

Yixintongmai Inhibits Proliferation, Migration and Promotes Apoptosis of Vascular Smooth Muscle Cells Cultured with High Glucose

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Research

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Abstract

Background: This study was designed to evaluate the effects of yixintongmai on proliferation, migration, and apoptosis of vascular smooth muscle cells (VSMCs) cultured with high glucose.

Methods: VSMCs of the thoracic aorta from 5–8 weeks male Sprague-Dawley rats were cultured with normal (4.5 mM) or high (25 mM) glucose, respectively. The effects of yixintongmai on proliferation, migration, and apoptosis of VSMCs cultured with high glucose were evaluated. The concentration of yixintongmai powder at 360 µg/ml was chosen for this study according to pre-experimental results.

Results: Yixintongmai inhibited the proliferation of VSMCs (CCK-8 assay: 0.75 ± 0.04 versus 0.98 ± 0.09 OD, $P < 0.001$; cell counting: 37533 ± 1861 versus 56009 ± 3779 cells/well, $P < 0.001$) and the expression of PCNA (0.74 ± 0.08 folds, $P < 0.001$) as compared with high glucose. Yixintongmai inhibited the migration of VSMCs (transwell assay: 146 ± 16 versus 265 ± 62 cells; $P < 0.001$; scratch wound assay: 2.69 ± 0.22 folds, $P < 0.001$) and the expression of MMP-9 (0.87 ± 0.03 folds, $P < 0.001$) as compared with high glucose. Yixintongmai promoted the apoptosis of VSMCs (0.36 ± 0.12 folds, $P < 0.001$) and inhibited the expression of bcl-2 (0.83 ± 0.07 folds, $P < 0.01$) as compared with high glucose. Yixintongmai inhibited ROS generation (0.58 ± 0.01 folds, $P < 0.001$) and the expression of NF-κB (0.71 ± 0.07 folds, $P < 0.001$) of VSMCs as compared with high glucose.

Conclusions: Yixintongmai inhibits the proliferation, migration and promotes the apoptosis of VSMCs cultured with high glucose, which suggests the potential anti-atherosclerotic effects of this traditional Chinese medicine.

Background

Cardiovascular complications are the major reason of death for patients with type 2 diabetes mellitus (T2DM). The role of hyperglycemia and hyperinsulinemia in the pathogenesis of diabetic atherosclerosis is still largely unclear (Michaela and Carlo, 2016). Chronic hyperglycemia disturbs the balance of vascular smooth muscle cells (VSMCs) between proliferation and apoptosis, and facilitates migration from the media of vessel into the intima, which leads to neointimal hyperplasia and fibrous cap formation. Hyperglycaemia-induced overproduction of reactive oxygen species (ROS) may be the key molecular mechanisms for diabetes mediated vascular damage (Katakami, 2018). ROS accumulation is critical for nuclear factor-kappa B (NF-κB) activation. NF-κB activation in VSMCs represents a key mechanism for the accelerated vascular disease observed in diabetes and is a pivotal stimulator for VSMCs dedifferentiation, proliferation and migration (Sun, et al., 2016)^[3, 4]. Additionally, the increased activities of MMP-2 and MMP-9 play a role in extracellular matrix degradation thereby accelerating atherogenesis and potentially reducing plaque stability in diabetes (Death, et al., 2003).

Yixintongmai is a traditional Chinese medicine and is used in patients with coronary artery diseases in China. Yixintongmai is consist of astragalus, chuanxiong, salvia miltiorrhiza, northern samphire, radix ginseng, radix salviae, yujin and roasted licorice. Several studies demonstrated that yixintongmai could

inhibit restenosis after coronary angioplasty in patients with coronary artery diseases and T2DM (Li, 1996). Angelica sinensis, astragalus (Chen and Zhang, 1998) and salvia miltiorrhiza (Li, et al., 2010), the specific ingredient of yixintongmai, inhibited the progress of atherosclerosis through affecting the proliferation, migration and apoptosis. Moreover, yixintongmai decreased the level of serum total cholesterol and triglycerides (Li, 1996).

We hypothesized that yixintongmai regulated the proliferation, migration, and apoptosis of VSMCs. Therefore, this study was designed to evaluate the effects of yixintongmai on proliferation, migration, and apoptosis of VSMCs cultured with high glucose. Additionally, we determined the effects of yixintongmai on ROS generation and the expression of NF- κ B of VSMCs cultured with high glucose.

Methods

1. Reagents

Fetal bovine serum (FBS) was purchased from Biological Industries (Beit-Haemek, Israel). Dulbecco's Modified Eagle's Medium (DMEM) and phosphate buffer saline (PBS) were purchased from Hyclone Laboratories Inc (Logan, Utah, USA). Rabbit polyclonal antibody against NF- κ B p65, matrix metalloprotein 9 (MMP-9) and rat polyclonal antibody against proliferation cell nuclear antigen (PCNA) were purchased from Proteintech Group Inc. (Wuhan, China). Secondary antibodies were purchased from CWBio (Beijing, China). Cell counting kit-8 and mitochondrial membrane potential detection kit was purchased from Solarbio Science & Technology (Beijing, China). N-acetyl-L-cysteine (NAC) 2', 7'-dichlorofluorescindiacetate (DCFH-DA) was purchased from Beyotime Biotechnology (Shanghai, China). Collagenase-II was purchased from Biosharp (Nanshan, Guangdong, China). Trans-well plates were purchased from Millipore (Bedford, MA, USA).

2. Animals

Male Sprague-Dawley rats (5-8 weeks) were purchased from HFK Bioscience Company (Beijing, China). All procedures were performed in accordance with the guidelines set by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Henan University of Science and Technology, which is in compliance with the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines on animal research.

3. Cell Culture

VSMCs were prepared from the thoracic aorta of Sprague-Dawley rats. Whole thoracic aorta was isolated from sacrificed rats. The thoracic aorta was cut open longitudinally and the endothelial cells were removed with a sterile Elbow tweezer scraping back and forth twice. The vascular adventitia was carefully stripped with ophthalmic tweezers, and then was rinsed with PBS twice. The aortic tissue was cut into small pieces (1 mm^2). The pieces of aortic tissue were digested with collagenase (125 U) for 2h (shaking once every 30 min) at 37°C in a humidified 5% CO₂ air atmosphere. When a great mass of cells

slipt, five folds compelet medium added into tube and centrifuged at 300 g for 5 min; At last, the cells were plated in T25. The cells were grown to confluence in DMEM with 5.5 mM glucose, 10% FBS, at 37°C in a humidified 5% CO₂. Cells were grown to 80% confluence and cells were used up to 8th (Ren, et al., 2007).

4. Cell Treatment Protocol

VSMCs were divided into four groups. Control group (NG): DMEM medium with 5.5 mM D-glucose; high glucose group (HG): DMEM medium with 25 mM D-glucose; mannitol group (MG): DMEM medium with 5.5 mM D-glucose and 19.5 mM mannitol; yixintongmai group (HG+Y): DMEM medium with 25 mM D-glucose and 360 µg/ml yixintongmai.

5. CCK-8 Assay

VSMCs proliferation was assayed with CCK-8 cell viability kit (Solarbio, Beijing, China). Firstly, VSMCs were randomly seeded at the density of 5×10^3 cells/well and cultured in a 96-well plate. After cells reached 20-30% confluence, VSMCs were starved for 24 h with free serum DMEM medium, and then incubated for 24 h to stimulate cell proliferation in the different conditions as mentioned above. Subsequently, CCK-8 reagent (10 µL) was added and cells were further incubated for 2 h at 37°C. The absorbance wavelength was read at 450nm using spectrophotometric plate reader (Jeong, et al., 2011).

6. Western Blot

Total protein was extracted using an extraction kit (Solarbio, Beijing, China) according to the manufacturer's protocols, and the protein concentration was determined by a BCA protein assay kit (Solarbio, Beijing, China). Protein samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (40 mg per lane, 80 V, 2 h); then transferred to polyvinylidene fluoride membranes (90 V, 120 min). After blocking with 5% non-fat dried milk in Tris-buffered saline with Tween-20 (PBST) for 1 h, the membranes were separately incubated with the appropriate primary antibody, β-actin (1:5000), PCNA (1:3000), NF-κB P65 (1:3000), MMP-9 (1:3000). They were then incubated with horseradish peroxidase-conjugated secondary antibody (Bioss, Beijing, China) and visualized using the ECL plus detection kit (Pierce Protein Research Products, Rockford, IL, USA). Protein expression was quantified by densitometry using Image J (Bio-Rad, Hercules, CA, USA) with β-actin as an internal loading control.

7. Wound Healing Assay

Wound healing assay was used to evaluate the migration of VSMCs. VSMCs (4×10^4 cells/well) were seeded and cultured in a six-well plate. After VSMCs grown to 80% confluence, VSMCs were incubated in serum-deprivation media for 24 h, and then a sterile plastic 1 ml micropipette tip was used to create a 1 mm scratch wound. Cells were continually cultured in the different culture media as mentioned above. The scratched region was photographed immediately and at 48h after scratching, and the migrating distance was thus calculated using image pro plus 6.0 (Shi, et al., 2017).

8. Trans-well Assay

VSMCs migration was analyzed by Trans-well assay. The cells were cultured in the different culture media as mentioned above. After 24 h, cells were trypsinized with 0.25% (v/v) trypsin and re-suspended in the serum-free DMEM. Cell were counted and seeded in the upper chamber of each Trans-well at the concentration of 1×10^5 cells in 0.2 ml serum-free DMEM. 0.8 ml of DMEM supplemented with 20% FBS ([Huang, et al., 2013](#)) was added to the lower chamber of each Trans-well. Chambers were incubated for 12 h at 37°C with 5% CO₂. Cells that migrated to the underside of the Trans-well filter were fixed with 4% formaldehyde (w/v) for 20 min at room temperature and then stained with DAPI (1:10) for 10 min. The staining was examined by fluorescence microscopy at 200× magnification. The numbers of cells were calculated using image pro plus 6.0.

9. Mitochondrial Membrane Potential Assay

VSMCs (4×10^5 cells/well) were seeded in 6-well plates. After VSMCs grown to 30% confluence, VSMCs were incubated in serum-deprivation media for 24h. And then the cells were cultured in the different culture media as mentioned above. Subsequently, mitochondrial membrane potential was analyzed by mitochondrial membrane potential assay kit with JC-1 according to manufacturer's instructions (Solarbio, Beijing, China).

10. Measurements of Intracellular ROS

Intracellular ROS was detected using the oxidant-sensitive probe DCFH-DA. VSMCs were seeded on six-well plates. After starving with serum-free DMEM medium for 24 h, the cells cultured in the different culture media as mentioned above for 48 h. And then cells were washed twice with PBS and were incubated with 5 μmol/L of DCFH-DA for 30 min. The relative DCF fluorescence intensity was detected by fluorescent microscopy (Nikon, Tokyo, Japan). The examination wavelength was 488 nm and the emission wavelength was 530 nm respectively.

11. Statistics analysis

All statistical analysis of the data was performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). The results are presented as the means ± standard deviation (SD) of at least three independent experiments. One-way analysis of variance was used for statistical analysis of the data. In all cases *P* values less than 0.05 were considered significant.

Results

1. Yixintongmai Inhibits the Proliferation of VSMCs and the Expression of PCNA of VSMCs Cultured with High Glucose

As shown in Figure 1 (a, b), yixintongmai inhibited the proliferation of VSMCs by CCK-8 (0.75 ± 0.04 versus 0.98 ± 0.09 OD, *P*<0.001) and cell counting (37533 ± 1861 versus 56009 ± 3779 cells/well, *P*<

0.001) as compared with high glucose, respectively.

As shown in Figure 1 (c, d), yixintongmai significantly inhibited the expression of PCNA of VSMCs as compared with high glucose (0.74 ± 0.08 folds, $P < 0.001$).

2. Yixintongmai Inhibits the Migration of VSMCs and the Expression of MMP-9 of VSMCs Cultured with High Glucose

As shown in Figure 2 (a, b), in transwell assay, yixintongmai significantly inhibited the migration of VSMCs as compared with high glucose (146 ± 16 versus 265 ± 62 cells, $P < 0.001$).

As shown in Figure 2 (c, d), in scratch wound assay, yixintongmai significantly inhibited the migration of VSMCs as compared with high glucose (2.69 ± 0.22 folds, $P < 0.001$).

As shown in Figure 2 (e, f), yixintongmai inhibited the expression of MMP-9 of VSMCs as compared with high glucose (0.87 ± 0.03 folds, $P < 0.001$).

3. Yixintongmai Promotes the Apoptosis of VSMCs and Inhibits the Expression of Bcl-2 Cultured with High Glucose

As shown in Figure 3 (a, b), high glucose inhibited the apoptosis of VSMCs as compared with normal glucose (1.45 ± 0.07 folds, $P < 0.001$), shown as the increased ratio of orange-red fluorescence and green fluorescence indicating the elevation of mitochondrial membrane potentials. Yixintongmai promoted the apoptosis of VSMCs as compared with high glucose (0.36 ± 0.12 folds, $P < 0.001$), shown as the decreased ratio of orange-red fluorescence and green fluorescence indicating the decrease of mitochondrial membrane potentials.

As shown in Figure 3 (c, d), yixintongmai inhibited the expression of bcl-2 of VSMCs as compared with high glucose (0.83 ± 0.07 folds, $P < 0.01$).

4. Yixintongmai Inhibits ROS Generation and the Expression of NF-κB of VSMCs Cultured with High Glucose

Yixintongmai inhibited ROS generation (0.58 ± 0.01 folds, $P < 0.001$) shown in Figure 4 (a, b) and the upregulation of NF-κB expression (0.71 ± 0.07 folds, $P < 0.001$) of VSMCs shown in Figure 4 (c, d) as compared with high glucose.

Discussion

In the present study, we provided the evidence that yixintongmai inhibited high glucose-induced VSMCs proliferation, migration, and promoted apoptosis, suggesting the potential anti-atherosclerotic effects of this Chinese [traditional medicine](#). Kobayashi et al ([Kobayashi Shinjiro, et al., 1993](#)) reported that ligustilide had anti-proliferative effects on VSMCs. Yuan et al ([Yuan, et al., 2008](#)) found that astragaloside IV, extracted from radix astragali, rectified the imbalance of proliferation and apoptosis and regulated

phenotypic modulation of VSMC induced by high glucose. N-butylideneephthalide, the active extraction of angelica sinensis, suppressed of platelet aggregation relaxat of vessels and inhibited of SMC proliferation (Liu, et al., 2011). Furthermore, salvia miltorrhiza inhibited thrombosis, reduced the level of serum lipids, and inhibited the formation of atherosclerotic plaque (Wang, et al., 2014). All these finding indicated that yixintongmai may have therapeutic potential in diabetes associated cardiovascular diseases.

VSMCs proliferation is an early key event in the formation of atherosclerotic plaques. Astragalus-angelica (Hou, et al., 2005), ligisticum, angelica sinensis (Yang, et al., 2003) inhibited VSMCs proliferation by decreasing the percentage of S and G2/M phase cells and increasing the number of cells in G0/G1 phases. Hiromu et al (Hiromu, et al., 2005) reported that PCNA, the pro-proliferation protein, regulated cell cycle progression from G1 phase to S phase. In this study, we found yixintongmai inhibited high glucose-induced VSMCs proliferation and the expression of PCNA, which suggested that yixintongmai inhibited high glucose-induced VSMCs proliferation via inhibiting cell cycle progression.

Upregulation of MMP-9 mediated the migration of VSMCs. ROS/NF κ B/MMP-9 signaling pathway may involve in the migration of VSMCs (Sun, et al., 2016). Hyperglycemia increased intracellular formation of advanced glycosylation end products (AGEs) (Michaela and Carlo, 2016). AGE-the receptor for AGE (RAGE) binding activated the expression of MMP-9 (Bongarzone, et al., 2017). Yan et al (Yan, et al., 2018) reported that astragalus-angelica combination inhibited the expression of MMP-9. In this study, yixintongmai inhibited the migration of VSMCs and the expression of MMP-9 induced by high glucose, suggesting the inhibition of MMP-9 expression may be one of the potential mechanisms of anti-atherosclerotic effects of yixintongmai.

Interestingly, yixintongmai promoted the apoptosis of VSMCs cultured with high glucose and inhibited the expression of Bcl-2 of VSMCs. Bcl-2 protein expressed in VSMCs regulated the antioxidant pathway (Hockenberry, et al., 1993, Leszczynski, et al., 1994, Schaper, 1996) and prevented apoptosis (Folli, et al., 2013). Hyperglycemia inhibited the apoptosis of VSMCs through the upregulation of Bcl-2 family (Bcl-2, Bcl-xL and Bfl-1) (Rudijanto, 2007). High glucose increased the expression of antiapoptotic proteins that may be important in the development of atherosclerosis in diabetic patients (Bennett, 1999, Kockx and Knaapen, 2000). Sara et al (Sara, et al., 2015) found that astragaloside and ligisticum downregulated the expression of Bcl-2. Shahzad et al (Shahzad, et al., 2015) reported that astragaloside IV and tetramethylpyrazine, one of the active ingredients of ligisticum, increased the expression of Bcl-2 in brain injury. Apoptosis was typically associated with loss of membrane polarization, leading to permeability changes (Ryter, et al., 2007). Mitochondria participated in apoptosis through opening of the mitochondrial membrane permeability transition pore and releasing Bcl-2 family. Yixintongmai decreased mitochondrial membrane potential in this study. The loss of mitochondrial membrane permeability represented the occurrence of the membrane permeability transition. The effects of yixintongmai on expression of Bcl-2 and mitochondrial membrane permeability were coincident to these findings, which may mediated the effects of yixintongmai on apoptosis of VSMCs cultured with high glucose.

It is known that oxidative stress are commonly implicated as major factor in the initiation and progression of diabetes-associated cardiovascular diseases (Ryter, et al., 2007). Hyperglycemia could promote ROS accumulation (Brownlee, 2001), which were similar to our data. Hyperglycemia induce ROS overproduction and subsequently activated of the redox sensitive transcription factor NF-κB (Bierhaus, et al., 2001). NF-κB activation is involved in proliferation, migration, inflammation, and oxidative stress of VSMCs (Gao, et al., 2018, Lu, et al., 2018). Therefore, hyperglycemia affected the proliferation, migration, and extracellular matrix synthesis, in addition to apoptosis of VSMCs (Folli, et al., 2013). Juan et al (Juan, et al., 2007) reported that chuangxiong effectively decreased the ROS formation, eliminated ROS to prevent lipid peroxidation, protected the mitochondrial function, and maintained mitochondrial membrane potential. As a potent ROS scavenger, Salvia miltiorrhiza Bunge inhibited high glucose-induced oxidative stress, reduced the generation of ROS and mitochondrial depolarization (Sun, et al., 2018). Astragalus polysaccharide had antioxidant, antiviral activities and promoted pro-apoptosis (Zhang, et al., 2018). These may partially explain the mechanisms of anti-atherosclerotic effects of yixintongmai. Inhibition of ROS/NF-κB signaling pathway may be one of anti-atherosclerotic mechanisms of yixintongmai.

Conclusions

Yixintongmai inhibits the proliferation, migration and promotes the apoptosis of VSMCs cultured with high glucose, suggesting the potential anti-atherosclerotic effects of this traditional Chinese medicine. These effects may be mediated by inhibition of ROS and NF-κB signal pathways.

Abbreviations

T2DM: type 2 diabetes mellitus

ROS: reactive oxygen species

NF-κB: nuclear factor-kappa B

VSMCs: vascular smooth muscle cells

FBS: fetal bovine serum

DMEM: Dulbecco's Modified Eagle's Medium

PBS: phosphate buffer saline

MMP-9: matrix metalloprotein 9

PCNA: proliferation cell nuclear antigen

NAC: N-acetyl-L-cysteine

DCFH-DA: 2', 7'-dichlorofluorescindiacetate

NG: control group

HG: high glucose group

MG: mannitol group

HG+Y: yixintongmai group

SD: standard deviation

Declarations

Ethics approval and consent to participate

All procedures were performed in accordance with the guidelines set by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Henan University of Science and Technology.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DJJ performed all the experiments and analyzed the data. DZ and DPS designed the study. DJJ and DZ was major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Figures

Fig. 1

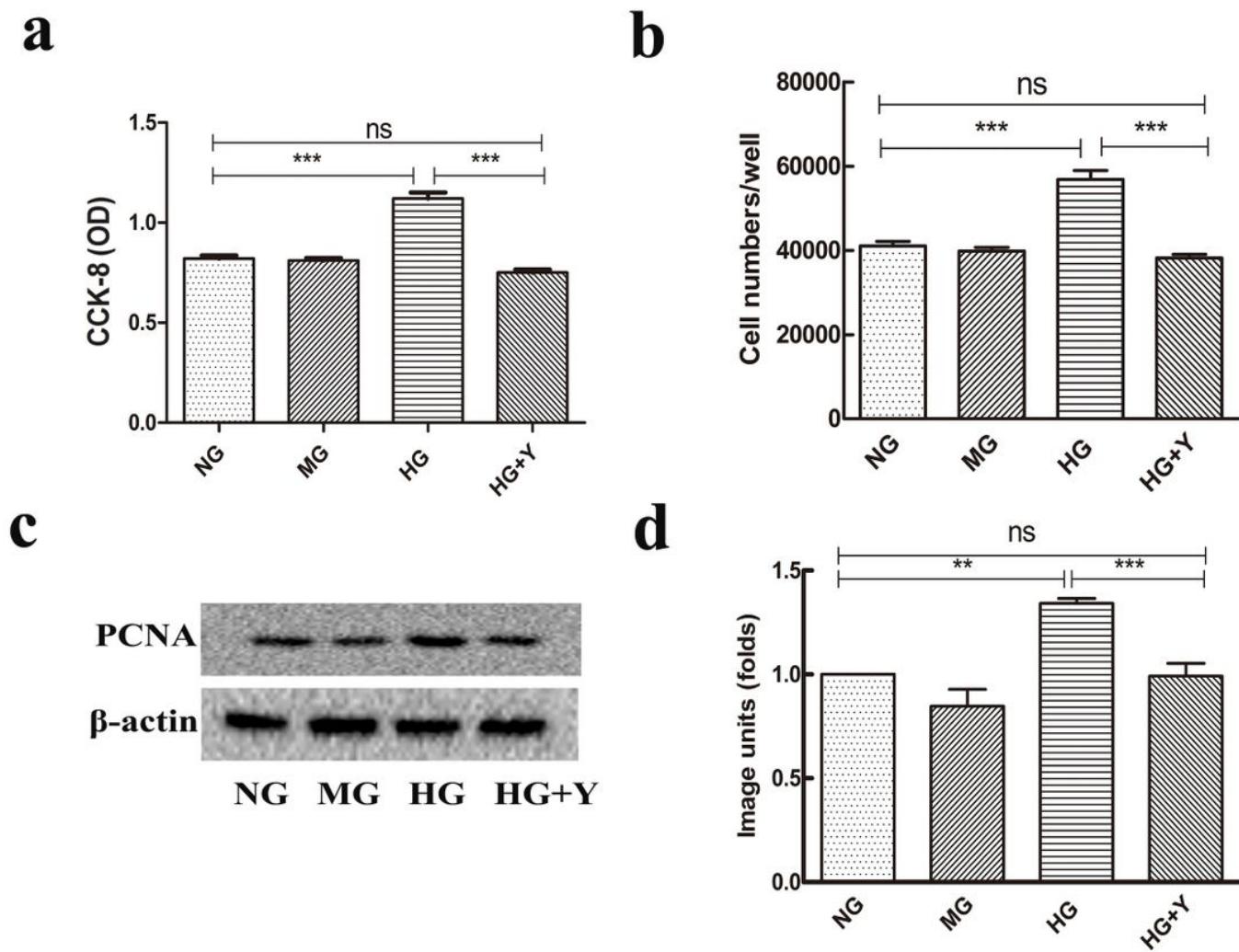


Figure 1

Effects of Yixintongmai on Proliferation and PCNA Expression of VSMCs Cultured with High Glucose. NG: normal glucose (5.5 mM); MG: normal glucose (5.5 mM) and mannitol (19.5 mM); HG: high glucose (25 mM); HG+Y: high glucose (25 mM) and yixintongmai powder (360 µg/ml); VSMCs proliferation were evaluated by CCK-8 (a) and cell counting (b). Western blot of PCNA expression (c, d); Densitometry quantification values were shown by fold over NG (control) for each time point. Values were expressed as means ± SD of sextuplicate experiments. **p<0.01; ***p<0.001; ns: not significant (p>0.05).

Fig. 2

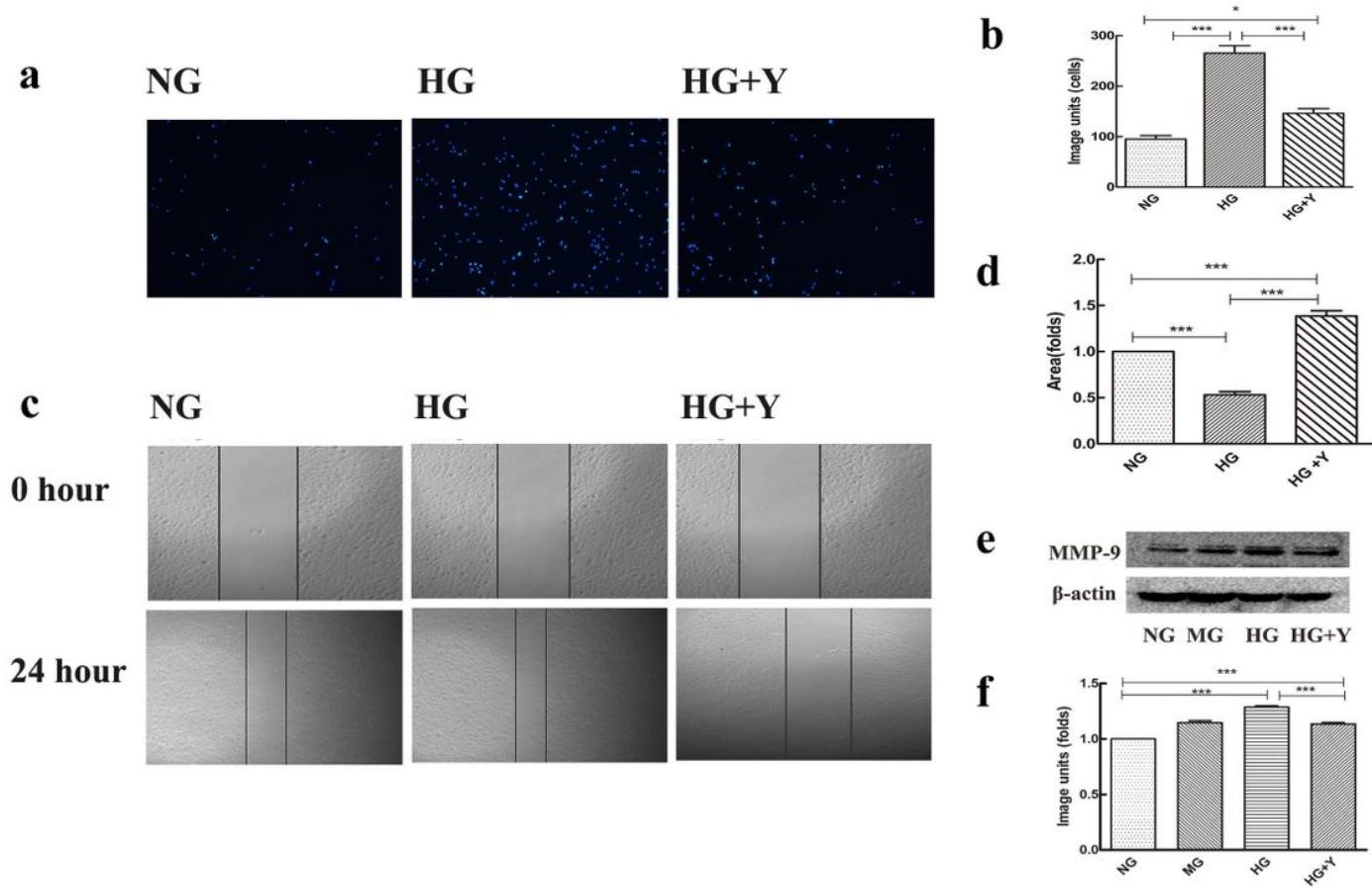


Figure 2

Effects of Yixintongmai on Migration and MMP-9 Expression of VSMCs Cultured with High Glucose. NG: normal glucose (5.5 mM); HG: high glucose (25 mM); HG+Y: high glucose (25 mM) and yixintongmai powder (360 µg/ml); MG: normal glucose (5.5 mM) and mannitol (19.5 mM); The migration of VSMCs was assessed using transwell assay (a, b) and scratch wound assay (c, d). In the transwell assay, the staining was examined by fluorescence microscopy at 100× magnification in three random fields. a: Fluorescent image; b: The mean numbers of VSMCs in three random fields; In wound healing assay, VSMCs migration was shown at 0 h, and 24 h (c). Migration rate (d); Western blot of MMP-9 (e, f); Densitometry quantification values were shown by fold over NG (control) for each time point. Values are expressed as means ± SD of triplicate experiments. *p<0.05; ***p<0.001.

Fig. 3

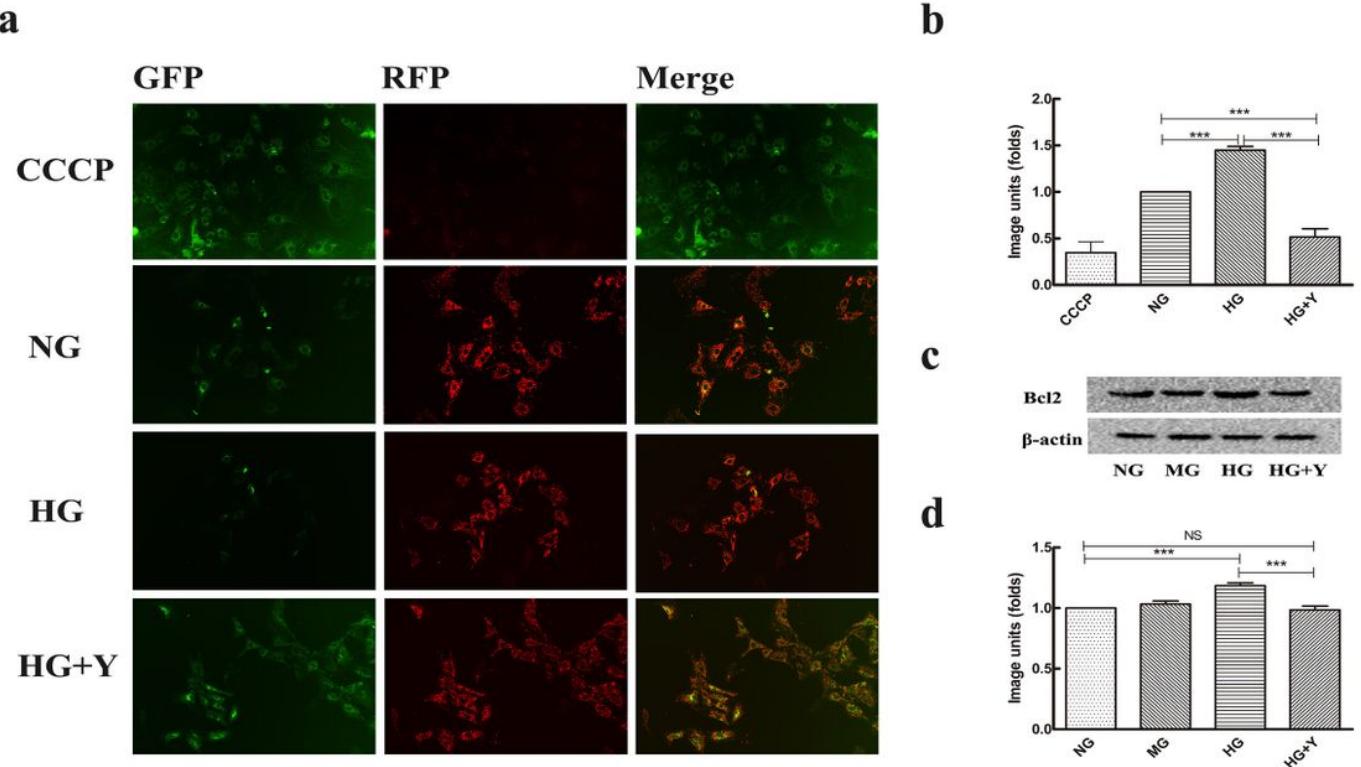


Figure 3

Yixintongmai Promotes VSMC Apoptosis and Inhibits the Expression of Bcl-2 of VSMCs Cultured with High Glucose. NG: normal glucose (5.5 mM); HG: high glucose (25 mM); HG+Y: high glucose (25 mM) and yixintongmai powder (360 µg/ml); MG: normal glucose (5.5 mM) and mannitol (19.5 mM); Normal VSMCs stained with JC-1 emitted orange-red fluorescence with a little green fluorescence, reflecting the condition of mitochondrial membrane potentials. The increased ratio of orange-red fluorescence and green fluorescence indicates the elevation of mitochondrial membrane potentials, due to the increase of aggregated JC-1 within mitochondria. Fluorescence intensity in VSMCs was quantified using Image-Pro Plus/IOD. a: The fluorescence intensity of VSMCs at different conditions; b: Densitometry quantification values shown by fold over NG (control) for each time point at different conditions; c and d: Western blot of Bcl2; ***p<0.001; ns: not significant (p>0.05); CCCP: Carbonyl Cyano-to-Chlorobenzene hydrazone (positive control).

Fig. 4

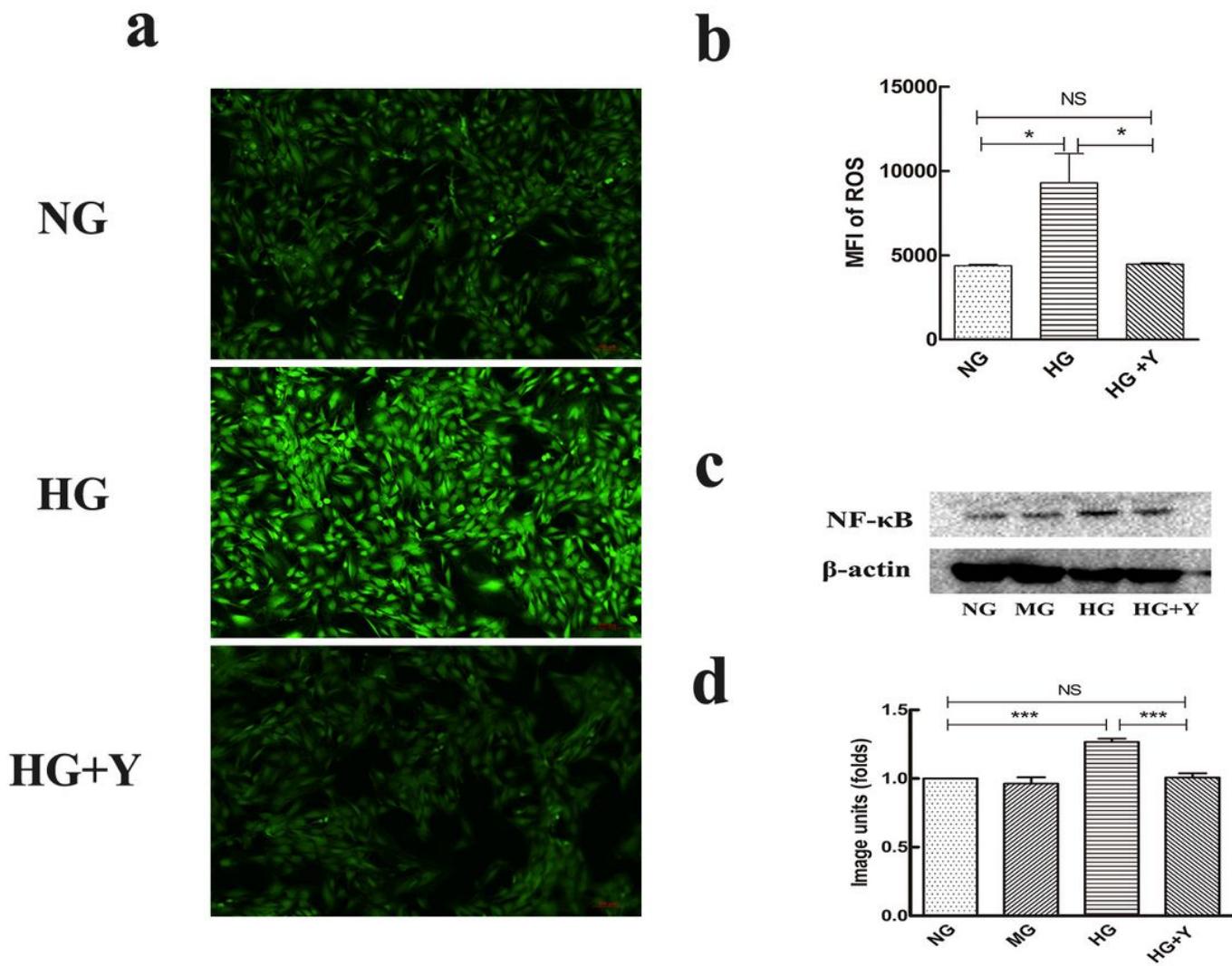


Figure 4

Yixin Tongmai Inhibits ROS Generation and Expression of NF- κ B P65 of VSMCs Cultured with High Glucose. NG: normal glucose (5.5 mM); HG: high glucose (25 mM); HG+Y: high glucose (25 mM) and yixin tongmai powder (360 μ g/ml); MG: normal glucose (5.5 mM) and mannitol (19.5 mM); a: The fluorescence intensity of VSMCs at different conditions; b: Densitometry quantification values of VSMCs at different conditions; C and d: Western blot of NF- κ B P65; Densitometry quantification values were shown by fold over NG (control) for each time point. *p<0.05; ***p<0.001; ns: not significant (p>0.05); MFI: Median Fluorescence Intensity.