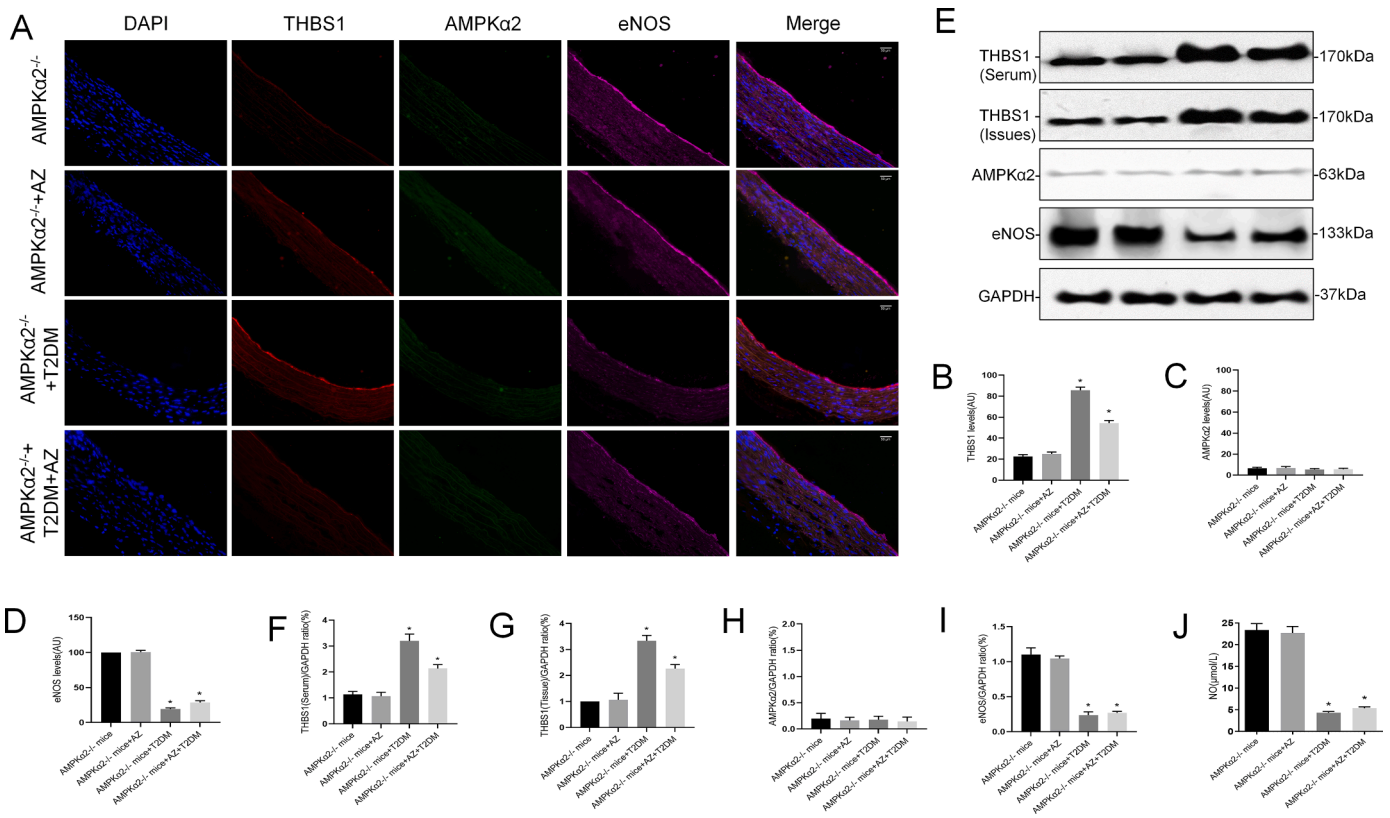

Fig. 7. AZ had effect on the expression of THBS1, AMPKα2, eNOS protein in AMPKα2 knockout mice. (A) The immunofluorescence detection of AMPKα2$^{-/-}$ in vascular endothelium (THBS1, red; AMPKα2, green; eNOS, purple). (B-D) Quantitative analysis of fluorescence intensity of THBS1, AMPKα2, eNOS. (E-I) Western blot analysis of THBS1 in serum and tissue, AMPKα2, eNOS (n = 3). All data were expressed as mean ± SD. $^{*}p<0.05$, vs. control;$^{\#}p<0.05$ vs. T2DM group.

that AMPK phosphorylates and activates eNOS in endothelial cells (Chen et al., 1999; Levine et al., 2007; Morrow et al., 2003; Reihill et al., 2007).

Baenziger et al. originally reported THBS1 (Baenziger et al., 1971), a multi-modular glycoprotein belonging to the thrombospondin family, almost fifty years ago. A glucose response element has been found to be present in the THBS1 promoter region (Huang et al., 2017b), and hyperglycemia is both a cause and an effect of metabolic stress (Huang et al., 2017a). We speculate that the abnormal metabolic environment of type 2 diabetes may lead to elevated THBS1 affecting eNOS expression and exacerbating endothelial dysfunction. Increased THBS1 transcription in endothelial cells in response to high glucose has been documented in a number of investigations (Raman et al., 2011). The C-terminal region of the receptor CD47 is where THBS1 binds (Kuijpers et al., 2014; Resovi et al., 2014; Roberts et al., 2012).

CD47, often referred to as integrin-associated protein (IAP), has a mass of 50 kDa. Given its strong affinity for THBS1 and its ability to bind at picomolar concentrations, it most likely serves as the primary signaling moiety *in vivo*. Every cell in the body, including red blood cells, expresses it. Its solitary extracellular immunoglobulin-like domain is followed by five transmembrane helices, making it an uncommon member of the immunoglobulin (IgG) family of proteins. (Brown, 2001). To start downstream signaling, the extracellular N-terminal domain of CD47 is ligated by the C-terminal domain of THBS1. (Oldenborg, 2013). Over the past ten years, a large body of research has shown how important THBS1-CD47 signaling is for both inflammation (Huang et al., 2017b) and cancer (Roberts et al., 2012). Our research focuses on their role in cardiovascular biology (Rogers et al., 2014).

We first screen for potential targets of alizarin at a macroscopic level using network pharmacology, which is able to assess the connections between medications, targets, and diseases in biological networks (Xia et al., 2020). Additionally, in light of network pharmacology analysis findings, we focused on the regulation of THBS1 and AMPK by alizarin. Next, we found that HE staining and immunohistochemistry were performed in the diabetes group, and vascular damage occurred.

At therapeutic concentrations, AZ did not show a significant irritating effect on animals. Next, we compared the results of AZ with the known positive drug metformin. The mechanism of action of metformin, a first-line drug for the treatment of T2DM (Mahgoub et al., 2023), is extremely complex and still not fully understood (Foretz et al., 2023). It is accepted more generally that it acts through activation of AMPK-dependent kinases (Zhou et al., 2001). It has also been shown that metformin has cardiovascular protective effects. John W Calvert et al. suggested that activation of AMPK during myocardial ischaemia-reperfusion, an endogenous protective mechanism, could enhance the cardioprotective effects of metformin (Calvert et al., 2008); CX Zhang et al. showed that long-term treatment with metformin could mediate the cardioprotective effects of AMPK and eNOS-dependent kinases through activation of AMPK and eNOS-dependent kinases (Foretz et al., 2023). AMPK and downstream signalling pathways involving eNOS-NO to attenuate pressure overload-induced ventricular hypertrophy (Zhang et al., 2011); and the work of Gang Wang et al. suggests a role for metformin-mediated regulation of miRNA signaling pathways controlling angiogenesis and vasculoprotective effects (Wang et al., 2021). Therefore, we chose to use metformin as a positive drug. The results showed that although the effect of AZ was weaker than that of metformin, it also had a significant ameliorating effect on vascular injury, and the synergistic effect of the two was the strongest.

These observations suggest that AZ induces AMPK activation in the prevention of vascular injury in T2DM, but whether AMPK is required for AZ-mediated protection of vascular function in T2DM, especially the AMPKα2 subunit, needs to be tested with AMPKα2-KO mice. We further validated the necessity of the AMPK pathway by AMPKα2 knockout mice. Our results suggest that without activation of the AMPK pathway, AZ is unable to achieve the combined effect of the two mechanisms.

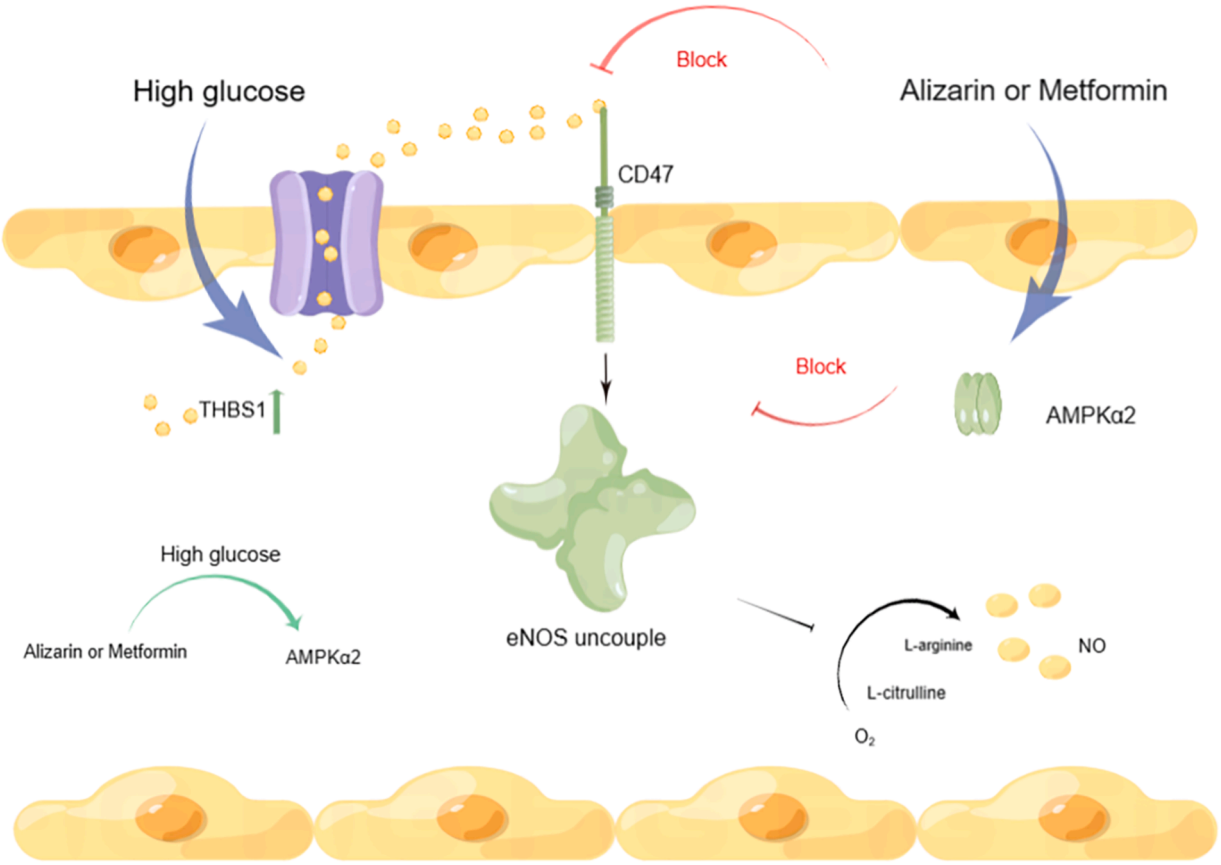

Fig. 8. Proposed mechanism of AZ against vascular endothelial dysfunction in T2DM.

Interestingly, alizarin itself did not affect changes in THBS1 and eNOS.

In conclusion, the primary discovery of this study indicates that alizarin might be involved in many vascular damage pathways brought on by T2DM. Mechanistically, alizarin reduces CD47 expression by activating the AMPK pathway and inhibits the eNOS activity of the THBS1-CD47 complex. Alizarin also blocks the high glucose-induced increase in THBS1 synthesis. Overall, alizarin ameliorates vascular injury by increasing NO levels through both of these pathways. This is very important for disease remission, improves endothelial dysfunction in type 2 diabetes, provides new targets for treating the disease and develops new potential drugs.

CRediT authorship contribution statement

Mo-Li Zhu: Methodology, Investigation, Writing – original draft. **Jia-Xin Fan:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Ya-Qi Guo:** Software, Visualization. **Li-Juan Guo:** Investigation. **Hua-Dong Que:** Resources, Project administration. **Bao-Yue Cui:** Resources, Project administration. **Yin-Lan Li:** Software, Data curation. **Shuang Guo:** Resources, Methodology, Investigation. **Ming-Xiang Zhang:** Visualization, Software, Project administration. **Ya-Ling Yin:** Validation, Supervision, Funding acquisition, Conceptualization. **Peng Li:** Writing – review & editing, Visualization, Validation, Funding acquisition.

Declaration of competing interest

We solemnly declare that there is no conflict of interest of any kind with any other organization/company/individual in this article. The consent of all authors has been obtained for this manuscript titled "Protective effect of alizarin on vascular endothelial dysfunction via inhibiting the type 2 diabetes-induced synthesis of THBS1 and activating the AMPK signaling pathway". All other authors declare they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.155557.

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