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RESEARCH ARTICLE

Retinoic Acid Improves Vascular Endothelial Dysfunction by Inhibiting PI3K/AKT/YAP-mediated Ferroptosis in Diabetes Mellitus

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Abstract: ***Background*:** Vascular endothelial dysfunction is the initial factor involved in cardiovascular injury in patients with diabetes. Retinoic acid is involved in improving vascular complications with diabetes, but its protective mechanism is still unclear. This study aimed to evaluate the effect and mechanism of All-trans Retinoic Acid (ATRA) on endothelial dysfunction induced by diabetes.

***Methods*:** In the present study, streptozotocin (STZ)-induced diabetic rats and high glucose (HG)-induced human umbilical vein endothelial cells (HUVECs) were observed, and the effects of ATRA on HG-induced endothelial dysfunction and ferroptosis were evaluated.

***Results*:** ATRA treatment improved impaired vasorelaxation in diabetic aortas in an endothelium-dependent manner, and this effect was accompanied by an increase in the NO concentration and eNOS expression. Ferroptosis, characterized by lipid peroxidation and iron overload induced by HG, was improved by ATRA administration, and a ferroptosis inhibitor (ferrostatin-1, Fer-1) improved endothelial function to a similar extent as ATRA. In addition, the inactivation of phosphoinositol-3-kinase (PI3K)/protein kinases B (AKT) and Yes-associated Protein (YAP) nuclear localization induced by HG were reversed by ATRA administration. Vascular ring relaxation experiments showed that PI3K/AKT activation and YAP inhibition had similar effects on ferroptosis and endothelial function. However, the vasodilative effect of retinoic acid was affected by PI3K/AKT inhibition, and the inhibitory effects of ATRA on ferroptosis and the improvement of endothelial function were dependent on the retinoic acid receptor.

***Conclusion*:** ATRA could improve vascular endothelial dysfunction by inhibiting PI3K/AKT/YAP-mediated ferroptosis induced by HG, which provides a new idea for the treatment of vascular lesions in diabetes.

Keywords: Retinoic acid, endothelial dysfunction, phosphoinositol-3-kinase, ferroptosis, diabetes, nitric oxide.

1. INTRODUCTION

Diabetes is a chronic metabolic disease worldwide that affects more than 460 million people, and its incidence continues to increase annually [1, 2]. Cardiovascular complications of diabetes are the leading cause of death, and vascular endothelial injury is the main cause of cardiovascular complications [3-5]. It is well known that blood vessels are composed mainly of endothelial cells, smooth muscle cells, and fibroblasts. As they come into direct contact with the blood, endothelial cells are the first cells to be destroyed by hyperglycemia [6-9]. As highly differentiated cells, vascular endothelial cells have specific morphology, functions, and gene expression patterns. However, under hyperglycemia, vascular endothelial vasodilation reactivity decreases, contractility increases, and nitric oxide (NO) bioavailability decreases [10-12]. Moreover, lipid peroxidation and iron overload result in high secreted levels of 4-HNE, MDA, lipid ROS, and ferrous iron, further inducing vascular endothelial disease and ferroptosis and aggravating cardiovascular injury in diabetes mellitus [13-16]. Therefore, the inhibition of diabetes-induced lipid peroxidation and ferroptosis may be a promising pathway for restoring diabetic vascular endothelial dysfunction.

Retinoic acid (RA) is the main metabolite of vitamin A in the body and is an important active substance in the body. RA can activate retinoic acid receptors and retinol X receptors, thereby regulating the expression of a large number of genes in the body and participating in a variety of physiological processes [17-20]. All-trans retinoic acid (ATRA) and 9-cis RA are common ligands for RA signaling. ATRA is selective for RARs, whereas 9-cis RA binds both RARs and RXRs [21]. Recent research has shown that retinoic acid can effectively inhibit cell ferroptosis induced by lipopolysaccharide and erastin in a retinoic acid receptor-dependent manner [22, 23], and targeting ferroptosis contributes to ATRA-induced acute myeloid leukemia differentiation *via* ROS-autophagy-lysosomal pathway [24]. In terms of diabetes, ATRA could improve diabetes symptoms in mice by boosting insulin secretion and preventing the development of autoimmune diabetes by inducing a state of immune tolerance [25-27]. In addition, ATRA deficiency and a reduction of retinol dehydrogenase 10 (RDH10, the rate-limiting enzyme in retinol metabolism) aggravate heart damage in diabetes through ferroptosis, and ATRA supplementation restores myocardial ATRA levels and reduces myocardial hypertrophy and fibrosis, accompanied by a reduction in lipid peroxidation and inhibition of ferroptosis in the hearts of db/db mice [28, 29].

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Previous studies have reported that ATRA can increase basal and acetylcholine (ACh)-induced NO production in endothelial cells and ameliorate high-fat-induced atherosclerosis *via* regulating eNOS activity and NO level [30, 31], indicating the endothelial protective effect of ATRA. However, whether ATRA has a protective effect on vascular endothelial dysfunction caused by diabetes is unclear.

Yes-associated Protein (YAP), the nuclear effector of Hippo signaling, is involved in the regulation of ferroptosis in different tissues and cells [32-34]. YAP gain- and loss-of-function studies showed that YAP interacted with the phosphoinositol-3-kinase (PI3K)/protein kinases B (AKT) pathway and regulates cardiomyocyte proliferation and survival [35]. In addition, YAP and PI3K/AKT pathway participate in the regulation of ferroptosis [36, 37]. Considering the above, the improvement effects of ATRA on impaired endothelial dysfunction were evaluated in human umbilical vein endothelial cells (HUVECs) and diabetic rats. The underlying mechanism targeting PI3K/AKT/YAP-mediated ferroptosis by which ATRA improved endothelial cell injury was also assessed. The results highlight the protective effects of ATRA on HG-induced endothelial dysfunction and establish new strategies for diabetes treatment.

2. MATERIALS AND METHODS

2.1. Animal Experiments

SD male rats (180-220 g) were raised by the SPF Animal Laboratory of Jinzhou Medical University. The temperature was controlled at approximately 20-24°C, and the humidity was controlled at 45%~55%. All animal experiments were conducted in accordance with the principles approved by the Animal Ethics Committee of Jinzhou Medical University (2023032). Eighty-five rats received the standard pellet diet provided by Shenyang Maohua Technology Co., Ltd. Except for those in the control group (n=15), 70 rats in the other groups were intraperitoneally injected with STZ (CAS No.: 18883-66-4, Sigma-Aldrich) at a single dose of 65 mg/kg. STZ was dissolved in pre-chilled citrate acid buffer (0.1 mol/L, pH 4.5). Blood glucose was assessed on the 7^{th} day after the STZ injection. Rats with random blood glucose ≥16.7 mmol/L were considered as diabetic [38]. Seventy rats were injected with STZ, 2 rats died naturally, and the blood glucose of 60 rats was greater than 16.7 mmol/L. Sixty rats were randomly divided into the model group, low (2.5 mg/kg), medium (5 mg/kg), and high dose (10 mg/kg) ATRA groups, and Fer-1 group (n=12 in each group). During the drug intervention, 2 rats died in the model group and 1 in the Fer-1 group. ATRA (CAS No.: 302-79-4, purity ≥ 98%, Sigma-Aldrich) was dissolved in olive oil before use and then intraperitoneally injected into rats once a day for 4 weeks. The weight of each rat was measured and recorded weekly. After 4 weeks of administration, the rats were anesthetized intraperitoneally using sodium pentobarbital (50 mg/kg), and the adequacy of anesthesia was confirmed by the absence of a reflex response to a foot squeeze [39, 40]. Then, the chest of the rat was quickly opened, the aorta and heart were exposed, and blood was extracted from the apex of the heart before stripping the aorta for subsequent experiments.

2.2. Vascular Diastolic Capacity

After the rats were anesthetized, the aorta was fully exposed, quickly removed, and placed in cold saline. The aorta was cut into a circle of approximately 2 mm, and the fatty tissue was removed. This process requires quick action, and attention must be focused on the effects of temperature and oxygen content on blood vessels to avoid loss of activity. Changes in vascular ring tension were measured by a DMT microvascular tension measurement system as previously described [10]. After 37°C, the rings were cultured in DMEM containing 10% FBS, removed, placed in DMT equipment containing PSS, preshrunked with phenylephrine (PE, 10^{-5} M), and treated with different concentrations of acetylcholine (ACh, 10^{-8}-10^{-5} M) when the shrinkage was stable. The endothelium-dependent relaxation (EDR) response is the percentage of maximum PE-induced contraction, representing the diastolic rate of the ring. When the ring was incubated, the diastolic rate of the ring was measured according to the above method.

2.3. Transmission Electron Microscopy

The aortas were collected and cut into small pieces. The small pieces were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4^{o}C for 24 h. The samples were post-fixed in 1% osmium tetraoxide in a sodium cacodylate buffer, dehydrated in an escalating series of ethyl alcohol, embedded in Spurr's resin, and then cut into ultrathin sections. After being stained with uranyl acetate and lead citrate, the ultrathin sections were observed with a Hitachi HT7700 transmission electron microscope.

2.4. Cell Culture

Human umbilical vein endothelial cells (HUVECs) were acquired from BLUEFBIO (Shanghai, China) and cultured in DMEM/F12 medium supplemented with 15% FBS and 1% penicillin-streptomycin antibiotics in an incubator at 37^{o}C and 5% CO_2. ATRA was dissolved in DMSO and diluted in DMEM/F12 for cell treatment. HUVECs were pretreated with different concentrations of ATRA(1, 2, and 4 μM) for 1 h and then exposed to 40 mM glucose. According to different experimental requirements, HUVECs were added with 1 μM Fer-1 (CAS No.: 347174-05-4, MedChemExpress), 10 μM LY294002 (CAS No.: 154447-36-6, MedChemExpress), 10 μM sorafenib (CAS No.: 284461-73-0, MedChemExpress), 1 μM verteporfin (CAS No.: 129497-78-5, MedChemExpress), 2 μM Ro41-5253 (CAS No.: 144092-31-9, MedChemExpress), or 1 μM LE-135 (CAS No.: 155877-83-1, MedChemExpress) before high glucose treatment. The selection of ATRA and other reagent concentrations was based on previous references [41-43]. Cells were seeded in 60 mm dishes at an initial density of 1×10^5 cells/well and grown to approximately 80% confluence, and the medium was changed every two days. Subsequently, cells will be cultured into different containers according to experimental needs.

2.5. Enzyme-linked Immunosorbent Assay

The levels of 4-HNE, MDA, lipid ROS, ferrous iron, GSH-Px, and NO in the serum of rats and HUVEC supernatant were detected *via* ELISA. The ELISA was performed according to the manufacturer's instructions.

2.6. CCK-8 Assay

The same number of HUVECs were inoculated into a 96-well plate, and different concentrations of drugs were added after the cells were attached to the wall. After 24 hours, CCK-8 solution was added, and the cells were incubated at 37°C for 1 hour. The absorbance of each well was measured at 450 nm using a microplate reader.

2.7. Immunofluorescence

The slide/24-well cell plate was immersed in PBS containing 0.5% Triton X-100 for 30 minutes and incubated in PBS containing 5% bovine serum albumin for 30 minutes. The slides were incu-

bated overnight with YAP antibody (Cat No.: A19134, rabbit, ABclonal, 1:100) or eNOS antibody (Cat No.:A15075, rabbit, ABclonal, 1:200) at 4°C. The next day, the slide/24-well cell plates were incubated with secondary antibodies using anti-rabbit IgG Alexa 594 (Cat No.: A-32740, Invitrogen, 1:1000) or anti-rabbit Alexa 488 (Cat No.: A-21441; Invitrogen, 1:1000), stained with DAPI solution and observed under a fluorescence microscope.

2.8. Western Blot

Total protein was extracted from rat tissues and HUVECs using RIPA buffer (Beyotime). The protein concentration was measured using the BCA Protein Assay Kit (Beyotime). The same amount of protein was then separated by a 10% SDS-PAGE gel, and the imprint was then transferred to a nitrocellulose membrane. The membrane was blocked at room temperature for 1 h using a 5% milk solution from TBST and incubated with the corresponding primary antibody at 4°C overnight. Signaling pathway proteins PI3K (Cat No.: 67071-1-Ig, Proteintech, mouse, 1:2000), AKT (Cat No.: A22770, ABclonal, rabbit, 1:1000), p-AKT (Cat No.: AP1208, ABclonal, rabbit, 1:1000), and YAP (Cat No.: A19134, ABclonal, rabbit, 1:1000) were observed by WB. TFRC (Cat No.: 65236-1-Ig, Proteintech, mouse, 1:2000) and ACSL4 (Cat No.: 66617-1-Ig, Proteintech, mouse, 1:2000) protein expression were analyzed as indicators of ferroptosis. The next day, the membrane was incubated with the appropriate secondary antibody conjugated with HRP for 1 h. After washing with TBST 3 times, the immunoreaction was detected with the ECL reagent.

2.9. Statistics

SPSS20.0 statistical software was used for data processing, and the data are expressed as the means ± SD. All images were taken using GraphPad Prism 8.0. When $p < 0.05$, the data were considered to be significantly different.

3. RESULTS

3.1. ATRA Treatment Attenuates Vascular Endothelial Dysfunction in Diabetic Rats

Endothelial dysfunction is mainly manifested by decreased vasodilation reactivity and increased contractility, essentially a decrease in NO bioavailability. Previous studies have confirmed that diabetes can lead to aortic ring diastolic dysfunction. In this study, 4 weeks of ATRA (2.5, 5, or 10 mg/kg) administration significantly improved impaired vasorelaxation in diabetic aortas, as evidenced by an increased vascular relaxation response to ACh (Figs. **1A**, **B**). However, the vasodilatory effect of ATRA on ACh was completely abolished after the endothelium was removed from the aorta or after the aorta was incubated with L-NAME (an eNOS inhibitor) (Fig. **1C**). In addition, the vasodilation effect of the ATRA was accompanied by an increase in the NO concentration and eNOS expression (Figs. **1D-F**). All the results confirmed that ATRA could improve endothelial-dependent vasodilation dysfunction caused by diabetes.

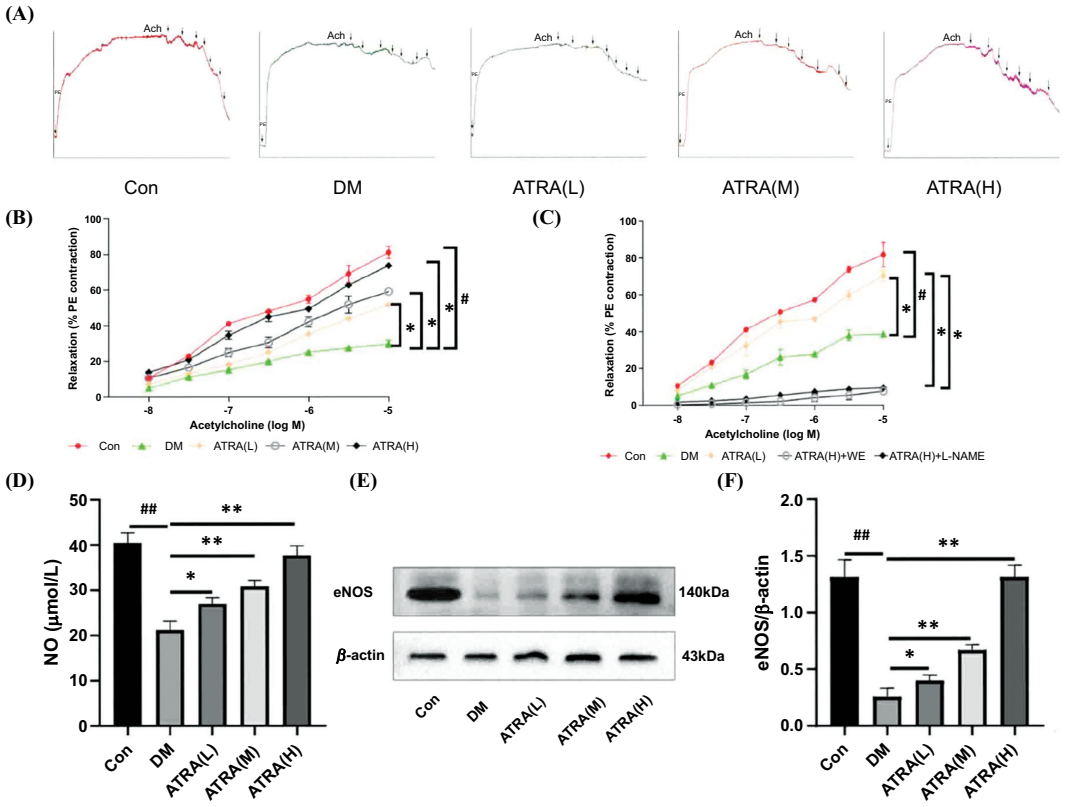

Fig. (1). ATRA treatment attenuates vascular endothelial dysfunction in diabetic rats. (**A-C**) Aortic vascular relaxation response to Ach. (**D**) The level of NO in the serum of rats was determined using a kit. (**E**, **F**) Western blotting was used to detect the protein expression of eNOS in the thoracic aorta. The data are expressed as the mean ± SD (n=3 for A-C and E-F, n=10 for D). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* the Con group; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* the DM group. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

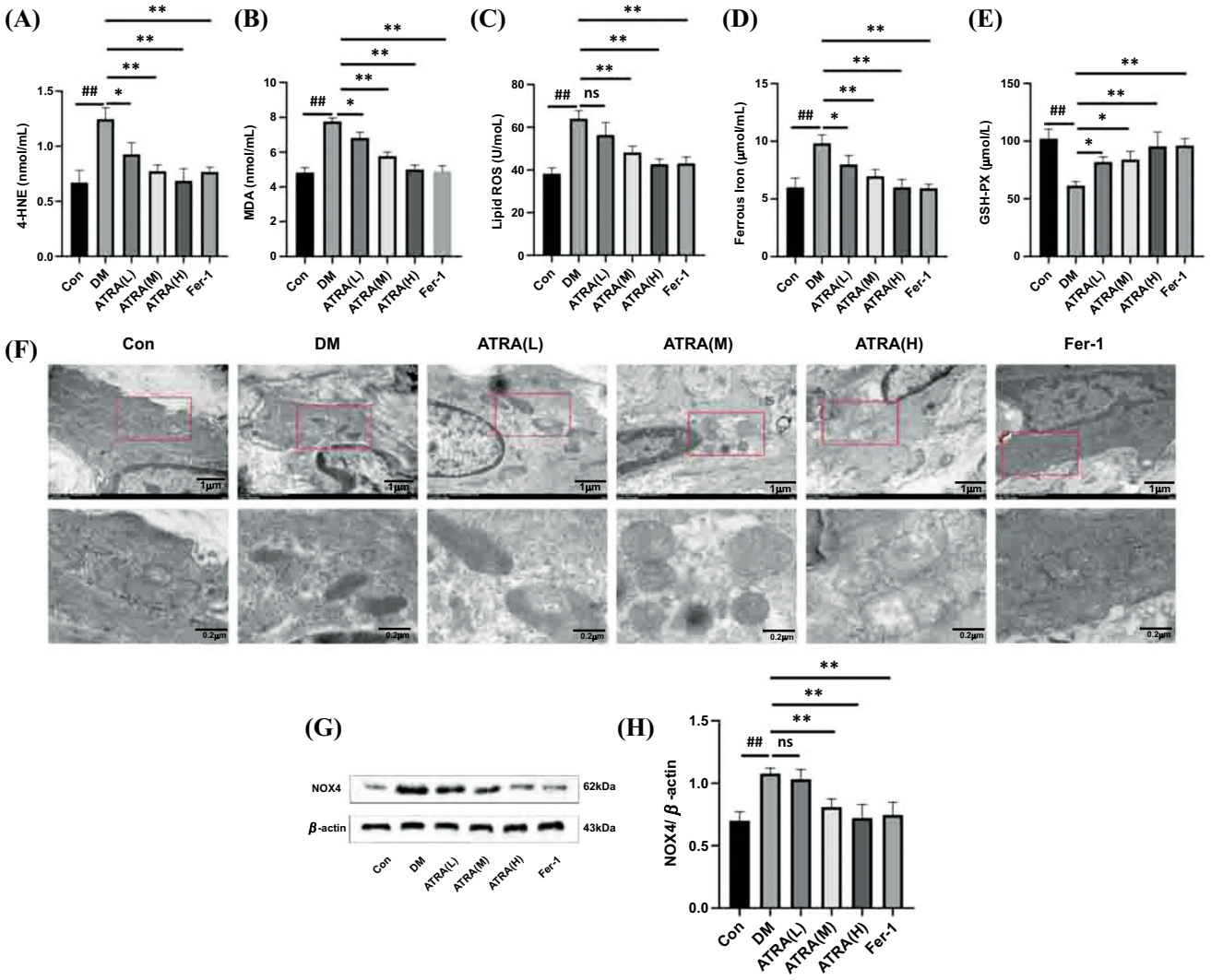

Fig. (2). ATRA treatment alleviates ferroptosis induced by diabetes. (**A**-**E**) The levels of lipid ROS, GSH, GSH-px, MDA, and 4-HNE in the serum of the rats were determined using kits. (**F**) The thoracic aorta was measured *via* TEM (10,000 X). (**G**, **H**) Western blotting was used to detect the expression of the NOX4 protein in the thoracic aorta. The data are expressed as the mean ± SD (n=10 for A-E, n=3 for F-H). $^{\#\#}p <$ 0.01 *vs.* the Con group; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* the DM group. **Abbreviation:** ns: no significance. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

3.2. ATRA Treatment Alleviates Ferroptosis Induced by High Glucose

Ferroptosis, characterized by lipid peroxidation and iron overload, participates in the process of cardiovascular injury in diabetes patients. 4-HNE and MDA levels in vascular tissue were determined to evaluate lipid peroxidation in the present study. The results showed that the levels of 4-HNE and MDA were greater in the diabetic rats than in the control rats. In addition, the production of lipid ROS, NOX4 expression, and ferrous iron significantly increased, while the GSH-Px content decreased in the diabetic vasculature, suggesting that diabetes induced obvious lipid peroxidation and iron overload in vascular tissue. Treatment with ATRA attenuated lipid peroxidation and iron overload induced by diabetes, as indicated by decreased levels of 4-HNE, MDA, NOX4, lipid ROS, and ferrous iron (Figs. **2A-E**, **G**, **H**). Notably, TME analysis revealed obvious morphological features of mitochondrial ferroptosis, which were attenuated by ATRA administration, as indicated by improvements in mitochondrial shrinkage and membrane density (Fig. **2F**). To further clarify the effect of ATRA on ferroptosis induced by high glucose (HG), ferroptosis inhibitors (Fer-1) and inducers (sorafenib) were used at the HUVEC level. Consistent with the *in vivo* results, the increase in 4-HNE, MDA, NOX4, lipid ROS, and ferrous iron levels induced by HG was reversed by ATRA administration at the cellular level (Figs. **3A-G**). However, the effects of ATRA on lipid peroxidation and iron overload were abolished by sorafenib (Figs. **3H-N**). These results suggested that ATRA attenuated ferroptosis induced by HG.

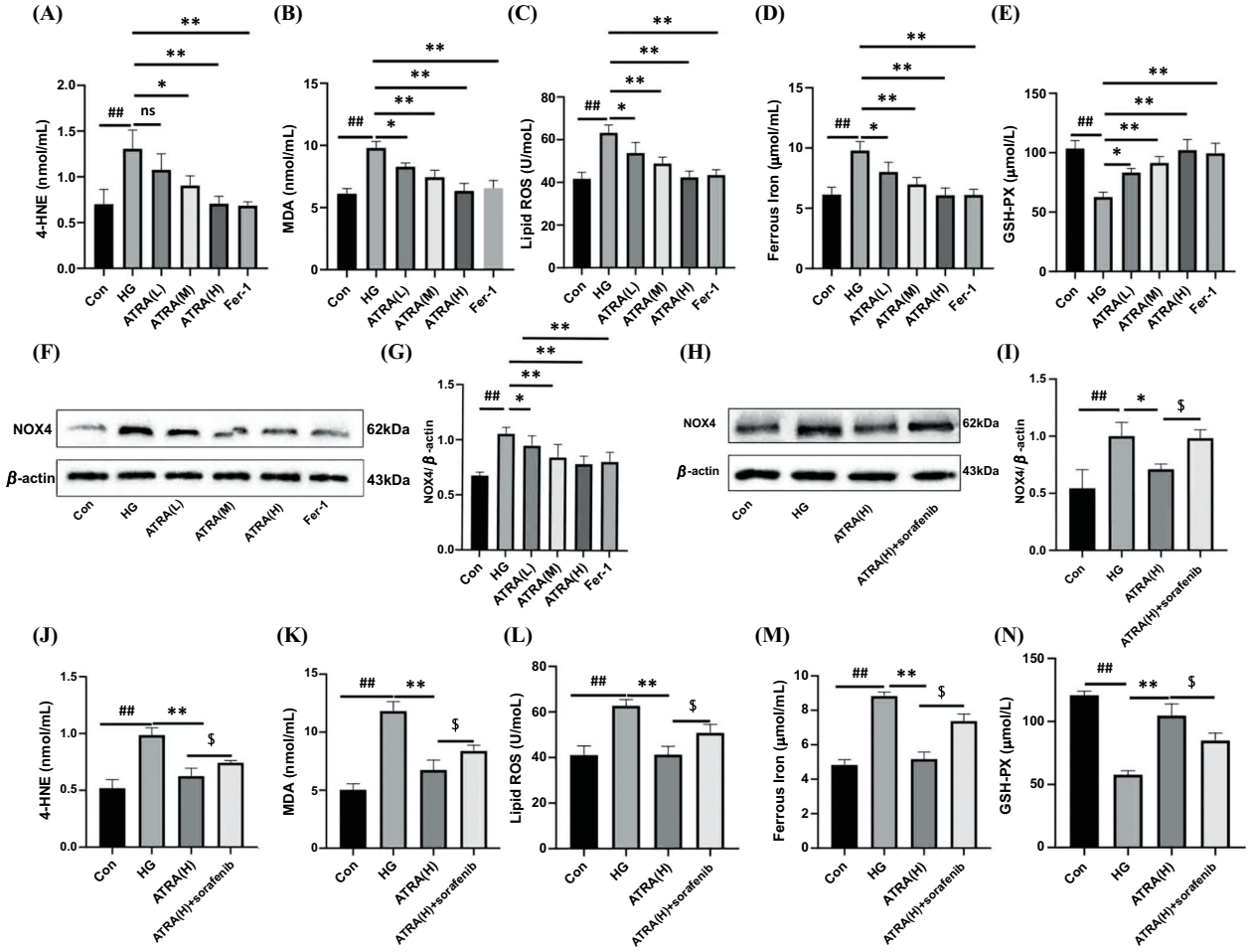

Fig. (3). ATRA treatment alleviates ferroptosis induced by HG. (**A**-**E**, **J**-**N**) The levels of lipid ROS, GSH, GSH-PX, MDA, and 4-HNE in HUVEC supernatant were determined using kits. (**F-I**) The expression of NOX4 protein in HG-induced HUVECs was detected by Western blot analysis. The data are expressed as the mean ± SD (n=10 for A-E and J-N, n=3 for F-I). $^{\#\#}p < 0.01$ compared with Con group, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with HG group, $^{\$}p < 0.05$ compared with ATRA(H) group. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

3.3. ATRA Treatment Attenuates Vascular Endothelial Dysfunction by Suppressing Ferroptosis

It has been demonstrated that ATRA can alleviate heart damage in diabetes through the intervention of ferroptosis. Here, we speculate that ATRA intervention could improve endothelial dysfunction through the inhibition of ferroptosis. The results showed that the ferroptosis inhibitor Fer-1 improved the vasodilatory dysfunction caused by diabetes, similar to that observed in the ATRA group (Figs. **4A-F**). To examine the role of ferroptosis in the vasoprotective effect of ATRA, thoracic aortas from ATRA rats were treated with or without sorafenib or Fer-1 *in vitro* for 12 hours. The combination of ATRA and Fer-1 had no superimposed effect on vasodilation, but sorafenib abrogated the vasodilatory effect of ATRA. Collectively, these results suggest that suppressing ferroptosis is predominantly responsible for the vasodilation of ATRA under HG conditions.

3.4. ATRA Treatment Modulates HG-induced PI3K/AKT and YAP

PI3K/AKT inhibition and YAP activation are associated with ferroptosis. The results showed that compared with those in normal vascular tissues, the expression levels of PI3K and p-AKT proteins were lower in diabetic vascular tissues, and the nuclear localization of YAP was significantly increased; moreover, ATRA reversed the above changes in PI3K, p-AKT, and nuclear YAP (Figs. **5A-D**). Consistent with the *in vivo* experimental results, ATRA-treated HUVECs increased the level of HG-induced PI3K and p-AKT (Figs. **5E**, **G**, **H**), while decreasing the level of nuclear YAP (Figs. **5F**, **I**, **J**).

3.5. ATRA Treatment Alleviates Ferroptosis and Improves Endothelial Function in HUVECs by Regulating PI3K/AKT-mediated YAP

Next, we investigated the mechanism by which ATRA inhibits ferroptosis under HG conditions. YAP has been reported to induce the expression of two key mediators of ferritin kinase, namely, transferrin receptor 1 (TFRC) and acyl-CoA synthetase long-chain family member 4 (ACSL4). Our results showed that HG induction in HUVECs resulted in increased nuclear localization of YAP, significantly increased expression of the downstream genes TFRC and ACSL4, decreased cell viability, and decreased levels of NO and eNOS. After ATRA treatment, the PI3K/AKT pathway was activated, the protein expression of NO and eNOS increased, the protein

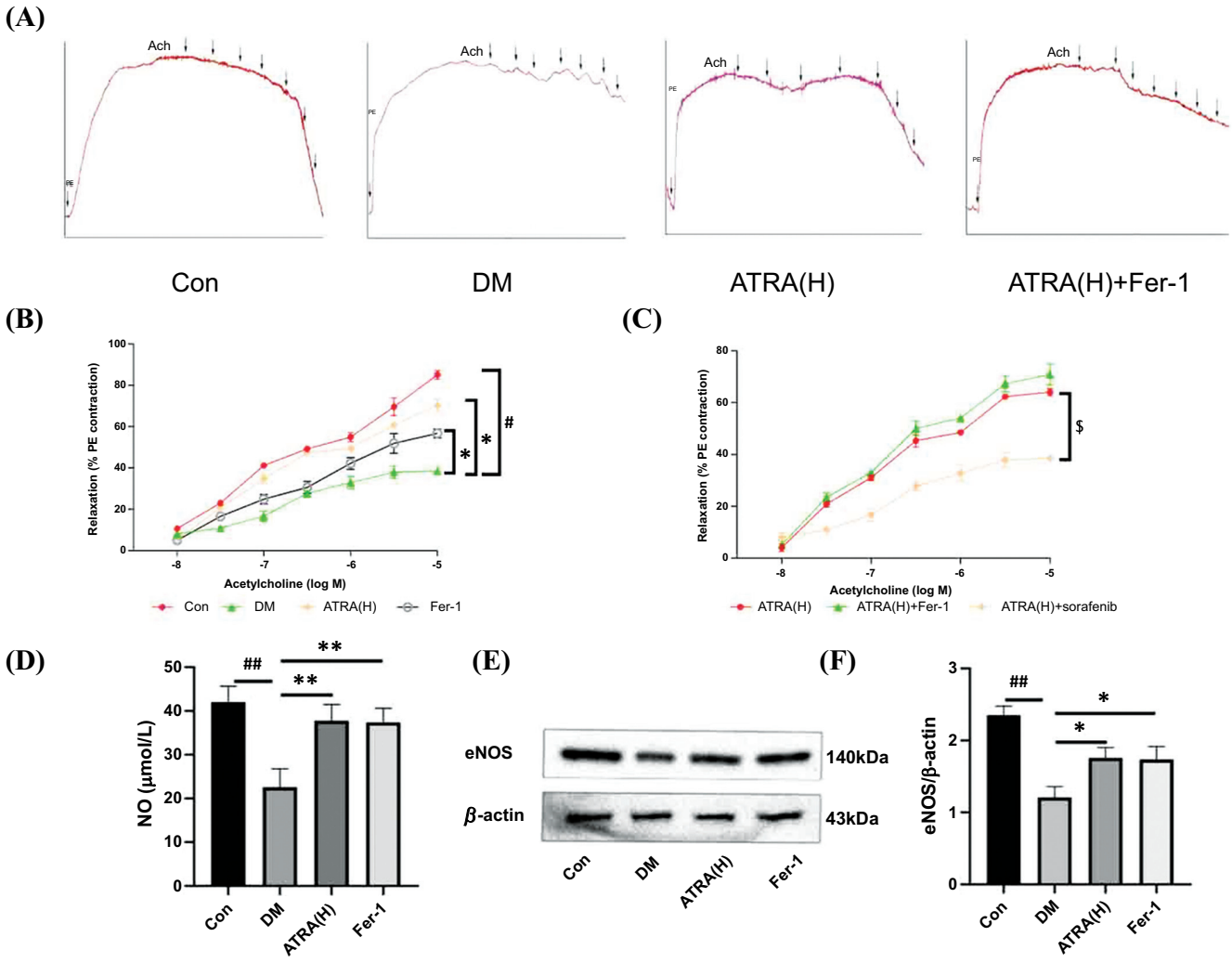

Fig. (4). ATRA treatment attenuates vascular endothelial dysfunction by suppressing ferroptosis. (**A**-**C**) PE precontracted aortas in response to Ach-induced vasodilation in all subgroups. (**D**) The level of NO in the serum of rats was determined using a kit. (**E**, **F**) Western blotting was used to detect the protein expression of eNOS in the thoracic aorta. The data are expressed as the mean ± SD (n=3 for A-C and E, F, n=10 for D). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* the Con group, $*p < 0.05$, $**p < 0.01$ *vs*. the DM group, $^{\$}p < 0.05$ *vs*. the ATRA(H) group. (*A higher resolution/-colour version of this figure is available in the electronic copy of the article*).

expression of TFRC and ACSL4 was reversed, cell viability was significantly increased, and endothelial function was improved. To further investigate this phenomenon, we treated cells with the PI3K/AKT inhibitor LY294002. LY294002 eliminated the inhibitory effect of ATRA on ferroptosis, reversed the expression of the TFRC, ACSL4, NO, and eNOS proteins, and significantly decreased the activity of HUVECs. In addition, the inhibitor of YAP, verteporfin, not only decreased the expression of TFRC and ACSL4 but also inhibited ferroptosis and attenuated endothelial injury induced by HG, similar to ATRA. These results suggest that ATRA treatment alleviates ferroptosis and improves endothelial function in HUVECs by regulating the PI3K/AKT/YAP signaling.

3.6. ATRA Therapy Alleviates Vascular Endothelial Dysfunction through the PI3K/AKT/YAP Pathway

We then investigated whether the inhibition of YAP-mediated ferroptosis was associated with vasodilation in ATRA under HG conditions. Thoracic aortas isolated from normal rats were treated with HG and different interventions. Vascular relaxation in response to ACh decreased after HG treatment, and activation of PI3K/AKT with 740Y-P and inhibition of YAP with verteporfin improved the vascular diastolic response to ACh, suggesting that PI3K/AKT and YAP are involved in HG-induced vascular endothelial dysfunction. In addition, ATRA improved the poor relaxation response to ACh induced by HG, but inhibiting PI3K/AKT with LY294002 counteracted the effect of ATRA (Figs. **6**, **7**).

3.7. ATRA Treatment Attenuates Vascular Endothelial Dysfunction in a RAR-dependent Manner

ATRA functions as a ligand for RARα and RARβ. To verify whether the ability of ATRA to improve vascular endothelial dysfunction is receptor-dependent, we added RAR inhibitors to vascular tissue and cells. Both RAR_{α} and RARβ inhibitors (Ro41-5253

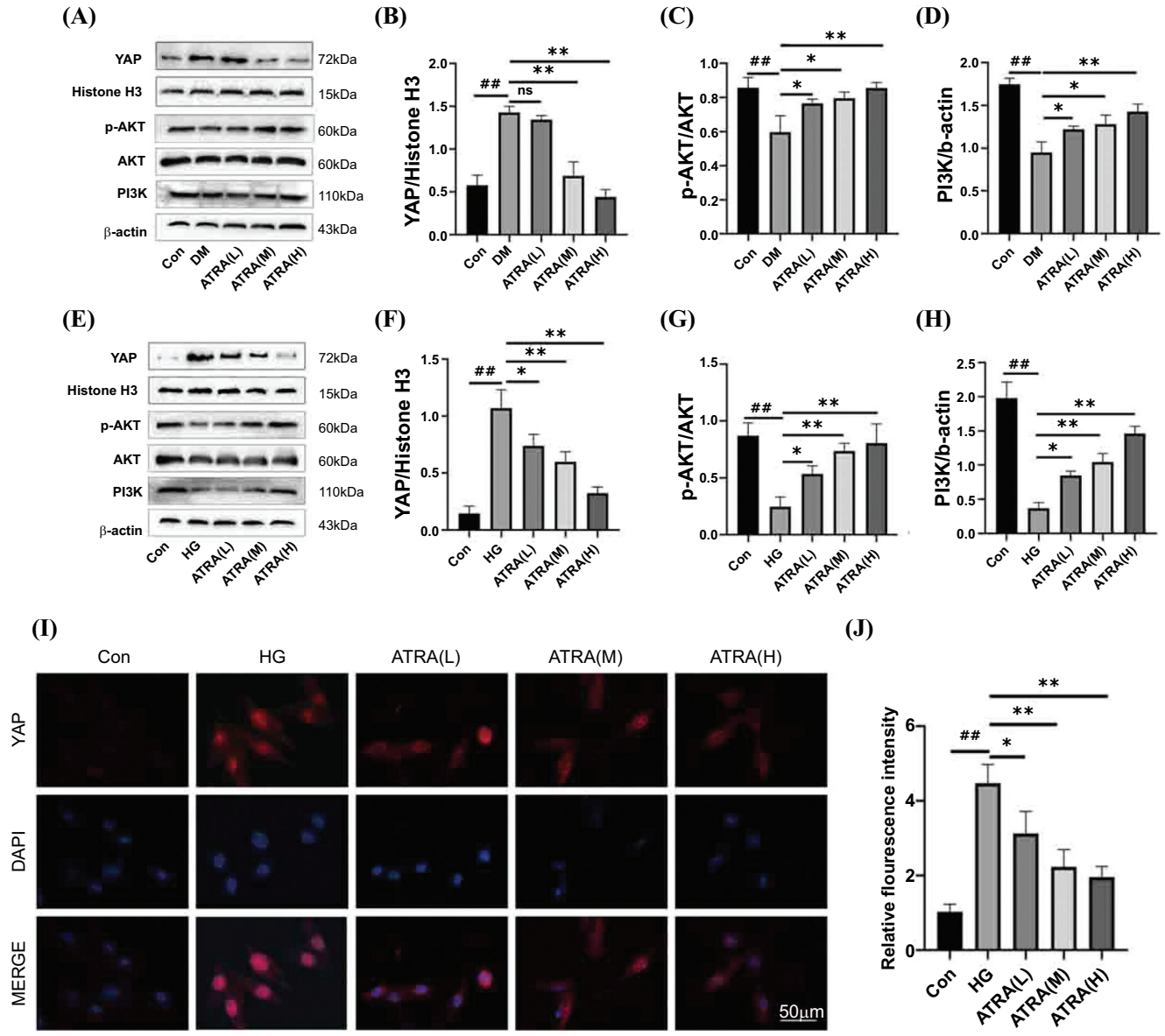

Fig. (5). ATRA treatment modulates HG-induced PI3K/AKT and YAP. (**A**-**H**) Western blotting was used to detect the expression of YAP, PI3K, p-AKT, and AKT proteins in thoracic aorta and HUVECs induced by STZ and HG. (**I**, **J**) The expression of YAP was detected by immunofluorescence staining. The data are expressed as the mean ± SD (n=3). $^{\#\#}p < 0.01$ *vs*. the Con group, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. the DM/HG group. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

and LE-135) abolished the protective effect of ATRA on vasodilation. The results from HUVECs showed that ATRA could not alleviate lipid peroxidation or iron overload caused by high glucose concentrations, nor could it restore NO and eNOS levels in the presence of RAR_{α} and RAR_{β} inhibitors (Figs. **8A-G**).

4. DISCUSSION

Diabetes is a metabolic disease caused by the secretion and utilization of insulin and can lead to severe cardiovascular disease in its advanced stages [44-46]. Chronic hyperglycemia can directly lead to endothelial cell damage, and endothelial dysfunction is a key cause and major factor leading to diabetic cardiovascular disease [47-49]. ATRA has been shown to reduce heart damage with diabetes by interfering with ferroptosis [29, 50]. Previous studies reported that diabetic cardiovascular disease is associated with abnormal vitamin A levels and metabolism and that ATRA intervention can improve diabetic cardiomyopathy [29, 51]; however, the specific mechanism is unclear. In this study, we demonstrated that ATRA can eliminate lipid peroxidation and iron overload, inhibit ferroptosis, and then improve high glucose-induced endothelial dysfunction. *In vivo* and *in vitro* results suggest that ATRA reverses HG-induced increase in 4-HNE, MDA, lipid ROS, and ferrous iron levels, and these effects are similar to those of Fer-1, a ferroptosis

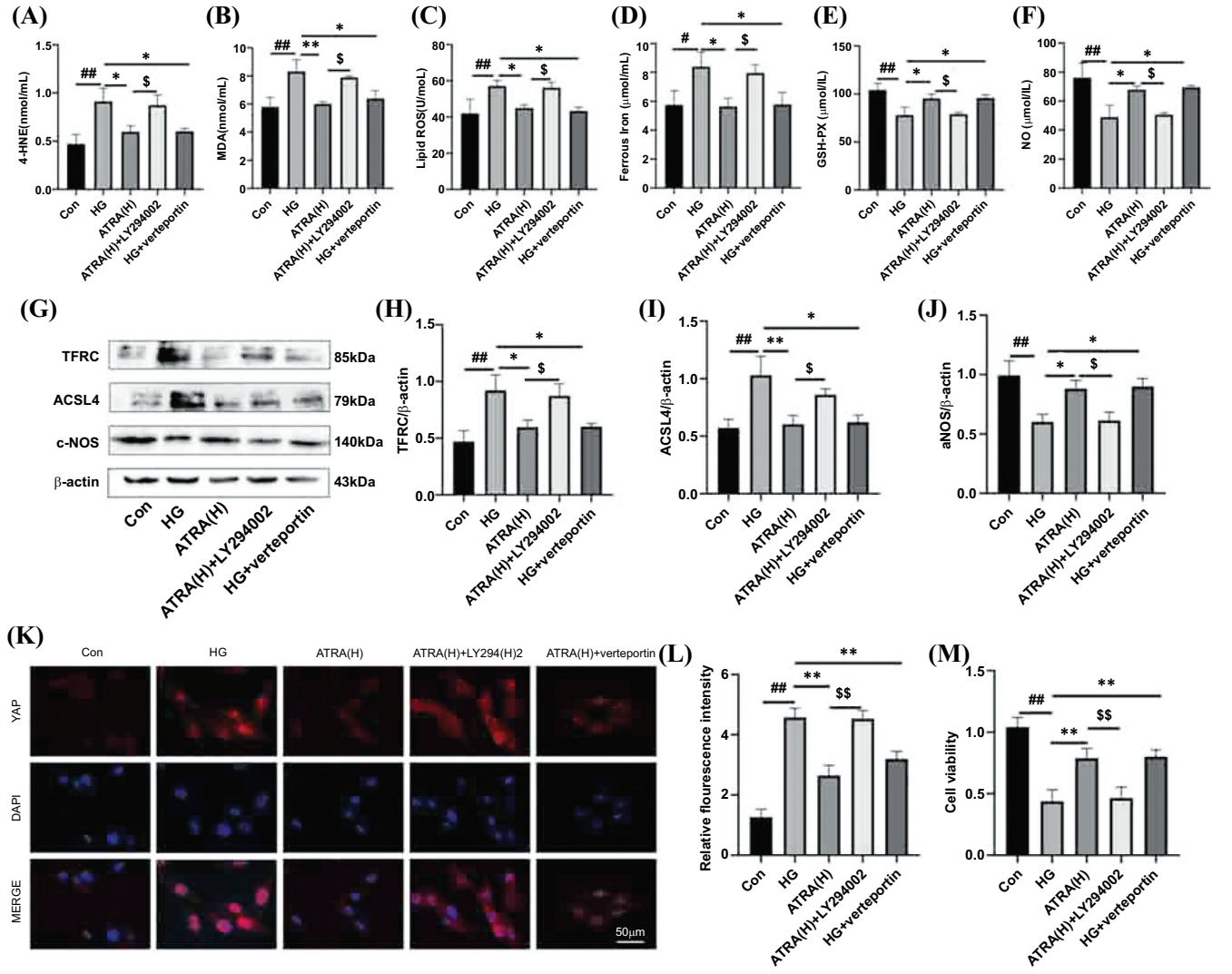

Fig. (6). ATRA treatment alleviates ferroptosis and improves endothelial function in HUVECs by regulating PI3K/AKT-mediated YAP. (**A-F**) The levels of lipid ROS, GSH, GSH-PX, MDA, and 4-HNE in HUVEC supernatant were determined using kits. (**G-J**) Western blotting was used to detect the expression of TFRC, ACSL4, and eNOS proteins in HUVECs induced by HG. (**K**, **L**) The expression of YAP was detected by immunofluorescence staining. (**M**) CCK-8 assay detection of cell viability. The data are expressed as the mean ± SD (n=10 for A-F, n=3 for G-M). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. the Con group, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. the HG group, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ *vs*. the ATRA(H) group. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

inhibitor. Both ATRA and Fer-1 can improve vascular relaxation in the diabetic aorta, and sorafenib can eliminate the effect of ATRA on vascular relaxation. In addition, the ameliorative effects of ATRA on HG-induced endothelial dysfunction and ferroptosis were accompanied by the activation of PI3K/AKT and inhibition of YAP, suggesting that PI3K/AKT and YAP are involved in the vasodilation of ATRA.

The PI3K/AKT pathway is one of the most important intracellular signal transduction pathways regulating cell proliferation and differentiation, and its abnormal regulation is involved in the pathogenesis of various cardiovascular diseases [52-55]. Many studies have reported that the therapeutic effect of the intervention on diabetic cardiomyopathy and endothelial cell damage is related to the activation of the PI3K/AKT signaling pathway [56-58]. Consistent with these findings, the present study showed that ATRA can ameliorate diabetes-induced impaired vasodilation and reverse the decrease in the expression of PI3K and p-AKT. Furthermore, the beneficial effect of ATRA on vasodilation was offset by LY294002, suggesting that the PI3K/AKT pathway is involved in the protective effect of ATRA against HG-induced endothelial dysfunction. Recently, it has been recognized that ATRA functions *via* regulating the PI3K/AKT pathway [59-61]. On the one hand, in the bleomycin-induced pulmonary fibrosis model in mice, ATRA alleviates pulmonary fibrosis by inhibiting the PI3K/AKT pathway [62]. On the other hand, ATRA inhibits the proliferation and migration of vascular smooth muscle cells through RARα-mediated PI3K/AKT and ERK signaling pathways [63]. These results suggest that the activation or inhibition of PI3K/AKT by ATRA may vary with different diseases and tissues.

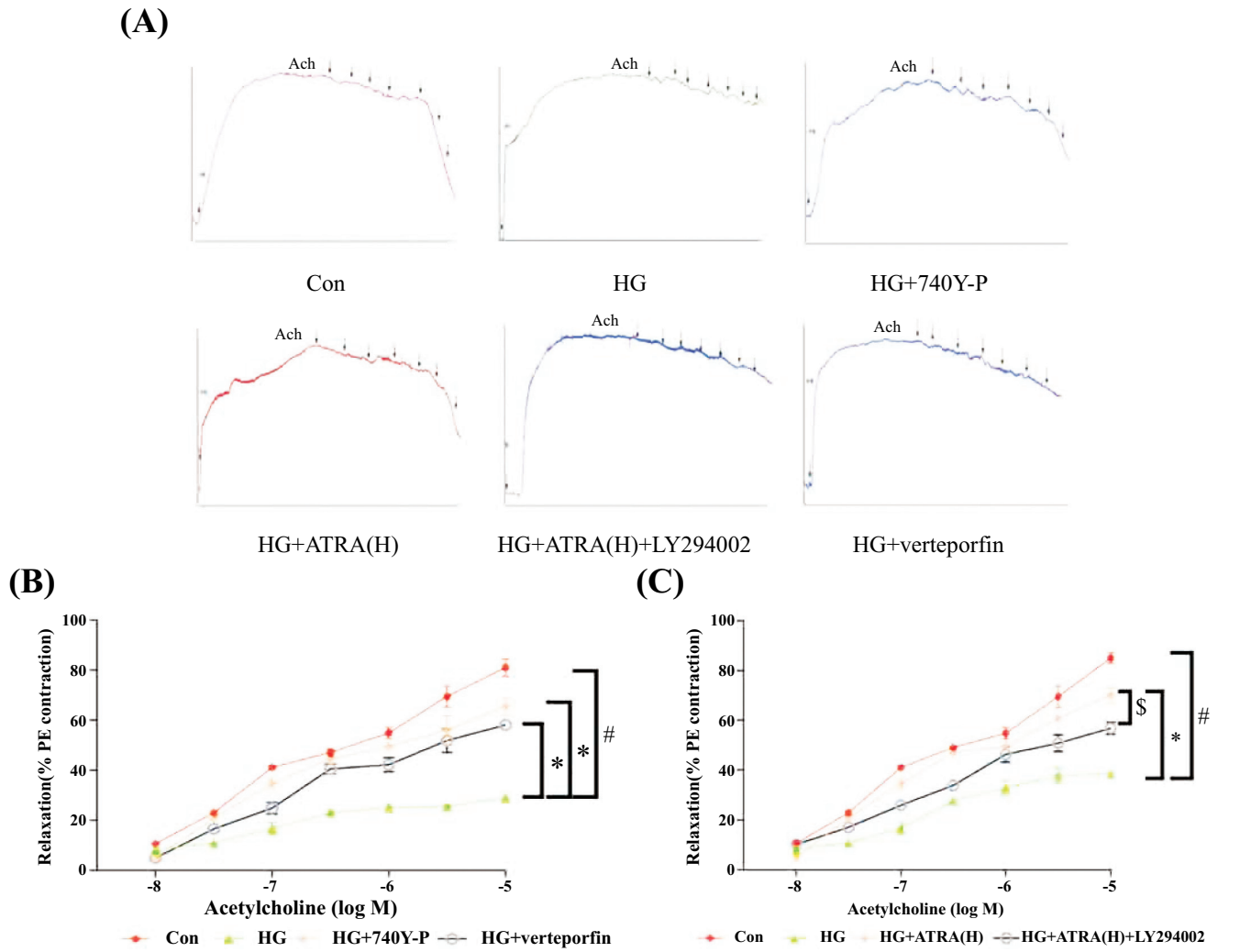

Fig. (7). ATRA therapy alleviates vascular endothelial dysfunction through the PI3K/AKT/YAP pathway. (**A-C**) PE precontracted aorta rings in response to ACh-induced vasodilation in all subgroups. The data are expressed as the mean ± SD (n=3). $^{\#}p < 0.05$ *vs*. the Con group, $^{*}p < 0.05$ *vs*. the HG group, $^{\$}p < 0.05$ *vs*. the HG+ATRA(H) group. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

As a transcriptional coactivator, YAP plays a crucial role in the Hippo signaling pathway. In addition to being an important member of the Hippo signaling pathway, YAP is involved in the regulation of ferroptosis in different tissues and cells, and it has been reported that YAP can induce the expression of two key mediators of ferritin kinase, namely, TFRC and ACSL4 [64-67]. In liver cancer cells, YAP can increase the iron concentration by increasing TFRC transcription [68]. YAP/ACSL4-mediated ferroptosis is an important factor in renal fibrosis induced by calcium oxalate crystal deposition. Another study showed that the inhibition of YAP reversed the upregulation of ACSL4 expression caused by matrix stiffness and alleviated intervertebral disc degeneration by inhibiting ferroptosis in nucleus pulposus cells [69]. Consistent with these previous studies, we found that the HG-induced upregulation of TFRC and ACSL4 was accompanied by increased nuclear localization of YAP, and the inhibition of YAP also reduced the upregulation of TFRC and ACSL4 expression. ATRA improved impaired endothelial function, inhibited lipid peroxidation and iron overload, and reversed the HG-induced upregulation of TFRC and ACSL4. Notably, all of these effects of ATRA were eliminated by inhibiting PI3K/AKT, suggesting that PI3K/AKT and YAP are involved in the vascular protective effects of ATRA. Previous studies have reported a complex feedback loop between YAP and PI3K/AKT that is stimulated by HG [70]. Upregulated YAP leads to hyperglycemic stress-mediated cardiomyocyte hypertrophy and fibrosis by increasing AKT phosphorylation and inhibiting GSK3β [71]. Qian reported [70] that YAP is activated through inhibition of the Hippo pathway under diabetic conditions and that PI3K/AKT is activated through the inhibitory effect of YAP on its repressor, PTEN. Activated PI3K/AKT induces YAP nuclear translocation by inhibiting the Hippo pathway, thereby participating in glomerular mesangial cell proliferation [72]. In addition, YAP and PI3K/AKT participate in the regulation of ferroptosis-related apoptosis. However, the current study explored the regulatory effect of these genes on ferroptosis but not the feedback loop involved, which is also a shortcoming of this study. A human cohort study showed that RA levels in obese and type 2 diabetes patients are significantly reduced, and low levels of RA in impaired fasting glucose can

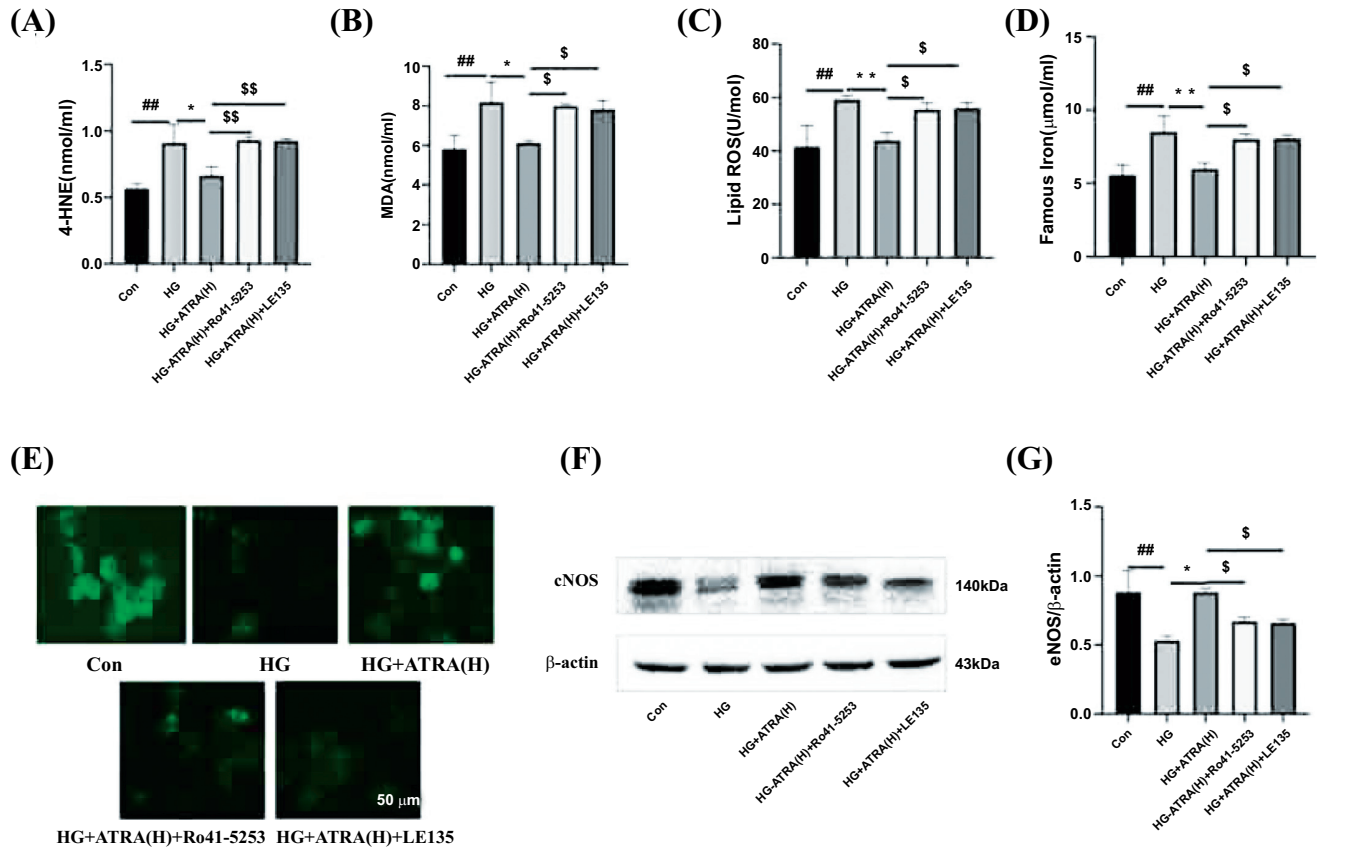

Fig. (8). ATRA treatment attenuates vascular endothelial dysfunction in a RAR-dependent manner. (**A**-**D**) The levels of lipid ROS, GSH, MDA, and 4-HNE in HUVEC supernatant were determined using kits. (**E**) Typical fluorescence pictures and fluorescence intensity of NO in different groups of HUVECs. (**F**, **G**) Western blotting was used to detect the expression of eNOS protein in HUVECs induced by HG. The data are expressed as the mean ± SD (n=10 for A-D, n=3 for E-G). $^{\#\#}p < 0.01$ *vs*. the Con group, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. the HG group, $^{\$}p < 0.05$ *vs.* the HG+ATRA(H) group. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

predict the development of type 2 diabetes [73]. Experimental research proves that RA and synthetic agonists of RAR can be used as a candidate drug for the treatment of diabetes [74-77], but the mechanism of action is still uncertain. The current study has observed the effect of ATRA on vasodilation dysfunction caused by type 1 diabetes, which was already validated in previous studies, and expounded its mechanism from the perspective of PI3K/AKT/YAP-mediated ferroptosis (Figs. **S1-7**).

CONCLUSION

In conclusion, the present study demonstrated that ATRA plays a novel role in protecting against diabetic vascular endothelial dysfunction by inhibiting PI3K/AKT/YAP-mediated ferroptosis. Furthermore, this study suggests a potential therapeutic pathway for diabetic vascular endothelial dysfunction and subsequent cardiovascular complications.

AUTHORS' CONTRIBUTIONS

MZ and ML conceived and wrote the original draft manuscript. MZ and YL conceived and conducted the experiments. YL and BT participated in the experiments of the study. HW and ML provided funding support. MZ and HW participated in data acquisition. All authors reviewed and revised the final version and approved the manuscript submission.

LIST OF ABBREVIATIONS

ACh	=	Acetylcholine
EDR	=	Endothelium-dependent Relaxation
HG	=	High Glucose
HUVECs	=	Human Umbilical Vein Endothelial Cells
NO	=	Nitric Oxide
PI3K	=	Phosphoinositol-3-kinase
RA	=	Retinoic Acid

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal procedures were conducted under the principles approved by the Animal Ethics Committee of Jinzhou Medical University, China (2023032).

HUMAN AND ANIMAL RIGHTS

The experimental procedures were in accordance with the National Institutes of Health Guide. This study adhered to internationally accepted standards for animal research, following the 3Rs principle. The ARRIVE guidelines were employed for reporting ex-

periments involving live animals, promoting ethical research practices.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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