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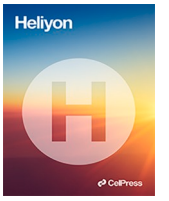

Research article

Cardioprotective role of oleanolic acid in patients with type 2 diabetes mellitus

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ABSTRACT

Background: Patients with type 2 diabetes mellitus (T2DM) experience a decline in cardiac function, resulting in poor prognosis. Therefore, restoration of cardiac function and improvement of myocardial fibrosis is an important treatment goal for patients with T2DM.
Material and methods: The chemical structure of oleanolic acid(OA) was downloaded from PubChem and uploaded to PharmMapper. GeneCards and OMIM databases were searched for genes related to OA and disease and plotted into a Venn diagram. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using R software. Then, a mouse model of diabetes mellitus was established, and ELISA, echocardiographic analysis of cardiac function, TUNEL assay, and reactive oxygen species assay were performed.
Results: Network pharmacology analysis identified the related targets and potential molecular mechanisms underlying the effects of OA in T2DM. ELISA, echocardiographic analysis of cardiac function, and TUNEL assay results showed that OA inhibits apoptosis and improves apoptotic indexes in mice with T2DM-induced myocardial injury.
Conclusion: The results demonstrate the myocardial protective effect of OA in this mouse model.

1. Introduction

According to the World Health Organization, there are an estimated 200 million individuals with diabetes worldwide, and diabetes is ranked the third top cause of global mortality after cancer and cardio-/cerebrovascular diseases [1,2]. The increasing annual incidence of diabetes aligns with the increase in aging among society. Patients with diabetes experience multiple systemic complications including cardiovascular, renal, ocular, and peripheral nerve complications as the disease progresses. Diabetic cardiomyopathy (DCM) is one such cardiac complication that can seriously affect the health of patients, and it is the leading cause of death among patients with diabetes [3]. Myocardial ischemia and myocardial fibrosis occur due to atherosclerosis in patients with diabetes. These changes lead to myocardial systolic and diastolic dysfunction. Epidemiological surveys have found that patients with diabetes have a fourfold higher prevalence of concomitant cardiovascular disease and fivefold higher mortality than individuals without diabetes, after

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adjusting for age and sex. Diabetes research has focused more on type 1 diabetes and less on type 2 diabetes mellitus (T2DM). The substantial prevalence of T2DM among the middle-aged and elderly population is a serious concern. It accounts for about 90 % or more of the total number of individuals with diabetes and is a common occurrence in the perioperative period [4,5]. Therefore, it is imperative to explore methods to improve cardiac function in patients with T2DM. Early intervention for patients with T2DM is important to address the clinical and social burden, especially in reducing the incidence of perioperative cardiac dysfunction [6].

Autophagy/lysosomal degradation is a process by which senescent organelles and proteins are transformed into amino acids and some other small molecules, which are reused to supply energy for maintaining cellular homeostasis and renewal in eukaryotes [7]. During autophagy, the cytoplasm and organelles are wrapped by a bilayer membrane and converted into an autophagosome. Fusion of this autophagosome with lysosomes results in the formation of autophagic lysosomes, which enter the lysosomal lumen. Here, the wrapped cytoplasm and organelles are degraded, allowing the circulation of macromolecules and energy contained in the autophagosome. The autophagy/lysosome pathway has been suggested to be involved in the development and progression of diabetic cardiomyopathy [8]. Reduced AMPK activity and concomitantly decreased levels of myocardial autophagy are important for the development of type I diabetic cardiomyopathy, and diabetic model mice that received pharmacological treatment exhibited enhanced expression of autophagic signature proteins and improved cardiac function [9]. Targeting Beclin1-Bcl2 binding promoted autophagosome synthesis in the diabetic myocardium [10]. However, autophagy is a "double-edged sword", as myocardial fibrosis significantly stimulates autophagy and contributes to the massive production of autophagosomes [11]. Myocardial fibrosis decreases myocardial autophagy or accelerates the production of autophagosomes without fusion with lysosomes, which is manifested by autophagosome accumulation, excessive damage to important organelles, disruption of the intracellular environment and energy depletion, and induced autophagic death. Therefore, autophagosome accumulation seems to play a major role in myocardial fibrosis in T2DM [12].

In our network pharmacology analysis, the potential molecular mechanism underlying the effects of oleanolic acid (OA) was uncovered. Echocardiography findings substantiated the cardioprotective effects of OA in diabetic model mice. We found that OA inhibits apoptosis and improves apoptotic indexes in model mice with diabetes-induced myocardial injury. The results indicated the good myocardial protective effect of OA.

2. Materials and methods

2.1. Target prediction of OA

The 2D and 3D chemical structures of OA were searched in the PubChem database, and the SDF files containing 3D structure information were downloaded. These files were uploaded to PharmMapper analysis platform to predict the targets of OA. The UniProt knowledge base was used to add the gene symbols to the predicted targets to obtain the targets of OA.

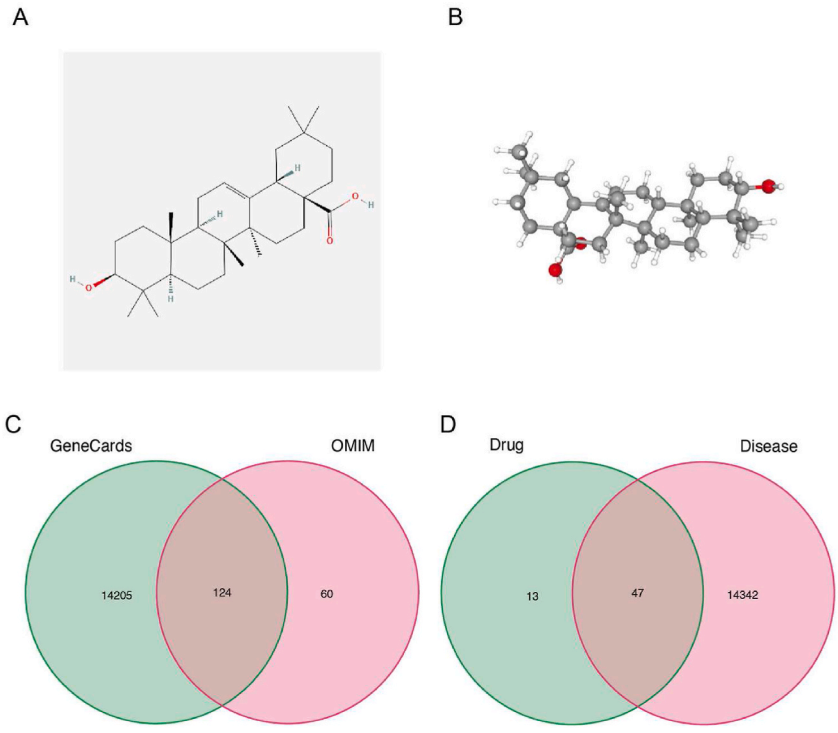

Fig. 1. Chemical structure of OA and composition of its downstream targets. (A) 2-D structure of OA. **(B)** 3-D structure of OA. **(C)** Venn diagram of T2DM targets based on GeneCards and OMIM databases. **(D)** Venn diagram of OA and T2DM targets.

2.2. Target screening for T2DM

The GeneCards database (https://genealacart.genecards.org/) and OMIM database (https://www.omim.org/) were searched for "Type 2 Diabetes Mellitus" as the keyword for disease-related targets, and the results were concatenated to obtain T2DM-related targets.

2.3. Construction of a regulatory network of OA against T2DM

OA targets and T2DM disease targets were mapped individually using Perl software to obtain the intersection targets of OA and T2DM. CytoScape software (v3.7.1) was used to visualize the network and obtain the active ingredient (node)-intersection (target) regulatory network (Fig. 1).

2.4. Construction of a protein-protein interaction (PPI) network of intersectional targets and core targets

The targets of OA were intersected with the targets related to T2DM, and the intersected targets were used as the predicted targets of OA against T2DM. The predicted targets were imported into STRING online website, limiting the study species to *Homo sapiens*, hiding the unlinked targets, and adopting other default settings to obtain the PPI network [13,14].

2.5. GO functional enrichment analysis

The intersecting targets of active ingredients and disease were processed using the Bioconductor bioinformatics package of R software to perform GO functional enrichment analysis (Fig. 3a and b). Statistically significant differences were considered at $p < 0.05$ [15].

2.6. KEGG pathway enrichment analysis

The intersection targets of active ingredients and disease were processed using the Bioconductor bioinformatics package of R software to conduct the KEGG pathway enrichment analysis (Fig. 4a and b). Statistically significant differences were considered at $p < 0.05$.

2.7. Mice

Mature male C57BL/6J mice were housed in five individual cages with free access to food and water in regular captivity settings (22 °C, 55 % relative humidity, 12 h light/dark). The Soochow University Animal Ethics Committee approved the animal experiments, which were conducted following the Standards for the Use of Animals in Toxicology adopted by the highly regarded Society of Toxicology in 1999.

2.8. Drugs and treatment

OA (#HY-N0156, MCE, USA) was dissolved in 10 % DMSO, 40 % PEG300, 5 % Tween-80, and 45 % saline. As a control treatment, only vehicles were used i.e., 10 % DMSO, 40 % PEG300, 5 % Tween-80, and 45 % saline. Preoperative intraperitoneal injection of OA 80 mg/kg once daily for 30 days.

2.9. Establishment of the T2DM mouse model

Throughout the experiment, the mice were provided access to rodent chow and had free access to distilled water. Mice in the control group consumed a normal diet, while insulin resistance and obesity were induced in the mice in the model group through supplementation of a high-fat diet (HFD). The HFD consisted of a mixed diet containing 63 % calories, 1 % (w/w) sucrose, 1 % (w/w) cholesterol, a high protein level, and 25 % extra virgin olive oil.

To induce T2DM in rats, streptozotocin (STZ; 30 mg/kg) was injected intraperitoneally once after 24-h fasting along with subsequent feeding of HFD. STZ was repeatedly injected intraperitoneally into the model diabetic mice for five days at a low dose of 55 mg/kg in citrate buffer. Access to both water and food was restricted for 12 h before injection. Five days straight saw one daily injection of STZ. One week after the last STZ injection, mice with non-fasting glucose levels of more than 15 mmol/L were chosen for further experimentation.

2.10. ELISA

Blood was withdrawn and centrifuged at 1000*g* for 10 min at 4 °C to obtain serum samples. According to the manufacturer's instructions, serum IL4, IL10, IL1β, IL6, CK, and LDH levels were quantified using the respective commercial ELISA kits (Sigma-Aldrich, USA).

2.11. Echocardiographic analysis of cardiac function

Mice were subjected to isoflurane anesthesia after reperfusion and then to sonography using a small animal model in vivo ultrasonography equipment (VEVO 2100, Visual Ultrasound, Canada). In the short-axis perspective, M-mode recordings were made at the level of the left ventricular papillary muscle. The recordings were used to measure the internal left ventricular end-diastolic (LVIDd) and end-systolic (LVIDs) dimensions.

The equations used to determine the left ventricular ejection fraction (EF) and fractional shortening (FS) are as follows: EF = [(LVIDd) [3]-(LVIDs) [3]]/(LVIDd) [3]x100 %; FS=(LVIDd-LVIDs)/LVIDdx100 %.

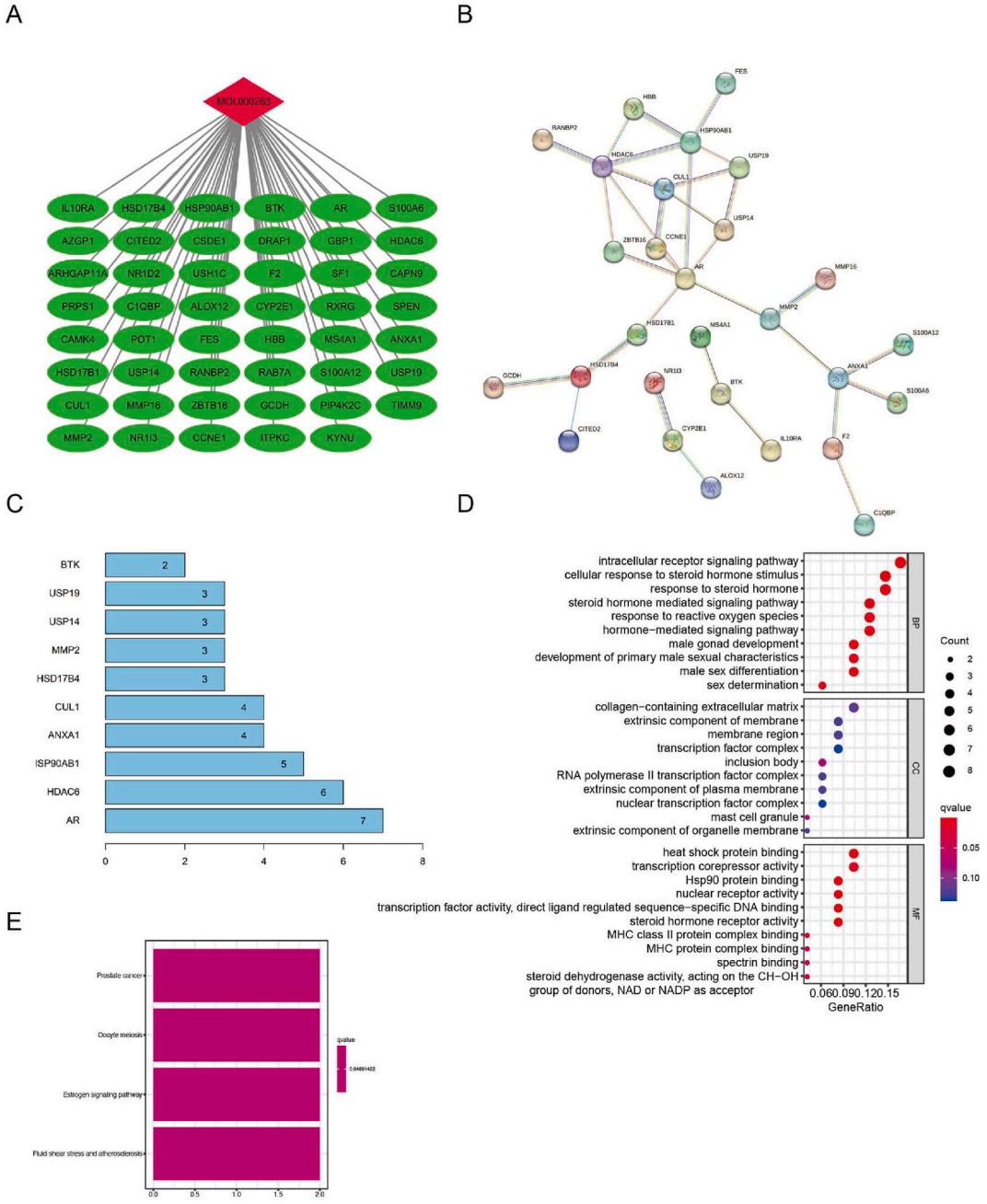

Fig. 2. Molecular biological functions of downstream targets of OA. (A) The ingredient-target regulatory network of OA. **(B)** Protein-protein interaction network of key targets of OA. **(C)** Bar plot of key targets of OA. **(D)** Bubble plot showing results for GO function enrichment analysis of OA. **(E)** KEGG pathway enrichment analysis of key markers of OA.

2.12. TUNEL assay

The TUNEL assay was performed using the One-Step TUNEL Apoptosis Detection Kit (Beyotime, China). In this experiment, the left ventricles of mice were harvested, fixed with 4 % paraformaldehyde, dehydrated with sucrose, embedded in OCT, and cut into 8 μm thick sections. Tissue sections were permeabilized with 0.5 % Triton X-100 and incubated for 60 min at 37 °C in a working buffer.

2.13. Quantification of malondialdehyde (MDA), glutathione (GSH), and total antioxidant capacity (T-AOC)

Using respective commercial assay kits, MDA, GSH, and T-AOC levels were quantified in the left ventricle tissue homogenates (Sigma-Aldrich, USA). The readouts were made through spectrophotometry (Qualcomm). The results of the experiment were expressed as MDA, GSH, and T-AOC units per milligram of protein.

2.14. Assessment of mitochondrial reactive oxygen species (ROS)

Mitochondrial ROS assay was performed using a ROS assay kit (Beyotime, China). The fluorescence intensity of dichlorodihydrofluorescein diacetate was measured using a microplate reader (Synergy H1, BioTek, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

3. Results

3.1. Target prediction results for OA

The 2D and 3D structures of OA were searched in the PubChem database (Fig. 1A and B). Sixty targets of OA were obtained.

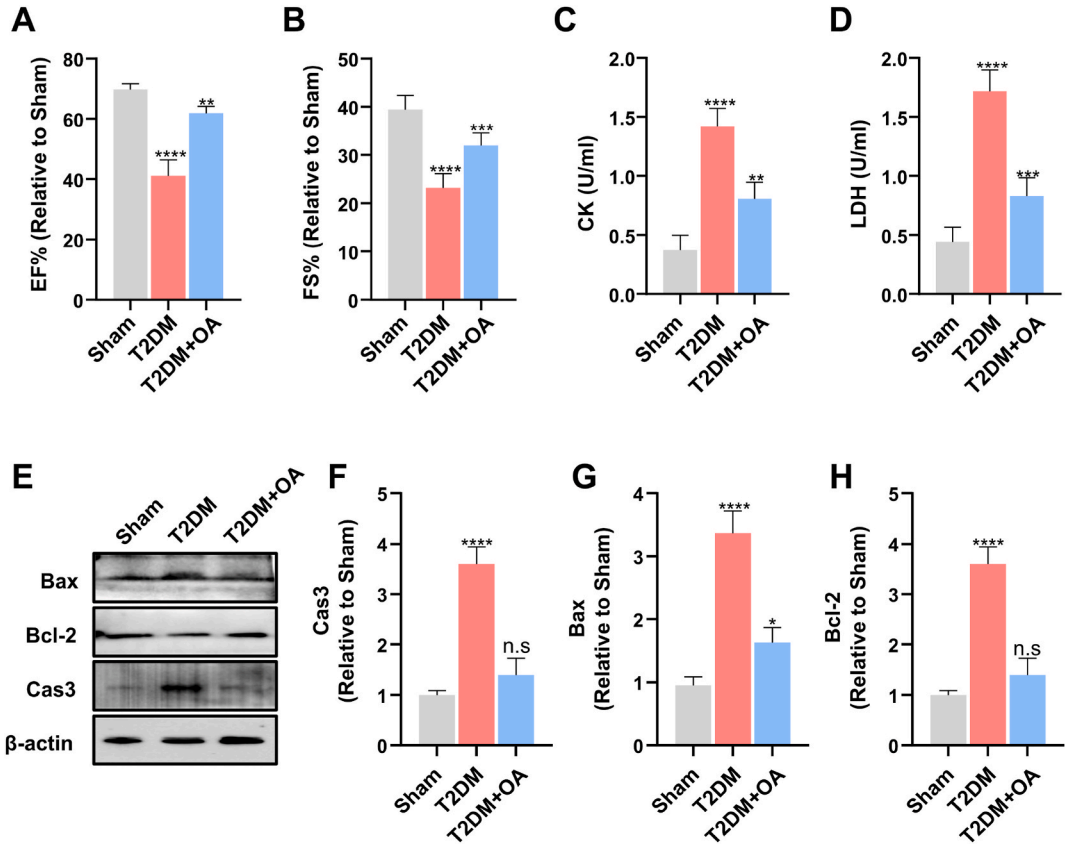

Fig. 3. Effect of OA on myocardial injury and cardiac function related to T2DM. (A–B) Quantitative analysis showed that OA prevented diabetes-induced reduction in ejection fraction and fractional shortening. **(C–D)** OA significantly suppressed diabetes-induced myocardial damage, as evidenced by decreased myocardial CK and LDH levels. Results of quantitative analysis are shown. **(E)** Representative Western blot images of apoptosis indicators in the damaged myocardium. OA significantly suppressed the levels of apoptosis-related proteins in the myocardium. There was a decrease in the levels of BAX and Caspase3 and an increase in the level of BCL2. **(F–H)** Quantitative analysis of the expression levels of BAX, Caspase3, and BCL2. n = 6 per group. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

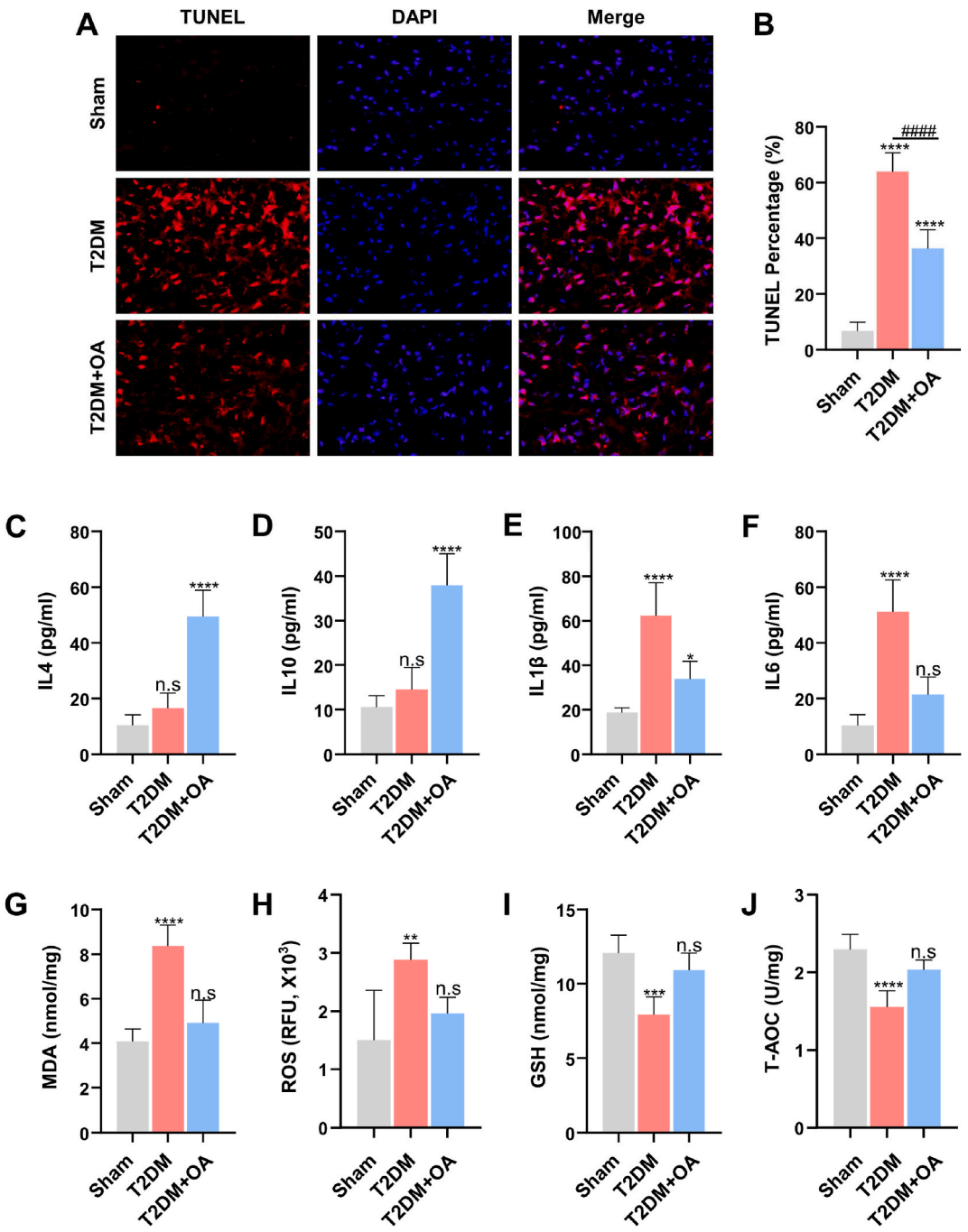

Fig. 4. Effect of OA on apoptosis, inflammation levels, and oxidative stress levels elevated by myocardial injury related to T2DM. (A–B) Representative images of myocardial apoptotic cells in the injury zone, and quantitative analysis of the results. OA significantly reduced the number of TUNEL-positive cells in the myocardium. **(C–D)** Detection and quantification of serum inflammation-associated cytokine levels in diabetic model mice. OA significantly upregulated the expression of anti-inflammatory cytokines in the myocardium. **(E–F)** Detection and quantification of serum inflammation-associated cytokine levels in diabetic model mice. OA significantly downregulated the expression of pro-inflammatory cytokines in the myocardium. **(G–J)** Detection of oxidative stress indicators in the damaged myocardium of diabetic model mice and quantitative analysis. OA significantly inhibited the upregulation of diabetes-induced indexes of myocardial oxidative stress. n = 5 per group. *p < 0.05 , ***p < 0.001 , ****p < 0.0001.

3.2. Target screening results of T2DM

After searching the GeneCards and OMIM databases using the keyword "Type 2 Diabetes Mellitus", 14,389 targets were obtained after concatenation (Fig. 1C). Using Perl software, 60 OA targets and 14,389 T2DM disease targets were intersected to obtain 47 intersection targets, and these 47 intersection targets corresponded to OA (Fig. 1D). The regulatory network is shown in Fig. 2A; the red diamonds represent OA, and the green ovals represent 47 intersecting target genes.

3.3. Construction of target PPI network and key target screening

PPI networks offer the possibility to screen key targets for drug modulation of disease onset. The PPI network and histogram of 47 intersecting targets (Fig. 2B and C) showed that AR, HDAC6, and ISP90AB1 are located in the core of the network with the highest number of neighboring nodes, which may be the core genes involved in the effect of OA against T2DM.

3.4. GO functional enrichment analysis

The GO function enrichment analysis mainly includes biological process, cellular component, and molecular function. The results of the GO function enrichment analysis using the Bioconductor bioinformatics package of R software are shown in Fig. 2D.

3.5. KEGG pathway enrichment analysis

The top four pathways enriched in the KEGG pathway enrichment analysis, performed using the Bioconductor bioinformatics package of R software, are shown in Fig. 2E.

3.6. Preventive effect of OA on myocardial injury in diabetic model mice

We first used echocardiography to investigate the cardioprotective effects of OA in diabetic mice (Fig. 3A–B). We found that OA treatment significantly increased left ventricular EF and FS in mice with diabetes-induced myocardial injury. Pretreatment with OA treatment significantly reduced serum CK and LDH levels in ischemia/reperfusion (I/R) mice (Fig. 3C–D). OA also inhibited apoptosis in mice with diabetes-induced myocardial injury, as shown by the decrease in the levels of BAX and Caspase-3 and the increase in the levels of BCL-2 (Fig. 3E–H).

TUNEL assay results showed that OA significantly reduced the percentage of apoptotic cells in the damaged myocardium (Fig. 4A–B). OA also reduced inflammatory indexes in the serum samples, indicating its good anti-inflammatory effect (Fig. 4C–F). Levels of MDA, ROS, GSH, and T-AOC also reflected the good antioxidant activity of OA (Fig. 4G–J).

All these results indicate the good myocardial protective effect of OA.

4. Discussion

Autophagy has recently been shown to play an important role in the pathogenesis of diabetic complications, and improving cardiac function and myocardial fibrosis by interfering with autophagic activity holds potential as a novel approach to the treatment of cardiac complications of T2DM [16,17]. Treatment options for T2DM that lower both glucose and lipid levels along with exhibiting an antioxidant effect are expected to break the vicious cycle formed by oxidative stress and disorders of glucose metabolism.

OA has a 30-carbon skeleton, including a five-ring structure consisting of one cyclopentane ring and four cyclohexane rings. It contains a hydroxyl group (-OH) and several other functional groups, making it important in terms of biological activity. OA reduces both glucose and lipid levels, acts as an antioxidant, and exhibits anti-atherosclerotic effects. Long-term toxicity data have shown that the drug is safe and reliable, without carcinogenic or mutagenic effects. Its antioxidant effect is irreversible, and OA has a stable structure [18]. It exhibits anti-inflammatory, antioxidant, anticancer, antiviral, and antibacterial activities. It also has the potential for liver protection and lowering blood glucose and lipid levels. These properties make OA a hot topic in the field of diabetes research.

The precise regulation of autophagic signaling is essential for cells to respond to different external stimuli. The occurrence of both basal and induced autophagy is under tight cellular regulatory mechanisms that allow the cells to maintain a stable internal environment and protect themselves in response to unexpected stimuli. TOR (target of rapamycin), a central molecule in autophagy regulation, allows sensing of multiple signals of cellular changes and enhances or decreases autophagosome formation. Signaling pathways that are activated in response to intracellular ATP levels and hypoxia can be integrated directly or indirectly through TOR, thus altering the formation of autophagosomes in response to different external environmental stimuli and protecting the cells. In our study, we found that OA has a good anti-apoptotic effect, observed through improved apoptotic indexes in model mice with diabetes-induced myocardial injury.

Based on the findings, we conclude that OA can improve myocardial autophagic activity in T2DM and significantly improve cardiac function and myocardial fibrosis in T2DM. A better understanding of the physiological functions and pharmacological effects of OA will have a broad influence on its therapeutic application. Our study explores the pathogenesis of T2DM-related cardiomyopathy and provides new targets for the prevention and treatment of cardiovascular complications in T2DM. The results obtained provide a base for other studies on cardiovascular prevention and treatment and further enrich and improve our understanding of the pathogenesis of cardiovascular complications in T2DM.

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Taicang Municipal Science and Technology Bureau Key Research and Development Program(TC2020JCYL15) and Suzhou Gusu Health Talent Research Project(GSWS2023067)

Data availability

The raw measurements are available in the Supplemental Files. Other data can be access via correspondence authors.

Ethics statement

This study was approved by the Ethics Committee of the Soochow Univeristy Affiliated Taicang Hospital.

CRediT authorship contribution statement

Chengrui Li: Writing – original draft, Software, Formal analysis, Data curation, Conceptualization. **Jing He:** Writing – original draft, Software, Resources, Formal analysis, Data curation, Conceptualization. **Yongjun Li:** Writing – original draft, Data curation, Conceptualization. **Chengyang Zhang:** Writing – original draft, Data curation, Conceptualization. **Ziheng Wang:** Writing – original draft, Data curation, Conceptualization. **Xiaoman Wu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fuwei Qi:** Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Ziheng Wang is the assocaited editor of Heliyon If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31303.

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