# Quercetin relieves coronary atherosclerosis via regulating fibroblast growth factor 2

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## Abstract

# Objective

The aim of this study is to investigate the role of quercetin on coronary atherosclerosis and further explore its possible mechanisms.

# Methods

Hematoxylin-eosin (H&E), Immunohistochemical (IHC) and Aniline blue staining are to analyze the pathological changes of aorta cross section. Traditional Chinese Medicine Systems Pharmacology Database (TCMSP), Swiss Target Prediction and PubChem are used to predict and screen the bioactive ingredients of Traditional Chinese medicine (Haunglian, Yuxingcao and Jinyinhua) for coronary atherosclerosis. Inflammatory factors and vascular protection parameters were quantitatively detected via ELISA and western blot. Cell Counting Kit-8 (CCK-8), 5-ethynyl-2-deoxyuridine (EdU), wound healing are conducted to detect the proliferation and migration of vascular smooth muscle cells (VSMC). DisGeNET, Matascape, SWISSMODEL, cellular thermal shift assay (CETSA) and fluorescence titrimetric are used to predict the targets of quercetin.

# Results

We found that quercetin is the effective active component of Huanglian, Yuxingcao and Jinyinhua acting on AS. Further *in vivo* and *in vitro* data revealed that quercetin improved the pathological changes of model mice and inhibited VSMC cells proliferation, migration and inflammatory response. Mechanically, we demonstrated that fibroblast growth factor 2 (FGF2) is a direct target of quercetin, and overexpression of FGF2 attenuated the anti-AS function of quercetin.

# Conclusion

In our study, we confirmed the functional role of the quercetin-FGF2 axis in the progression of coronary atherosclerosis, which provides a latent target for coronary atherosclerosis treatment.


## Introduction

Coronary artery disease (CAD) is a leading cause of death from heart disease, and its incidence is rising due to an increasing population of obesity and diabetes [1, 2]. According to statistics, CAD accounts for approximately 40% of all deaths related causes in developed countries [3]. The mechanism of CAD is complex and involves many factors, among which oxidative stress and atherosclerosis are particularly closely related to the pathogenesis of CAD [5]. At present, chemotherapy, percutaneous coronary intervention, coronary artery bypass grafting and other treatment methods are the main treatment strategy of CAD [6]. Although we have made great progress in understanding the pathogenesis of CAD and finding treatment strategies, the complex mechanisms of CAD still lead to inaccuracy and incompleteness of diagnostic strategies. Therefore, the development of high-characteristic bio-diagnostic markers and more efficient therapeutic drugs is a problem that we urgently need to address, and it has high application value sensitivity and convenience.

Quercetin is a widely distributed, naturally occurring polyhydroxyl flavonoid found in many plant-based foods, such as red onions, apples, broccoli, parsley and berries [7, 8]. It has a wide range of pharmacological properties and has been recognized as a potential drug against diabetes, hypertension, cancer and neurodegenerative diseases [9]. Additionally, quercetin is also of great concern for its anti-atherosclerosis effects. Jia et al. showed that quercetin treatment significantly reduced atherosclerotic plaque area and lipid accumulation in the aorta [10]. Similarly, Cao et al. demonstrated that quercetin treatment improved atherosclerotic pathology and reduced inflammatory response in $ApoE^{-/-}$ mice [11]. Although the protective or preventive effects of quercetin on atherosclerosis have been reported, further mechanisms have not been reported yet.

In view of the fact that atherosclerosis is a common cause of CAD, we used high-fat-fed rats to simulate human atherosclerosis, exploring the protective effect of quercetin on coronary atherosclerosis in model rats. Besides, we studied the mechanism of quercetin in inhibiting proliferation, migration and inflammation in VSMC cells and hope to find a more characteristic bio-diagnostic marker for quercetin in the treatment of coronary atherosclerosis.

## Methods

# Animals and experimental design

A total of 60 $ApoE^{-/-}$ mice, weighing 180–250 g were enrolled in the research. Among them, 10 mice were fed with 1.25% high cholesterol to establish an atherosclerosis model, and the same number of mice was injected with physiological saline in equal volume as a control group. When atherosclerosis were measurable, mice were randomly divided into five groups: 1) model group (n = 10, fed with 1.25% high cholesterol); 2) 25 mg/kg group (n = 10, administrate with 25 mg/kg quercetin); 3) 50 mg/kg group (n = 10, administrate with 50 mg/kg quercetin); 4) 100 mg/kg group (n = 10, administrate with 100 mg/kg quercetin); 5) positive control group (n = 10, administrate with nitroglycerin). Quercetin (95% purity) was procured from Sigma-Aldrich Ltd (S2424, St. Louis, Missouri, USA). The present study was approved by the Ethics Committee of Changchun University of Chinese Medicine.

# Cell culture

Human aortic vascular smooth muscle cells (VSMC) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). After all the cells were resuscitated, they were cultured in RPMI-1640 medium (Sigma-Aldrich Co. LLC., USA) in a humidified environment, and the medium was changed every two days. When the cell growth density reached 80%, they were harvest for further analysis.

# Hematoxylin-eosin (H&E) staining

The aortic tissues were fixed with formaldehyde (10%, Sigma-Aldrich Co. LLC., USA) for 24 h, and then tissues were placed in a 5% nitric acid decalcification solution for 3–5 d. After washing with water, routine dehydration, transparency, paraffin immersion, embedding and sectioning, the tissue sections were stained with hematoxylin and eosin (Hubei Jusheng Technology Co., Ltd., China). Finally, the pathological changes of aortic tissues were observed under microscopy (Olympus, Tokyo, Japan).

# Immunohistochemical (IHC)

Paraffin-embedded aortic tissues slides were treated with 3% hydrogen peroxide containing methanol, following by incubation with 10% goat serum to block. After washing, the sections were incubated with primary antibody against CD68 (1:500, Abcam, USA) overnight, and then secondary antibody. Subsequently, the sections were stained with DAB and visualized by microscopy (Olympus, Tokyo, Japan).

# Aniline blue staining

First, aortic tissue sections were fixed in 3% glutaraldehyde in 0.2 M phosphate buffer for 30 min at room temperature. Then, the samples were then stained with 5% aqueous aniline blue mixed with 4% acetic acid (Shanghai Bojing Chemical Co., Ltd., China) for 5 min. Finally, the staining was observed under a microscope (Olympus, Tokyo, Japan).

# Measurement of interleukin (IL)-6 and IL-8

The concentrations of IL-6 and IL-8 in the model mice and VSMC cells were assessed using the available ELISA kit (USCN Business Co., Ltd, China).

# Western blot

Aortic tissues were lysed by RIPA Lysis Buffer (Solarbio, Beijing, China), and protein concentration was measured by the BCA protein assay kit (Solarbio, Beijing, China). The prepared protein was separated by polyacrylamide-SDS gels and then transferred onto PVDF membranes (Roche, Switzerland). Blocked with 5% skimmed milk for 2.5 h, the PVDF membrane was subjected to incubation with primary antibodies against VEGF, Hsp70 and β-actin (1:1000, Proteintech Group Inc., Wuhan, China) at 4°C overnight. On the following day, protein samples were incubated with the secondary antibody at 37°C for 45 min. The protein blots were visualized using the enhanced chemiluminescence (ECL) with exposure to X-ray films (Hyperfilm, GE Healthcare, UK, USA). β-actin was used as internal control, and the gray value ratio of the protein band to β-actin was deemed as relative protein expression.

# Cell Counting Kit-8 (CCK-8)

In short, VSMC cells adjusted to the appropriate concentration ($5\times10^4$ cells) were inoculated on 96-well plates and treated accordingly. Then, each well was added with CCK-8 solution (Sangon Biotech

(Shanghai) Co., Ltd., China) and incubated for 2 h in the dark. Finally, the optical density at 450 nm was measured.

## 5-ethynyl-2-deoxyuridine (EdU) assay

Briefly, VSMC cells were inoculated in 96-well plates for 48 h. Washed with PBS (Beyotime, Beijing, China), they were incubated with 10 μM EdU (Beyotime, Beijing, China) for 2 h at 37°C. EdU-positive cells were detected by Apollo staining and DAPI staining, and the percentage of positive cells was defined as proliferation rate.

## Wound healing assay

First, VSMC cells were inoculated in a 6-well plate for 24 h. When the cells were fully fused, the pipette tip was applied to create a scratch wound on the confluent cells in the center. The migration and cell movement of the entire wound area were observed with an inverted optical microscope (Oberkochen, Germany), and the images were taken at 48 h with a camera connected to the microscope (SonyCyber shot, Shanghai Suoguang Visual Products Co., Ltd., China). The cell migration ability was statistically analyzed according to the cell healing.

## Cell transfection

pc-NC and pc-FGF2 were constructed by GenePharma Technology Co., Ltd (Shanghai, China). pcDNA3.1 was used for FGF2 overexpression. When the VSMC cells confluence rate reached 80%, Lipofectamine 2000 (Invitrogen, USA) was applied for transfection.

## Bioinformatics

The Traditional Chinese Medicine Systems Pharmacology Database (TCMSP, http://ibts.hkbu.edu.hk/LSP/tcmsp.php) was used to screen the common active ingredients of Huanglian, Yuxingcao and Jinyinhua. Swiss Target Prediction (http://www.swisstargetprediction.ch/) was used to predict the common targets of quercetin and coronary atherosclerosis. DisGeNET (https://www.disgenet.org/) and Metascape (https://metascape.org/) were used to further screen targets of quercetin acting on coronary atherosclerosis. PubChem (https:// pubchem.ncbi.nlm.nih.gov/) was used to analyze the chemistry of quercetin, including molecular formula, 2D and 3D structural information. SWISSMODEL (https://swissmodel.expasy.org/) and Autodock (http://www.scripps.edu/mb/olson/doc/Autodock) were used for 3D models construction and docking study of FGF2, respectively.

## Cellular thermal shift assay (CETSA)

VSMC cells treated with quercetin or DMSO at 37°C for 24 h were collected, and the cell suspension was distributed into 0.2 ml PCR tubes, with 200 μl cell suspension in each tube. The PCR tubes were heated at the designated temperature (40, 60, 64, 67, 70, 72 and 75°C) for 3 min. They were then removed and incubated at 4°C immediately following heating. Cells were then lysed using cell lysis buffer for western

(Beyotime Institute of Biotechnology) and analyzed by western blotting as described in the western blotting methods above.

## Statistical analysis

All the data were analyzed by Statistical Package for Social Sciences19.0 (SPSS, Chicago, IL, USA) and presented as mean ± standard deviation (SD). Differences among the groups were analyzed by one One-way ANOVA followed by Dunnett's multiple comparisons. $P < 0.05$ were considered significant differences between groups.

# Results

## Construction of mice model of coronary atherosclerosis

As shown in Fig. 1A, H&E staining showed an increased necrotic area of cross-sectional aortic in the high-fat-fed mice compared to the normal-fed mice. Consistently, monocyte/macrophage content assessed by anti-CD68 IHC revealed that high-fat-fed significantly increased CD68 $^{+}$ cells (macrophages) in aortic lesions (Fig. 1B), indicating that the atherosclerosis model was successfully constructed.

## Quercetin is effective in the treatment of coronary atherosclerosis in vivo

From the TCMSP database, two common ingredients of Huanglian, Yuxingcao and Jinyinhua were obtained (Fig. 2A). According to the requirement of easy absorption of the drug, quercetin was selected. Next, we obtained a total of 37 common targets of quercetin and CAD through Swiss Target Prediction, suggesting that quercetin may act on coronary atherosclerosis (Fig. 2B). The molecular structure of quercetin was shown in Fig. 2C. To further study the role of quercetin on coronary atherosclerosis, we detected the changes in the expression of inflammatory factors and vascular protective factors under the intervention of quercetin. As expected, compared to the model group, quercetin intervention significantly reduced the levels of inflammatory factors IL-6 and IL-8, and vascular protection parameters such as VEGF and HSP70 were both evidently decreased (Fig. 3A and 3B). Of note, with the increase of quercetin concentration, its inhibitory effect on the above factors was also enhanced. Further, aortic cross-sections histological analysis with IHC and aniline blue showed that quercetin treatment significantly reduced inflammatory cell and collagen content in a concentration-dependent manner as a comparison to model mice (Fig. 3C and 3D).

Quercetin inhibits VSMC proliferation, migration, and secretion of inflammatory cytokines in a dose-dependent and time-dependent manner in vitro

Quercetin showed inhibition effects on the progression of coronary atherosclerosis in model mice. To further explore the biological function of quercetin, we conducted *in vitro* studies using VSMC cells. In the proliferation experiment, we found that quercetin significantly inhibited the proliferation activity of VSMC, especially at the concentration of 50 μM (Fig. 4A). In the migration experiment, the relative migration width of the 50 μM group was also evidently larger than that of the other three groups (model, 2 μM and 10 μM) (Fig. 4B). Similarly, the levels of inflammatory cytokines IL-6 and IL-8 were remarkably reduced after quercetin treatment, especially in the 50 μM group (Fig. 4C). The above-mentioned data confirmed that quercetin inhibited VSMC proliferation, migration and secretion of inflammatory cytokines in a dose-dependent manner. Considering that the inhibitory effect of 50 μM quercetin is more obvious, we chose 50 μM as a fixed dose to explore the influence of time on the treatment of coronary atherosclerosis. In the beginning, the weakened fluorescence activity was found in EdU staining with the increase of quercetin treatment time (Fig. 5A). Next, the wound healing data depicted that 50 μM quercetin reduced the cell migration rate in a time-dependent manner (Fig. 5B). Meanwhile, compared with other time periods (12 h and 24 h), the content of inflammatory factors IL-6 and IL-8 in VSMC cells was the lowest after 36 h of quercetin treatment (Fig. 5C).

# FGF2 is the target of quercetin on coronary atherosclerosis

To clarify the regulatory mechanism of quercetin on coronary atherosclerosis, we performed further bioinformatics analysis on the above screened 37 targets. We screened targets related to coronary atherosclerosis through DisGeNET enrichment analysis and found these targets are mainly related to hyperlipidemia, dyslipidemias, endothelial dysfunction, vascular diseases and atherosclerosis of the aorta (Fig. 6A). After that, Metascape was applied to select the target gene related to epithelial cell proliferation and apoptosis. As presented in Fig. 6B, these genes were primarily associated with cholesterol metabolism and regulation of plasma lipoprotein particles. Of the 37 genes, we focused on FGF2, as it is related to both coronary atherosclerosis and epithelial cell proliferation and apoptosis and has a higher expression. Its 3D structure and possible binding patterns with quercetin are shown in Fig. 6C. To further verify whether FGF2 is the direct target of quercetin acting on coronary atherosclerosis, CETSA was conducted, and the data confirmed that quercetin could bind to FGF2 and promote the degradation of FGF2 protein (Fig. 6D).

# FGF2 overexpression reverses the inhibitory effect of quercetin on coronary atherosclerosis

To further explore the relationship between quercetin and FGF2 in the progression of coronary atherosclerosis, we performed rescue experiments. As exhibited in Fig. 7A, compared with quercetin + pc-NC group, transfected with quercetin + pc-FGF2 partially offset the toxic effect of quercetin on VSMC cells. Consistently, EdU results showed that the number of cell clones in group quercetin + pc-FGF2 also increased relatively in comparison to quercetin + pc-NC group (Fig. 7B). In the verification of migration experiments, the wound healing results showed that the migration rate of VSMC cells transfected with quercetin + pc-FGF2 was significantly higher than that of cells transfected with quercetin + pc-NC (Fig. 7C). Furthermore, ELISA data depicted that overexpression FGF2 weakened the inhibition of quercetin on inflammatory response,manifested by an increase in inflammatory cytokines IL-6 and IL-8 (Fig. 7D).

## Discussion

Pathological manifestations of animal aorta showed obvious atherosclerosis plaque formation, lipid deposition, vascular wall thickening and hardening, reduced elasticity, and increased necrotic area [13]. Similarly, in the current research, we observed pathological changes and increased cross-sectional necrotic area of the aorta in model mice. Atherosclerosis is a complex chronic inflammatory disease involving the interaction of many inflammatory cells and cytokines, such as the up-regulation of adhesion molecules leading to the recruitment of immune cell vascular walls [14]. In atherosclerosis lesions, the most abundant immune cell type is the macrophage, which is involved in all pathological stages of atherosclerosis by secreting chemokines [15, 16]. Consistently, IHC data revealed a significant increase of $CD68^{+}$ cells (macrophage) in the aortic lesions of mice fed with high fat, indicating that our *in vivo* simulation of atherosclerosis modeling was successful.

Many studies suggested that inflammation plays an important role in all stages of atherosclerosis development and progression [17], among which IL-6 and IL-8 are two important factors that promote an inflammatory response. [18]. Our ELISA results showed that the model mice had higher expressions of IL-6 and IL-8. Conversely, the above two inflammatory factors in the quercetin group were significantly reduced. This conclusion is consistent with Cao et al. [11]. Meanwhile, we observed that quercetin inhibited the expression of inflammatory cells, again confirming the anti-inflammatory effect of quercetin. Vascular endothelial growth factor (VEGF) is a very effective pro-angiogenesis factor, and it can increase vascular permeability and recruit inflammatory cells to injury sites [19]. HSP70 is involved in the immune response during the formation of atherosclerosis plaques, which activates inflammatory cells and reduces the stability of atherosclerosis plaques [20]. Consistent with the reports, both VEGF and HSP70 were highly expressed in the model group. Terao et al. revealed that quercetin regulates blood pressure and maintains cardiovascular function by changing vascular compliance, anti-inflammatory and antioxidant effects [21]. Interestingly, our data also showed that quercetin decreased the expression of vascular protective parameters VEGF and HSP70. Collagen, which forms the main component of atherosclerosis plaques, can block blood vessels and promote lipid deposition [22]. Correspondingly, we observed that high-fat feeding led to higher collagen content in the aorta of model mice. Of note, quercetin improved coronary atherosclerosis by reducing aortic lesions and collagen levels, which is consistent with Li et al. [23].

Accumulated evidence clearly confirms that normal quiescent VSMC proliferates intensively and migrate to the inner subcutaneous intima in the progress of atherosclerosis. More than that, the proliferation and migration of VSMC to the intima are important components of atherosclerosis [24, 25]. Therefore, limiting the proliferation and migration of VSMC is contributed to inhibiting the progression of atherosclerosis. The dietary flavonoid quercetin is thought to promote health, in part due to its anti-inflammatory and antioxidant properties [26]. Consistent with previous studies [27, 28], quercetin, especially with a concentration of 50 μM and a treatment time of 48h, showed similar effects in inhibiting VSMC proliferation, migration and inflammation response. Taken together, quercetin plays an anti-atherosclerosis role by inhibiting the proliferation, migration and inflammatory response of VSMC.

Fibroblast growth factor 2 (FGF-2) is an effective growth factor that stimulates the migration and proliferation of endothelial cells and promotes mitosis of VSMC [29]. FGF2 is involved in the regulation of a variety of cellular functions in a variety of cell types, including cell proliferation, differentiation, viability, adhesion, and migration [30, 31]. According to reports, FGF2 and its receptors have a dual role in the cardiovascular system. In the walls of normal blood vessels, the high expression of FGF2 is conducive to vascular homeostasis, vascular protection and endothelial survival. In atherosclerosis lesions, FGF2 and its receptors contribute to the inflammatory process, intimal thickening and angiogenesis in the plaque [32]. Here, we screened FGF2 as a downstream target of quercetin involved in the progression of AS through biosynthesis analysis. Further, the rescue experiment data revealed that high expression of FGF2 reversed the inhibition of quercetin on proliferation, migration and inflammation of VSMC cells.

# Conclusion

In this study, we demonstrated the anti-atherosclerosis effect of quercetin both *in vivo* and *in vitro.* Mechanically, we confirmed FGF2 was the target of quercetin, and overexpression of FGF2 attenuates the anti-atherosclerosis effect of quercetin. The results provide insight into the potential use of quercetin to expand a therapeutic window in the prevention of coronary atherosclerosis.

# Declarations

### Conflicts of interest

The authors state that there are no conflicts of interest.

### Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Animal Ethics Committee of Changchun University of Chinese Medicine.

### Consent for publication

Not applicable.

### Availability of data and material

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

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### Authors' contributions

Rui Shi and Yue Deng conceived and designed the study. Rui Shi, Zhaozheng Liu and Jinzhu Yin conducted most of the experiments. Rui Shi analyzed the data. Jinzhu Yin performed the literature search and data extraction. Rui Shi drafted the manuscript. Rui Shi and Yue Deng finalized the manuscript. All authors read and approved the final manuscript.

### Acknowledgments

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# Figures

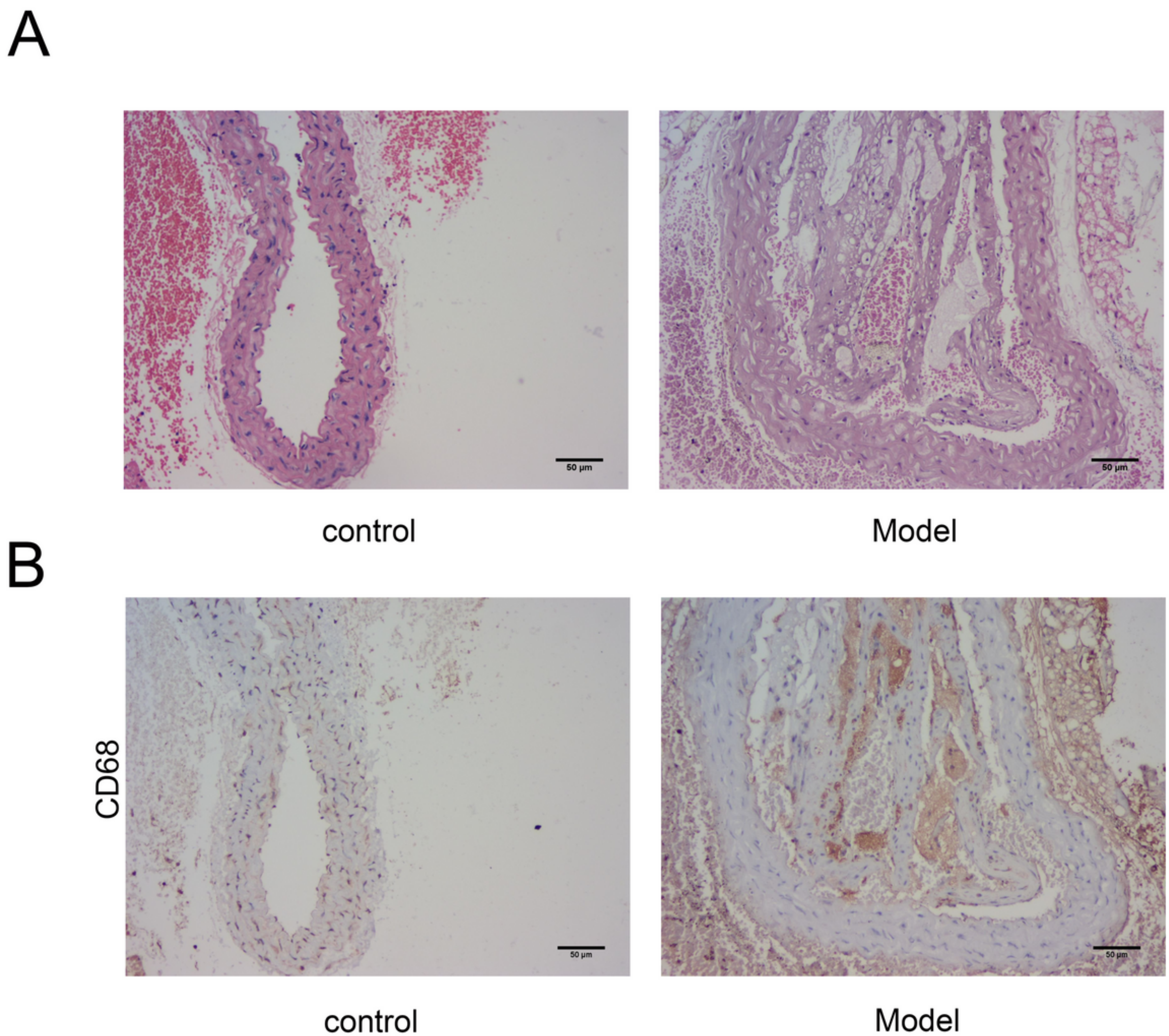



Figure 1

Construction of mice model of coronary atherosclerosis. A) H&E staining was used to observe the necrotic area of the aortic cross section; B) The changes of inflammatory cells in the cross section of the aorta were observed by Immunohistochemistry.

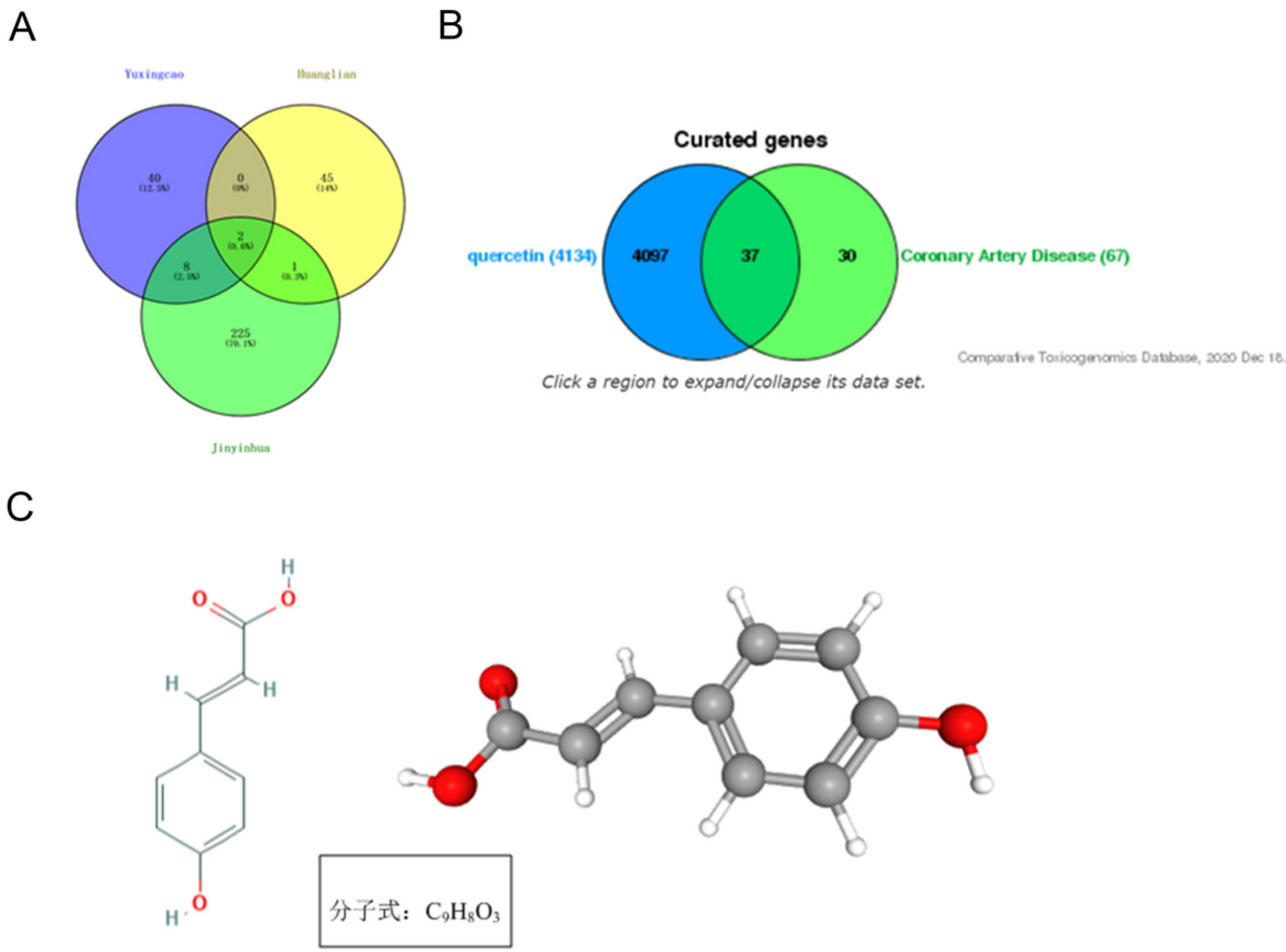
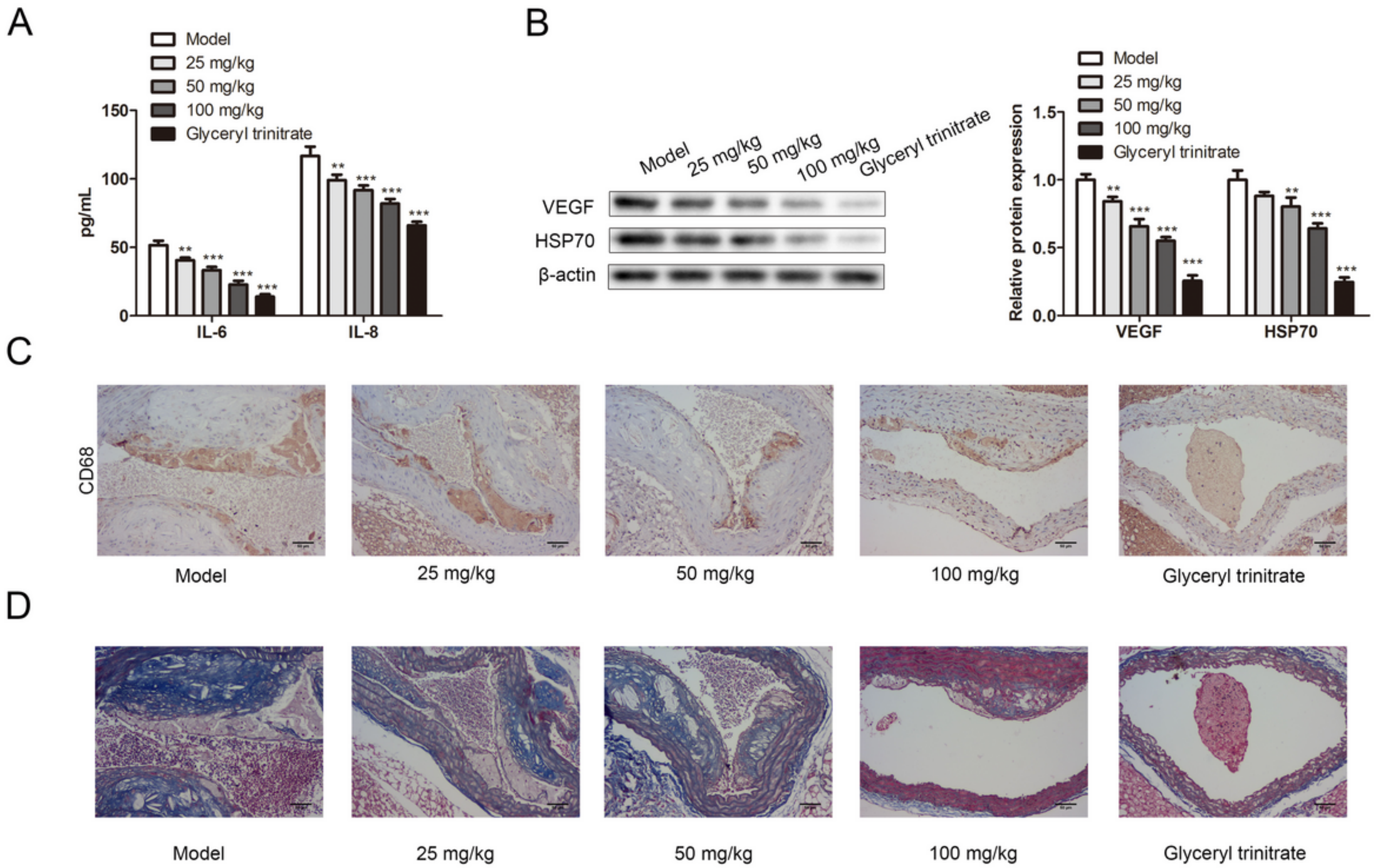


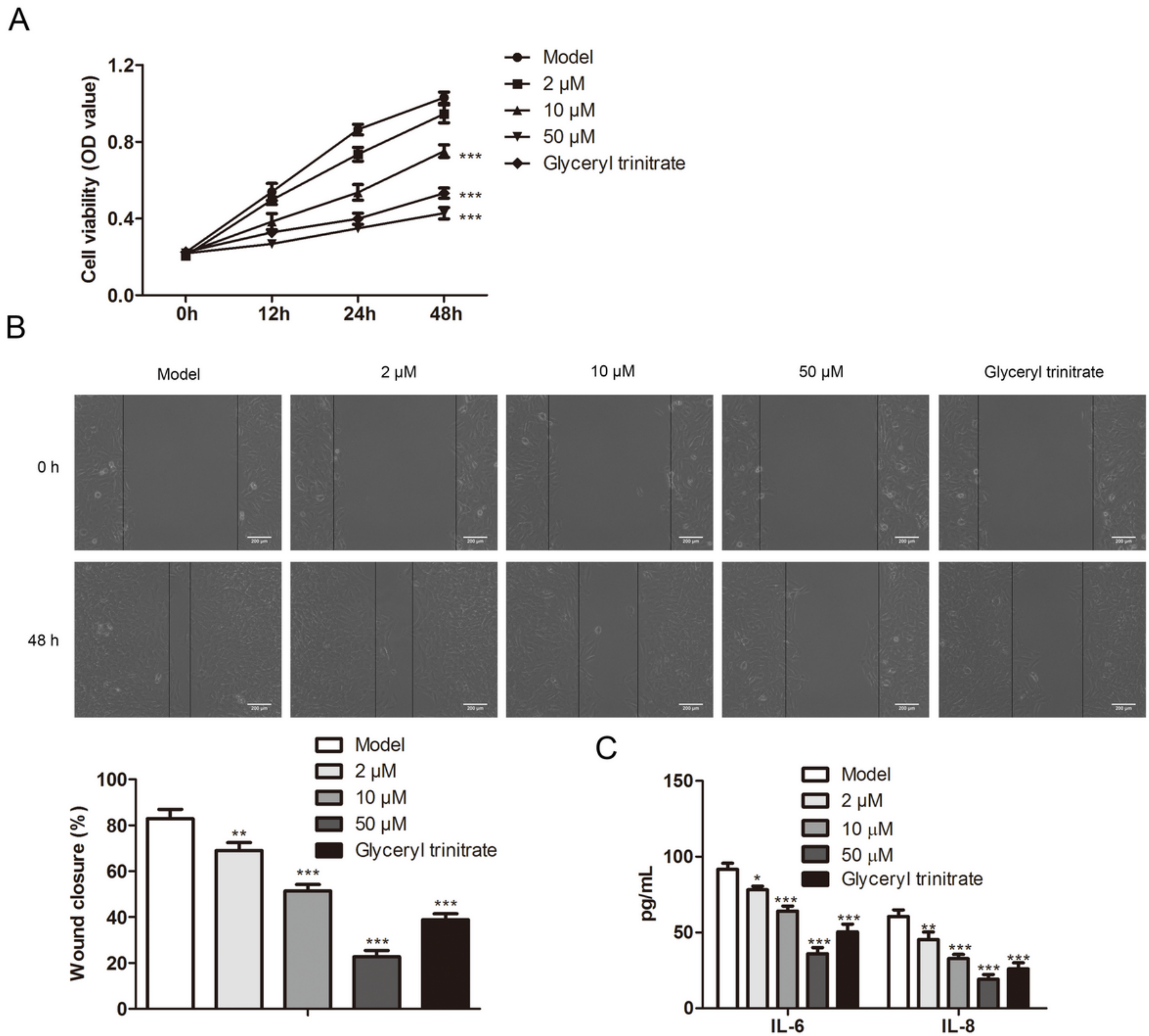

**Figure 2**

Quercetin is a common effective monomer of Huanglian, Yuxingcao and Jinyinhua in the treatment of coronary atherosclerosis. A) The major active components of Huanglian, Yuxingcao and Jinyinhua were predicted by TCMSP; B) The common targets of quercetin and coronary atherosclerosis are predicted by Swiss Target Prediction; C) The chemical structure of quercetin was analyzed via PubChem.



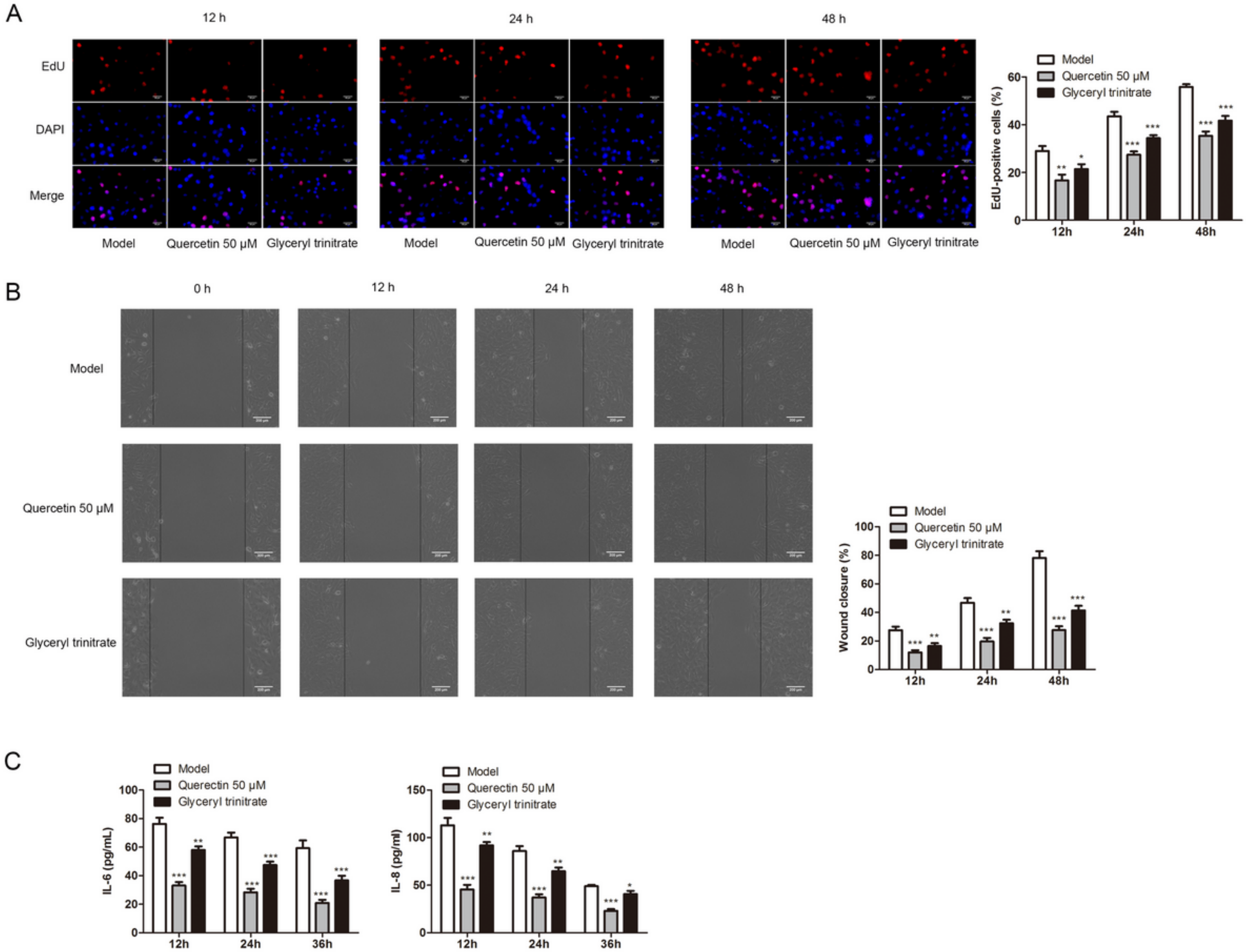

## Figure 3

Quercetin is effective in the treatment of coronary atherosclerosis in vivo. A) The content of IL-6 and IL-8 in serum was quantitatively determined by ELISA; B) Vascular protection parameters (VEGF and HSP70) were quantitatively evaluated via western blot; C) Aortic cross-sections histological analysis with Immunohistochemical; D) Aortic cross-sections histological analysis with aniline blue.



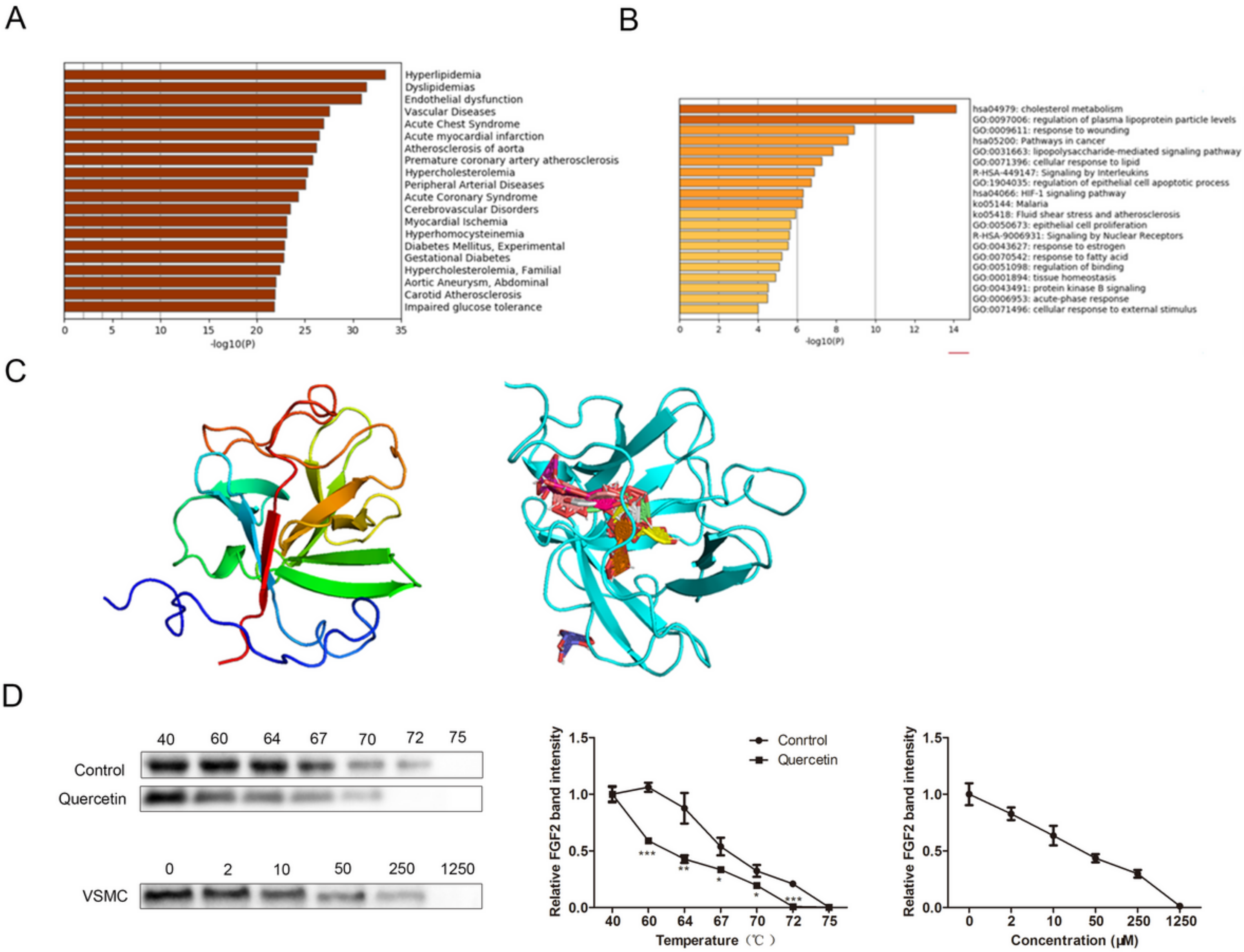

## Figure 4

Quercetin inhibits VSMC proliferation, migration and secretion of inflammatory cytokines in a dose-dependent manner in vitro. A) The proliferation ability of VSMC after treatment with quercetin (2 μM, 10 μM and 50 μM) was detected via CCK-8; B) The relative migration distance represents the migration rate of each group of VSMC cells; C) The content of IL-6 and IL-8 in serum was quantitatively determined by ELISA.



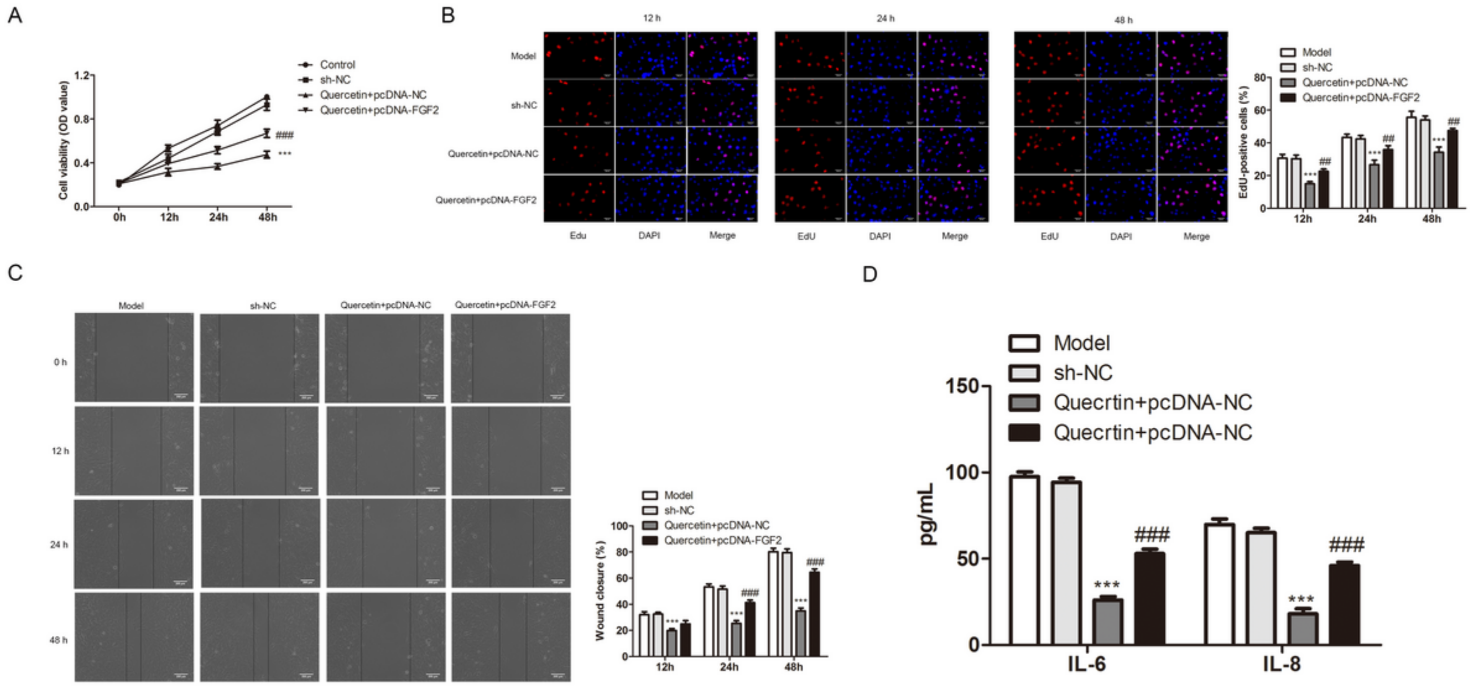

## Figure 5

Quercetin inhibits VSMC proliferation, migration, and secretion of inflammatory cytokines in a time-dependent manner in vitro. A) The proliferation ability of VSMC after treatment with quercetin (12 h, 24 h and 48 h) was detected via EdU; B) The relative migration distance represents the migration rate of each group of VSMC cells; C) The content of IL-6 and IL-8 in serum was quantitatively determined by ELISA.




## Figure 6

FGF2 is the target of quercetin on coronary atherosclerosis. A) The 37 targets were performed enrichment analysis via DisGeNET to further screen argets related to coronary atherosclerosis; B) Metascape was applied to select the target gene related to epithelial cell proliferation and apoptosis; C) 3D model of quercetin was constructed via SWISSMODEL, and molecule combination was calculated via Sand Autodock; D) CETSA analysis of the effects of different temperatures and different doses of quercetin on the expression of FGF2.




## Figure 7

FGF2 overexpression reverses the inhibitory effect of quercetin on coronary atherosclerosis. After co-transfection with quercetin and pc-FGF2, the proliferation, migration, and secretion of inflammatory cytokines ability of VSMC cells was detected by A) CCK-8, B) EdU, C) wound healing and D) ELISA.