

# The mitochondrial calcium uniporter induces apoptosis in cardiomyocytes cultured with high-glucose medium by affecting mitochondrial function

**Xia Chen**

The 940th Hospital of the Joint Logistic Support Force of the Chinese people's Liberation Army

<https://orcid.org/0000-0003-0669-1255>

**Wenyun Guo**

Attending physician in cardiology

**Zhe Jing**

Attending physician in cardiology

**Tao Zhang**

Master's degree

**Zhaoqi Wu**

Master's degree

**Hengqiang Cui**

Master's degree

**Wenxue Zheng**

postgraduate

**Yongqing Chen** (✉ [350214351@qq.com](mailto:350214351@qq.com))

**Hui Chen**

Chief physician of endocrinology , PhD, doctoral supervisor

---

## Study protocol

**Keywords:** mitochondrial calcium uniporter (MCU), mitochondrial dysfunction, oxidative stress, apoptosis, diabetic cardiomyopathy

**Posted Date:** May 29th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-27647/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

# Abstract

## Background

As the number of diabetics worldwide continues to increase, diabetic cardiomyopathy has become one of the main causes of cardiovascular disease risk in diabetic patients. Currently, the pathophysiological mechanism of DCM has not been fully elucidated. In the present study, relevant pathological changes of cardiomyocytes in the high glucose environment were simulated by in vitro culture of rat H9C2 cardiomyocytes, to explore the mechanism by which MCU induces apoptosis in cardiomyocytes.

## Method:

Cultured rat myocardium H9C2 cells in vitro and divided into high glucose group (glucose concentration 33 mmol/L), normal group (glucose concentration 5.5 mmol/L), experimental group (5.5 mmol/L glucose and transfected with MCU siRNA) and control group (5.5 mmol/L glucose and transfected negative control siRNA). Comparative analysis of MCU expression,  $\text{Ca}^{2+}$  uptake, mitochondrial function, oxidative stress and apoptosis of two groups of cells.

## Results

(1) Compared with normal group, in the high glucose group the MCU expression of myocardial cells in H9C2 rats decreased, The  $\text{Ca}^{2+}$  levels, membrane potential and mitochondrial ATP levels decreased, mitochondrial ROS levels increased,  $\text{NADH}^+/\text{NADPH}$  ratio in cardiomyocytes increased, GSH/GSSG ratio decreased, the expression levels of cleaved caspase-3 and cleaved caspase-9 increased, bcl-2 expression decreased, the number of cardiomyocytes apoptotic cells increases. (2) Compared with the normal group and the control group, the experimental group MCU expression of myocardial cells in H9C2 rats decreased, The  $\text{Ca}^{2+}$  levels, membrane potential and mitochondrial ATP levels decreased, mitochondrial ROS levels increased,  $\text{NADH}^+/\text{NADPH}$  ratio in cardiomyocytes increased, GSH/GSSG ratio decreased, the expression levels of cleaved caspase-3 and cleaved caspase-9 increased, bcl-2 expression decreased, the number of cardiomyocytes apoptotic cells increases.

## Discussion

This study suggested that MCU expression in rat H9C2 cardiomyocytes was decreased in the high glucose environment, causing abnormal mitochondrial calcium uptake and imbalanced calcium homeostasis, which may further contribute to mitochondrial dysfunction and enhanced oxidative stress in cardiomyocytes. Mitochondrial dysfunction and enhanced oxidative stress ultimately led to apoptosis in cardiomyocytes.

# 1. Background

Over the past two decades, diabetes mellitus (DM) prevalence has been increasing worldwide. Meanwhile, the risk of severe cardiovascular disease (CVD) is 2–4 fold higher in DM patients compared with individuals suffering from hypertension, coronary artery disease and heart failure (HF), and the overall mortality rate is 3 fold higher than that of cases without DM <sup>[1]</sup>. Diabetic cardiomyopathy (DCM) has become an increasingly serious public health issue <sup>[2]</sup>. Various clinical trials have shown that HF prevalence in DM patients is as high as 19–26% <sup>[3, 4]</sup>. It has been reported in the Framingham Heart Study that 19% of HF patients have type 2 diabetes mellitus (T2DM), which increases the risk of HF by 2–8 fold <sup>[5, 6]</sup>. It was also shown that DM may be an independent risk factor for HF <sup>[7]</sup>. Mitochondrial dysfunction constitutes an important pathological mechanism of DCM development. Recent studies have demonstrated that unbalanced calcium homeostasis caused by abnormal mitochondrial calcium transport is an important cause of mitochondrial dysfunction <sup>[8, 9]</sup>. Meanwhile, mitochondrial calcium homeostasis is crucial in regulating cytosolic calcium homeostasis, mitochondrial energy metabolism, ATP production, intracellular calcium signaling, oxidative stress, autophagy and apoptosis <sup>[8]</sup>. The mitochondrial calcium uniporter (MCU) is an important component of the mitochondrial calcium uniporter complex <sup>[10]</sup>, which is the main channel by which calcium ions enter the mitochondrial matrix from the intermembrane space and plays a key role in regulating mitochondrial calcium homeostasis <sup>[11, 12]</sup>. This study aimed to elucidate the mechanism by which MCU affects mitochondrial function in DCM, providing new strategies and targets for the clinical diagnosis and treatment of DCM.

## 2. Methods

### 2.1 Materials

H9C2 rat cardiomyocytes were provided by the Cell Bank of Chinese Academy of Sciences. DMEM, fetal bovine serum, streptomycin was purchased from Hyclone (USA) and Teizol from Thermo Fisher Scientific (USA). The mitochondrial membrane potential assay kit (JC-1), ATP assay kit, NADP<sup>+</sup>/NADPH detection kit (WST-8 method), GSH and GSSG detection kit and RIPA lysis buffer were manufactured by Beyotime Biotechnology Co., Ltd. (China). Skim milk powder for blocking was purchased from Solarbio Science & Technology Co., Ltd. (China). The calcium ion assay kit and MCU antibodies were provided by Abcam (USA). Goat anti-mouse and anti-rabbit polyclonal antibodies were purchased from Boster Biological Technology Co., Ltd. (China). PVDF membranes were from Millipore (USA). MCU primers were provided by TaKaRa (Japan). Reverse transcription kit and RT-PCR assay kit purchased from TIANGEN Biotech Co., Ltd. (China). TUNEL apoptosis detection kit was purchased from Riobio Technology Co., Ltd. (China). Annexin V-FITC / PI cell apoptosis detection kit was purchased from BD Biosciences Co., Ltd. (USA). siRNA purchased from GenePharma.

### 2.2 Methods

#### 2.2.1 Cell culture

H9C2 cardiomyocytes were cultured in DMEM (Hyclone) complete medium (20% fetal bovine serum, 1% streptomycin, and 100 U/L penicillin) for about 5 days (80% confluency), and used for experiments after 2–3 passages. The cultured H9C2 cardiomyocytes were assigned to the experimental and control groups. The experimental group was cultured with high glucose (33 mmol/L), and control cells were cultured with glucose (25 mmol/L). Experiments were conducted after culturing for 24 h.

## 2.2.2 Experimental grouping

High glucose group (HG): glucose concentration is 33 mmol/L.

Normal group (NG): Glucose concentration is 5.5 mmol/L.

Experimental group (NG + MCU siRNA): 5.5 mmol/L glucose and transfected with MCU siRNA.

Control group (NG + NC siRNA): 5.5 mmol/L glucose and transfected with negative control siRNA.

## 2.2.3 Research methods

Real-time quantitative polymerase chain reaction and FastKing One Step RT-qPCR Kit (TIANGEN) was used to detect MCU mRNA expression, and Western blot was used to detect MCU protein expression. JC-1 Mitochondrial membrane potential detection kit (Beyotime) detects mitochondrial membrane potential, ATP detection kit (Beyotime) detects cardiomyocyte mitochondrial ATP activity, mitochondrial calcium dye Rhod-2 (Abcam) was used to detect myocardial cell mitochondrial calcium uptake capacity, and Mito-SOX reagent (Thermo Fisher Scientific) was used to detect Changes of reactive oxygen species (ROS) in cardiac mitochondria. NADP<sup>+</sup>/NADPH detection kit (Beyotime) and GSH/GSSG detection kit (Beyotime) were used to detect myocardial cells NADP<sup>+</sup>/NADPH, GSH/GSSG. Western blot was used to detect the expression of caspase-3 (Abcam), cleared caspase-9 (Abcam) and Bcl-2 (Abcam). Flow cytometry combined with Annexin V-FITC/PI staining (BD) and TUNEL apoptosis detection kit (RIBO) was used to detect cardiomyocyte apoptosis.

## 2.3 Statistical treatments

Statistical analyses were performed with the SPSS 22.0 software. Measurement data conforming to normal distribution were presented as Mean  $\pm$  SD and compared by the t test. Count data were presented as absolute number or percentage.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 Effects of high glucose on MCU expression in H9C2 cardiomyocytes

MCU mRNA expression was detected by real-time polymerase chain reaction. The results showed that MCU mRNA expression in the high glucose group was lower than that of the normal group (Fig. 1a). The

protein expression of MCU was detected by Western blot. The results also showed that MCU protein amounts in the high glucose group were lower than normal group values (Fig. 1b).

### **3.2 Down-regulation of MCU in a high glucose environment causes a decrease in mitochondrial Ca<sup>2+</sup> uptake in H9C2 cardiomyocytes**

Rhod-2 was used to detect mitochondrial Ca<sup>2+</sup> in cardiomyocytes, and fluorescence intensity is directly proportional to Ca<sup>2+</sup> levels. The results showed that Ca<sup>2+</sup> levels in the high glucose group were lower than normal group (Fig. 2).

In order to clarify the relationship between Ca<sup>2+</sup> reduction and MCU downregulation in high glucose environment, we downregulated MCU expression in H9C2 cells and used Rhod-2 again to detect mitochondrial Ca<sup>2+</sup> in myocardial cells. The results showed that the mitochondrial Ca<sup>2+</sup> uptake in the experimental group was reduced compared with the normal group and the control group, but there was no difference in the mitochondrial Ca<sup>2+</sup> between the control group and the normal group (Fig. 3).

## **3.3 Mitochondrial dysfunction of H9C2 cardiomyocytes caused by down-regulation of MCU in high glucose environment**

Mitochondrial membrane potential (MMP) is one of the important parameters that reflect the functional status of mitochondria in cells. When mitochondrial function is abnormal, mitochondrial membrane potential decreases. JC-1 was used to detect mitochondrial membrane potential in H9C2 cardiomyocytes. A high mitochondrial membrane potential resulted in JC-1 aggregation in the mitochondrial matrix to form a polymer, which could produce red fluorescence; in case of mitochondrial membrane potential, JC-1 could not aggregate in the mitochondrial matrix, and, JC-1 as a monomer could produce green fluorescence. Usually, the relative proportion of red to green fluorescence is used to measure mitochondrial depolarization. In the high glucose group, red fluorescence signals were weak, while green fluorescence signals were strong, suggesting low membrane potential; in the normal group, red fluorescence signals were strong, and green fluorescence signals were weak, suggesting high membrane potential. There were significant differences between the two groups (Fig. 4a).

Mitochondria are the main sites for ATP production, and generally a decrease in ATP levels indicates impaired or decreased mitochondrial function. An ATP assay kit was used to detect mitochondrial ATP activity in cardiomyocytes. The results showed that mitochondrial ATP levels in H9C2 cardiomyocytes of the high glucose group were lower than normal group (Fig. 4b).

In order to clarify whether the decrease of mitochondrial membrane potential and the decrease of ATP level in high glucose environment were caused by the down-regulation of MCU, we down-regulated the expression of MCU in H9C2 cells and used JC-1 again to detect the mitochondrial membrane potential of myocardial cells. The potential level is lower than that in the normal group and the control group, and

there is no difference in the mitochondrial membrane potential level between the control group and the normal group, (Fig. 5a). Using the ATP detection kit to detect ATP activity of mitochondria in myocardial cells, the results showed that the ATP concentration in the experimental group was lower than that in the control group and the normal group, and there was no difference between the control group and the normal group (Fig. 5b).

### **3.4 Effects of MCU down-regulation in high glucose environment on oxidative stress of H9C2 cardiomyocytes**

Mito SOX™-Red reagent penetrates into living cells and selectively targets the mitochondria. It could be rapidly oxidized by superoxide ROS, and after binding of the oxidation product to nucleic acids, red fluorescence is produced. A strong fluorescence indicates large amounts of ROS, and a weak fluorescence indicates small amounts of ROS. The results showed that the high glucose group had stronger red fluorescence signals (elevated ROS amounts) compared with the normal group (Fig. 6a).

NADPH and GSH are both important components of the oxidized respiratory chain. NADPH is also used to maintain the reduced state of GSH. When the cell is under oxidative stress, both NADPH and GSH decrease, so the NADP<sup>+</sup>/NADPH ratio increases and the GSH/GSSG ratio decreases. The NADP<sup>+</sup>/NADPH assay kit was used to detect NADP<sup>+</sup>/NADPH in H9C2 cardiomyocytes. The results suggested that the NADP<sup>+</sup>/NADPH ratio in the high glucose group was higher than that of the control group (Fig. 6b). GSH and GSSG assay kits were used to detect GSH and GSSG (glutathione and oxidized glutathione), respectively, in H9C2 cardiomyocytes; the results demonstrated that the GSH/GSSG ratio in the high glucose group was lower than that of the control group (Fig. 6b).

To clarify the relationship between H9C2 cardiomyocyte oxidative stress enhancement and MCU, we down-regulated the expression of MCU in H9C2 cells and used Mito SOX™-Red reagent to detect the amount of ROS. The results showed that the experimental group had more ROS than the control group and the normal group, and there was no difference between the control group and the normal group (Fig. 7a). Using NADP<sup>+</sup>/NADPH detection kit to detect H9C2 cardiomyocytes NADP<sup>+</sup>/NADPH, the results showed that the experimental group NADP<sup>+</sup>/NADPH was higher than the control group and the normal group, the control group and the normal group were no different (Fig. 7b). The GSH and GSSG detection kits detect H9C2 cardiomyocytes GSH/GSSG, the results show that the experimental group GSH/GSSG is less than the control group and the normal group, there is no difference between the control group and the normal group (Fig. 7b).

### **3.5 Effect of MCU down-regulation on apoptosis of H9C2 cardiomyocytes in high glucose environment**

Western blot to detect the expression of apoptosis-related proteins caspase-3, caspase-9 and apoptosis antagonist protein Bcl-2. The results revealed that these apoptosis-related proteins were altered in the

high glucose group compared with the normal group (Fig. 8a).

Use the TUNEL apoptosis detection kit to detect the apoptosis of two groups of cells. Apoptotic cells will be incorporated into TAM-dUTP at the 3'-OH terminals of DNA by the catalysis of TdT enzyme, producing red fluorescence (DAPI dye will stain all nuclei). The results showed that the cells in the high glucose group had more red fluorescence than the normal group, and the cells in the high glucose group had more apoptosis than the normal group (Fig. 8b).

Flow cytometry combined with Annexin-V-FITC/PI staining was used to detect the apoptosis of H9C2 cardiomyocytes in the two groups. Annexin-V-FITC can combine with early apoptotic cells to emit fluorescence, and PI can combine with late apoptotic cells to emit fluorescence. In the figure, the LL quadrant represents living cells, the UL quadrant represents cell debris, the LR quadrant represents early apoptotic cells, and the UR quadrant represents late apoptotic cells. Adding the data of the LR and UR quadrants gives the number of apoptotic cells. The results still showed that the apoptosis of cardiomyocytes in high glucose culture was more than that in normal glucose group (Fig. 8c).

In order to clarify the relationship between increased apoptosis of H9C2 cardiomyocytes and MCU, we downregulated the expression of MCU in H9C2 cells, and used Western Blot again to detect the expression of caspase-3, caspase-9 and Bcl-2. The results showed that the expression of caspase-3 and caspase-9 in the experimental group was higher than that in the normal group and the control group, the expression of Bcl-2 was lower than that in the normal group and the control group. There was no statistical difference in protein expression between the control group and the normal group (Fig. 9a).

Using TUNEL apoptosis kit and flow cytometry combined with Annexin-V-FITC/PI staining to detect H9C2 cardiomyocyte apoptosis. The results showed that the apoptosis of myocardial cells in the experimental group was more than that in the normal group and the control group. There was no statistical difference between the control group and the normal group (Fig. 9b and Fig. 9c).

## 4. Discussion

DCM is a disorder of the cardiac muscle among DM patients in the absence of hypertension and structural heart diseases, such as valvular heart disease and CAD [13]. Its main clinical feature is abnormal systolic and diastolic function of the heart [14]. The high incidence of heart failure in DM patients has an important connection with DCM, and DCM has become one of the main causes of death in DM patients [15]. Currently, the pathophysiological mechanism of DCM has not been fully elucidated, and the existing treatment methods are limited, and the patient has a poor prognosis. In the multifactorial pathogenesis of DCM, the reduction of mitochondrial  $\text{Ca}^{2+}$  uptake, Mitochondrial calcium homeostasis leads to abnormal mitochondrial electron transport chain and mitochondrial membrane potential and Oxidative stress enhanced caused by imbalanced mitochondrial calcium homeostasis in the high glucose environment is considered one of the important pathological mechanisms of DCM [16, 17]. MCU is the main channel for mitochondrial  $\text{Ca}^{2+}$  uptake, playing an essential role in the regulation of mitochondrial

calcium homeostasis [18]. In the present study, relevant pathological changes of cardiomyocytes in the high glucose environment were simulated by in vitro culture of rat H9C2 cardiomyocytes, to explore the mechanism by which MCU induces apoptosis in cardiomyocytes.

MCU is the main channel for mitochondrial  $\text{Ca}^{2+}$  uptake. As a mitochondrial calcium uniporter, its downregulation directly leads to abnormal mitochondrial calcium uptake, resulting in imbalanced mitochondrial calcium homeostasis [19, 20]. We tested the expression level of MCU and mitochondrial  $\text{Ca}^{2+}$  in rat H9C2 cardiomyocytes cultured in simulated high glucose environment and normal glucose culture. The results showed that MCU mRNA expression and mitochondrial calcium uptake in the high glucose group was lower than that of the normal control group. However, is the decrease in mitochondrial calcium uptake in cardiomyocytes caused by a decrease in MCU expression? We culture H9C2 cardiomyocytes in a normal environment in vitro to down-regulate the expression of MCU, the results showed that the decreased MCU expression in H9C2 cardiomyocytes caused a decrease in mitochondrial calcium uptake. Therefore, it can be seen that the loss of MCU expression in cardiomyocytes in a high glucose environment significantly affects the mitochondrial calcium uptake of cardiomyocytes, which may be one of the important causes of myocardial damage caused by the imbalance of mitochondrial calcium homeostasis in DM patients.

As the second messenger factor,  $\text{Ca}^{2+}$  is an important signal in the transmission mechanism of mitochondrial energy activity which regulates multiple mitochondrial functions from metabolism to apoptosis [21]. In the complexes I, II, III and IV in the oxidative respiratory chain in mitochondria,  $\text{Ca}^{2+}$  can enhance the activity of oxidative phosphorylation, thereby increasing the production of ATP [22]. And when  $\text{Ca}^{2+}$  enters the mitochondrial matrix, it can activate three important  $\text{Ca}^{2+}$ -dependent dehydrogenases in the rate-limiting enzymes in the tricarboxylic acid cycle, i.e. pyruvate dehydrogenase (PDH),  $\alpha$ -ketoglutarate dehydrogenase ( $\alpha$ KGDH) and isocitrate dehydrogenase (IDH), furthermore, it strengthens the mitochondrial tricarboxylic acid cycle reaction and ATP production [23, 24]. At the same time, MCU is the most highly selective channel for mitochondrial  $\text{Ca}^{2+}$  uptake, and its transport to  $\text{Ca}^{2+}$  depends on the electrochemical gradient of mitochondrial membrane potential [25]. Previous studies have shown that after the application of MCU inhibitors, mitochondrial calcium homeostasis has been disturbed and further led to mitochondrial oxidative respiratory dysfunction and membrane potential loss [26]. Mitochondrial  $\text{Ca}^{2+}$  uptake depends on the mitochondrial membrane potential, and the myocardial cell mitochondrial membrane potential decreases in a high-glucose environment, thus forming a vicious cycle, which ultimately leads to cell apoptosis [27]. The present experimental results showed that the calcium homeostasis high glucose group H9C2 cardiomyocytes had reduced ATP production, decreased membrane potential, and increased apoptosis. We also cultured H9C2 cardiomyocytes in a normal environment in vitro to down-regulate the expression of MCU, which further proves that the increase in apoptosis caused by mitochondrial dysfunction of cardiomyocytes in high glucose environment is caused by the decrease in MCU expression.

Oxidative stress is considered to be one of the important factors that trigger the occurrence of DCM. Persistent hyperglycemia and fluctuations in blood glucose in patients with DM can cause acute oxidative stress, resulting in increased production of ROS, cell dysfunction, and death [28, 29]. Overproduction of ROS has been identified as one of the initial pathogenic factors of DCM. Related studies have shown that enhancing the endogenous antioxidant capacity of the myocardium can effectively reduce the myocardial damage in patients with DM [30]. In the electron transfer chain (ETC), superoxide anion radicals ( $O_2^{\cdot-}$ ) are produced at the regions I and III of the complex, and disproportionate to hydrogen peroxide ( $H_2O_2$ ) by  $Mn^{2+}$ -dependent superoxide dismutase [31–33]. Then,  $H_2O_2$  is detoxified by glutathione reductase (GSH), and thioredoxin and peroxiredoxin systems; all these reactions require NADPH, which is produced by the tricarboxylic acid cycle [34]. Therefore,  $Ca^{2+}$ -regulated tricarboxylic acid cycle not only provides energy, but also maintains the body's redox equilibrium. Once The imbalance of mitochondrial calcium homeostasis, it will lead to altered redox equilibrium in cardiomyocytes, enhanced oxidative stress and increased production of ROS, eventually it will lead to cardiomyocyte apoptosis [35, 36]. Through experiments, we have found that under high glucose environment, the mitochondrial  $Ca^{2+}$  uptake of H9C2 cardiomyocytes is reduced, and the production of cardiac NADPH and GSH is reduced, which leads to weakened antioxidant capacity, increased ROS production and myocardial apoptosis. Simultaneously, an appealing phenomenon was also found in H9C2 cardiomyocytes that regulated the expression of MCU under normal circumstances. Therefore, we speculate that the apoptosis caused by the increased oxidative stress of cardiomyocytes in high glucose environment is caused by the down-regulation of MCU. This may be an important cause of myocardial damage caused by DCM.

This study suggested that MCU expression in rat H9C2 cardiomyocytes was decreased in the high glucose environment, causing abnormal mitochondrial calcium uptake and imbalanced calcium homeostasis, which may further contribute to mitochondrial dysfunction (decreased mitochondrial membrane potential and reduced ATP production) and enhanced oxidative stress (increased ROS production, increased ratio of  $NADP^+$ /NADPH and reduced GSH/GSSG ratio) in cardiomyocytes. Mitochondrial dysfunction and enhanced oxidative stress ultimately led to apoptosis in cardiomyocytes. In conclusion, MCU may be vital in the development and progression of DCM. Previous studies have also demonstrated that recovery of MCU level can restore high glucose-induced metabolic changes and normalize  $Ca^{2+}$  [37]. Thus, MCU, as the main channel involved in mitochondrial  $Ca^{2+}$  uptake, could be considered a novel potential target for the treatment of DCM, and is worthy of further research.

## List Of Abbreviations

|                                       |               |
|---------------------------------------|---------------|
| <b>diabetes mellitus</b>              | <b>DM</b>     |
| cardiovascular disease                | CVD           |
| heart failure                         | HF            |
| Diabetic cardiomyopathy               | DCM           |
| type 2 diabetes mellitus              | T2DM          |
| mitochondrial calcium uniporter       | MCU           |
| mitochondrial membrane potential      | MMP           |
| reactive oxygen species               | ROS           |
| pyruvate dehydrogenase                | PDH           |
| $\alpha$ -ketoglutarate dehydrogenase | $\alpha$ KGDH |
| isocitrate dehydrogenase              | IDH           |
| electron transfer chain               | ETC           |
| glutathione reductase                 | GSH           |

## Declarations

## Ethics approval and consent to participate

Not applicable.

### Consent for publication

All the authors agree.

### 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.

## Funding

All sources of funding for the research reported was from the Gansu Natural Fund (Mitochondrial Calcium Uniporter Transporter Down-regulation Increases Myocardial Ischemic Vulnerability and Its Mechanism), Project code: 18JR3RA402.

## Authors' contributions

Xia Chen, Wenyun Guo and Zhe Jing designed the study, analysed and interpreted the data, and drafted the paper. Yongqing Chen and Hui Chen designed and directed research. Tao Zhang, Zhaoqi Wu, Hengqiang Cui and Wenxue Zheng contributed to the experimental data collection. All authors read and approved the final manuscript.

## Acknowledgements

Thanks to the members of our experimental team and instructors for their contributions in this research.

## References

1. Danaei G, Finucane MM, Lu Y, et al. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants[J]. *Lancet*. 2011;378(9785):31–40. doi:10.1016/S0140-6736(11)60679-X.
2. Wang ZV, Hill JA. Diabetic cardiomyopathy: catabolism driving metabolism[J]. *Circulation*. 2015;131:771–3. doi:10.1161/CIRCULATIONAHA.115.015357.
3. Ryden L, Armstrong PW, Cleland JG, et al. Efficacy and safety of high-dose lisinopril in chronic heart failure patients at high cardiovascular risk, including those with diabetes mellitus: results from the ATLAS trial[J]. *Eur Heart J*. 2000;21:1967–78. doi:10.1053/euhj.2000.2311.
4. Shindler DM, Kostis JB, Yusuf S, et al. Diabetes mellitus, a predictor of morbidity and mortality in the Studies of Left Ventricular Dysfunction (SOLVD) Trials and Registry[J]. *Am J Cardiol*. 1996;77:1017–20. doi:10.1016/s0002-9149(97)89163-1.
5. Wong AK, AlZadjali MA, Choy AM, et al. Insulin resistance: a potential new target for therapy in patients with heart failure[J]. *Cardiovasc Ther*. 2008;26:203–13. doi:10.1111/j.1755-5922.2008.00053.
6. Maisch B, Alter P, Pankuweit S. Diabetic cardiomyopathy: fact or fiction[J]? *Herz*. 2011;36:102–115. doi: 10.1007/s00059-011-3429-4.
7. Jia G, DeMarco VG, Sowers JR. Insulin resistance and hyperinsulinaemia in diabetic cardiomyopathy[J]. *Nat Rev Endocrinol*. 2016;2:144–53. doi:10.1038/nrendo.2015.216.
8. Duncan JG. Mitochondrial dysfunction in diabetic cardiomyopathy[J]. *Biochim Biophys Acta*. 2011;1813(7):1351–9. doi:10.1016/j.bbamcr.2011.01.014.

9. Abel 9BuggerH. ED. Mitochondria in the diabetic heart[J]. *Cardiovasc Res*. 2010;88(2):229–40. doi:10.1093/cvr/cvq239. Epub 2010 Jul 16. Review.
10. Fan C, Fan M, Orlando BJ, et al. X-ray and cryo-EM structures of the mitochondrial calcium uniporter[J]. *Nature*, 2018, 559(7715):575–579. DOI:10.1038/s41586-018-0330-9.
11. Baughman JM, Perocchi F, Girgis HS, et al. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter[J]. *Nature*. 2011;476:341–5. doi:10.1038/nature10234.
12. Choi S, Quan X, Bang S, et al. Mitochondrial calcium uniporter in transfers calcium between the endoplasmic reticulum and mitochondria in oxidative stress-induced cell death[J]. *J Biol Chem*. 2017;292(35):14473–85. doi:10.1074/jbc.M116.765578.
13. Aneja A, Tang WH, Bansilal S, et al. Diabetic cardiomyopathy: insights into pathogenesis, diagnostic challenges, and therapeutic options[J]. *Am J Med*. 2008;121:748–57. doi:10.1016/j.amjmed.2008.03.046.
14. Pham T, Loiselle D, Power A, et al. Mitochondrial inefficiencies and anoxic ATP hydrolysis capacities in diabetic rat heart[J]. *Am J Physiol Cell Physiol*. 2014;307(6):499–507. doi:10.1152/ajpcell.00006.2014. Epub 2014 Jun 11.
15. Bragg F, Holmes MV, Iona A, et al. Association between diabetes and causespecific mortality in rural and urban areas of China[J]. *JAMA*. 2017;317(3):280–9. doi:10.1001/jama.2016.19720.
16. Sorrentino A, Borghetti G, Zhou Y, et al. Hyperglycemia induces defective Ca<sup>2+</sup> homeostasis in cardiomyocytes[J]. *Am J Physiol Heart Circ Physiol*. 2017;312(1):H150–61. doi:10.1152/ajpheart.00737.2016.
17. Brownlee M. Biochemistry and molecular cell biology of diabetic complications[J]. *Nature*. 2001;414(6865):813–20. doi:10.1038/414813a.
18. Kamer KJ. The molecular era of the mitochondrial calcium uniporter[J]. *Nat Rev Mol Cell Biol*. 2015;16(9):545–53. doi:10.1038/nrm4039.
19. Kwong JQ, Lu X, Correll RN, et al. The Mitochondrial Calcium Uniporter Selectively Matches Metabolic Output to Acute Contractile Stress in the Heart[J]. *Cell Rep*. 2015;12(1):15–22. doi:10.1016/j.celrep.2015.06.002. Epub 2015 Jun 25.
20. Kwong JQ. The mitochondrial calcium uniporter in the heart: energetics and beyond[J]. *J Physiol*. 2017;15(12):3743–51. doi:10.1113/JP273059. Epub 2017 Feb 1. 595 ) .
21. Bravo-Sagua R, Parra V, López-Crisosto C, et al. Calcium Transport and Signaling in Mitochondria[J]. *Comprehensive Physiology*. 2017;7(2):623–34. doi:10.1002/cphy.c160013.
22. Plattner H. Inseparable tandem: evolution chooses ATP and Ca<sup>2+</sup> to control life, death and cellular signalling[J]. *Philos Trans R Soc Lond B Biol Sci*. 2016;371(1700). doi:10.1098/rstb.2015.0419.
23. Rizzuto R, De Stefani D, Raffaello A, et al. Mitochondria as sensors and regulators of calcium signalling[J]. *Nat Rev Mol Cell Biol*. 2012;13(9):566–78. doi:10.1038/nrm3412.

24. Denton RM. Regulation of mitochondrial dehydrogenases by calcium ions[J]. *Biochim Biophys Acta*. 2009;1787(11):1309–16. doi:10.1016/j.bbabi.2009.01.005.
25. De Stefani D, Raffaello A, Teardo E, et al. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter[J]. *Nature*. 2011;476(7360):336–40. doi:10.1038/nature10230.
26. Rossi CS, Vasington FD, Carafoli E, et al. The effect of ruthenium red on the uptake and release of  $\text{Ca}^{2+}$  by mitochondria[J]. *Biochem Biophys Res Commun*. 1973;50(3):846–52. doi:10.1016/0006-291x(73)91322-3.
27. Kain V, Sawant MA, Dasgupta A. A novel SOD mimic with a redox-modulating mn (II) complex, ML1 attenuates high glucose-induced abnormalities in intracellular  $\text{Ca}^{2+}$  transients and prevents cardiac cell death through restoration of mitochondrial function[J]. *Biochem Biophys Res Commun*. 2016;5:296–304. doi:10.1016/j.bbrep.2016.01.003.
28. Liu ZW, Zhu HT, Chen KL, et al. Protein kinase RNA-like endoplasmic reticulum kinase (PERK) signaling pathway plays a major role in reactive oxygen species (ROS)-mediated endoplasmic reticulum stress-induced apoptosis in diabetic cardiomyopathy[J]. *Cardiovasc Diabetol*. 2013;12:158. doi:10.1186/1475-2840-12-158.
29. Evangelista I, Nuti R, Piccioni T, et al. Molecular Dysfunction and Phenotypic Derangement in Diabetic Cardiomyopathy[J]. *Int J Mol Sci*. 2019;20(13):1–15. doi:10.3390/ijms20133264.
30. Guo Y, Zhuang X, Huang Z, et al. Klotho protects the heart from hyperglycemia-induced injury by inactivating ROS and NF- $\kappa$ B-mediated inflammation both in vitro and in vivo[J]. *Biochim Biophys Acta Mol Basis Dis*. 2018;1864(1):238–51. doi:10.1016/j.bbadis.2017.09.029.
31. Chen Y-R, Zweier JL. Cardiac mitochondria and reactive oxygen species generation[J]. *Circ Res*. 2014;114(3):524–37. doi:10.1161/CIRCRESAHA.114.300559.
32. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release[J]. *Physiol Rev*. 2014;94(3):909–50. doi:10.1152/physrev.00026.2013.
33. Murphy MP. Understanding and preventing mitochondrial oxidative damage[J]. *Biochem Soc Trans*. 2016;44(5):1219–26. doi:10.1042/BST20160108.
34. Nickel AG, von Hardenberg A, Hohl M, et al. Reversal of  $\text{Ca}^{2+}$  mitochondrial transhydrogenase causes oxidative stress in heart failure[J]. *Cell Metab*. 2015;22(3):472–84. doi:10.1016/j.cmet.2015.07.008.
35. Kohlhaas M, Nickel AG. Mitochondrial energetics and calcium coupling in the heart[J]. *J Physiol*. 2017;595(12):3753–63. doi:10.1113/JP273609.
36. Joseph SK, Booth DM, Young MP, et al. Redox regulation of ER and mitochondrial  $\text{Ca}^{2+}$  signaling in cell survival and death[J]. *Cell Calcium*. 2019;79:89–97. doi:10.1016/j.ceca.2019.02.006.
37. Diaz-Juarez J, Suarez J, Cividini F, et al. Expression of the mitochondrial calcium uniporter in cardiac myocytes improves impaired mitochondrial calcium handling and metabolism in simulated hyperglycemia[J]. *Am J Physiol Cell Physiol*. 2016;311(6):C1005–13. doi:10.1152/ajpcell.00236.2016.

# Figures

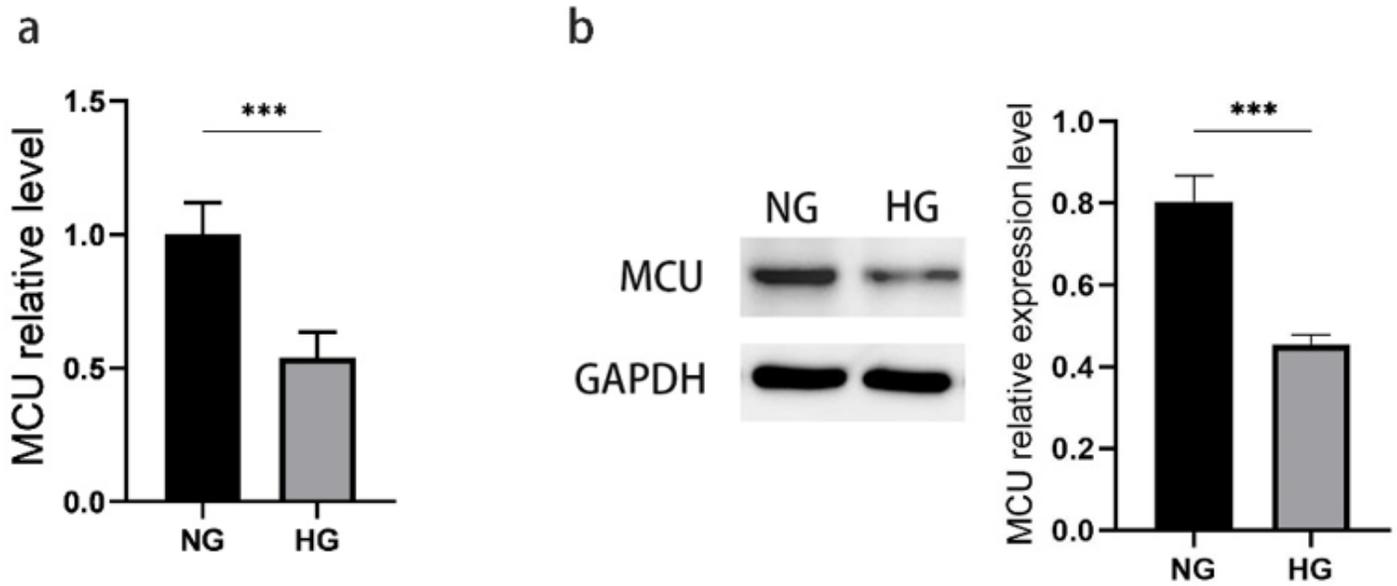


Figure 1

Expression of MCU in H9C2 cardiomyocytes under high glucose environment. a: RT-PCR to detect the MCU mRNA expression level of H9C2 cardiomyocytes (compared with NG group, \*\*\* $P < 0.001$ ). b: Western Blot detection of MCU protein expression (compared with NG group, \*\*\* $P < 0.001$ ).

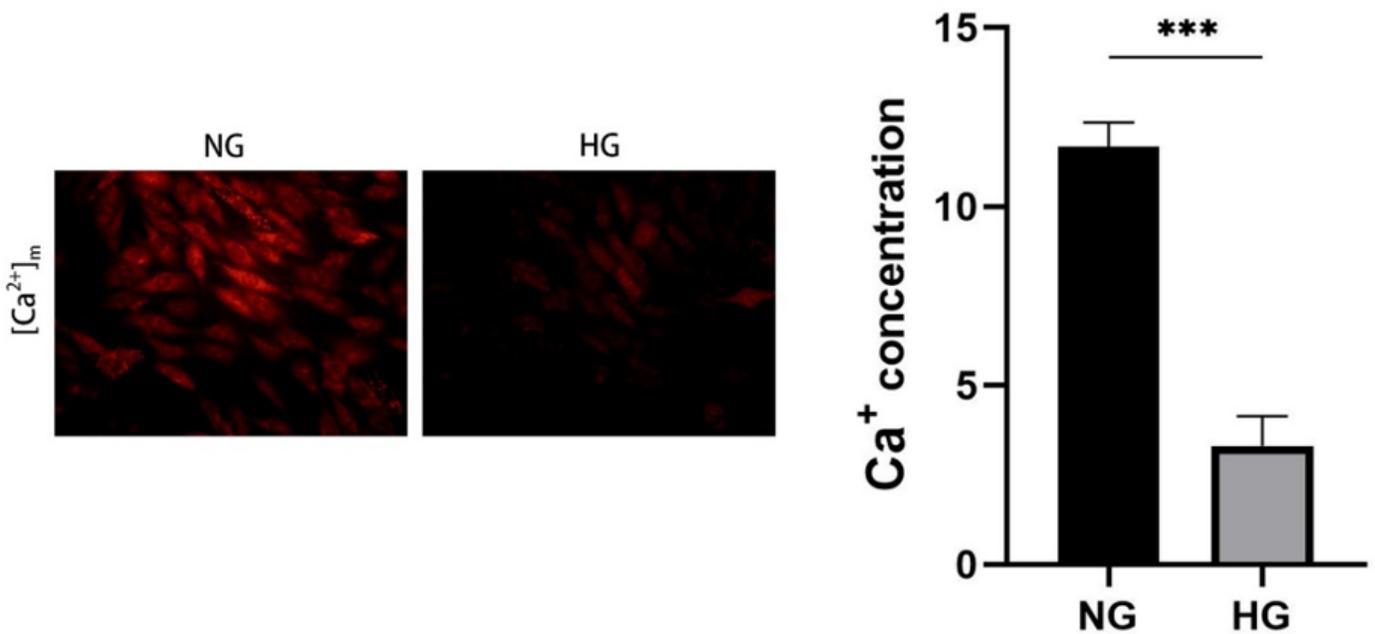
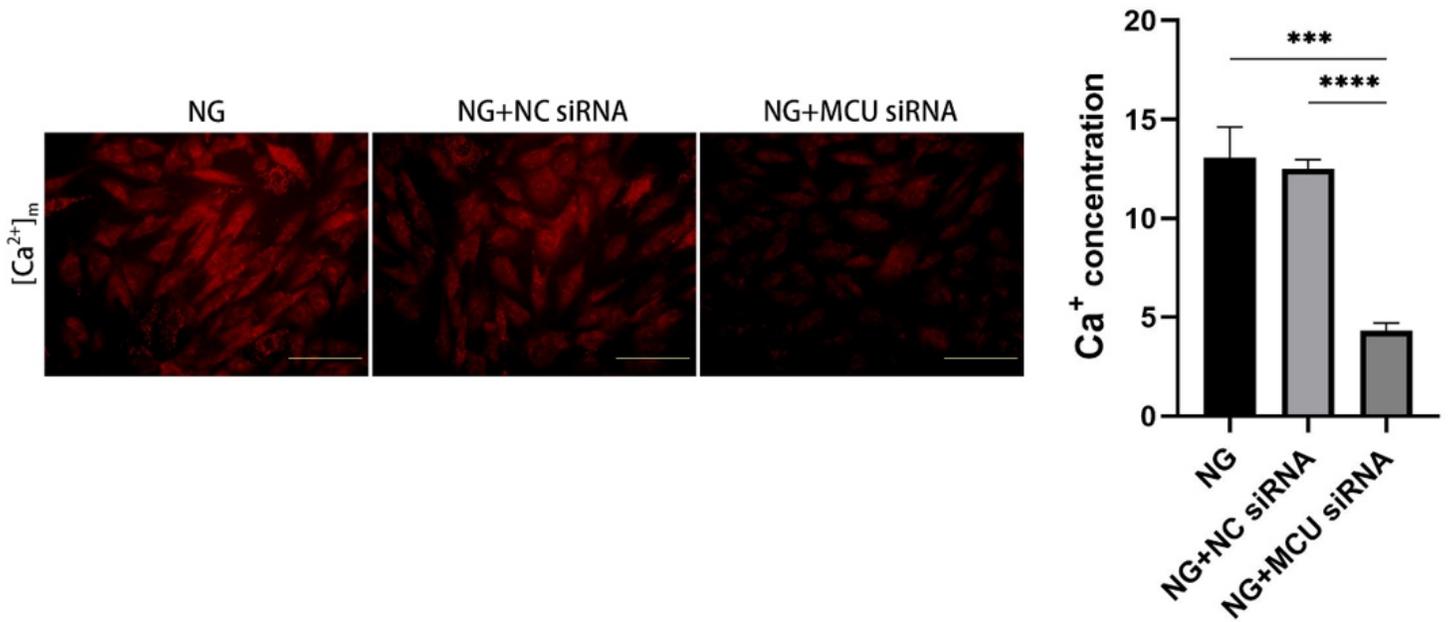


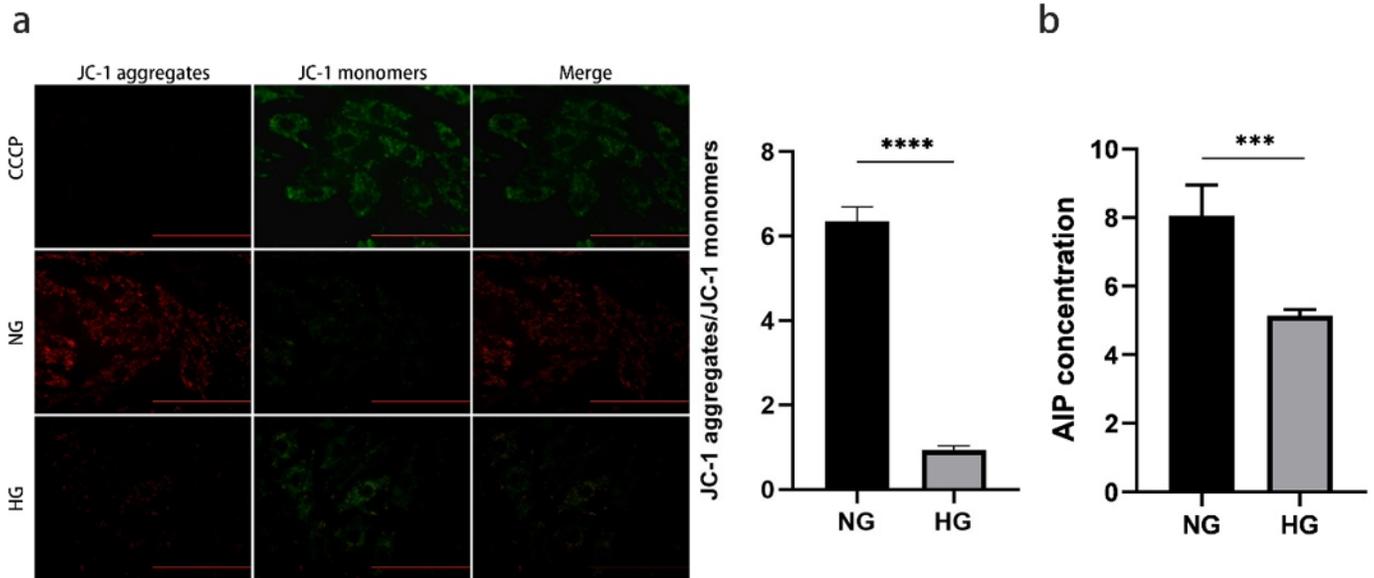
Figure 2

Cell mitochondrial  $Ca^{2+}$  assessment by Rhod-2.  $Ca^{2+}$  levels in the high glucose group were lower than those of the normal group (\*\*\* $P < 0.001$ ).



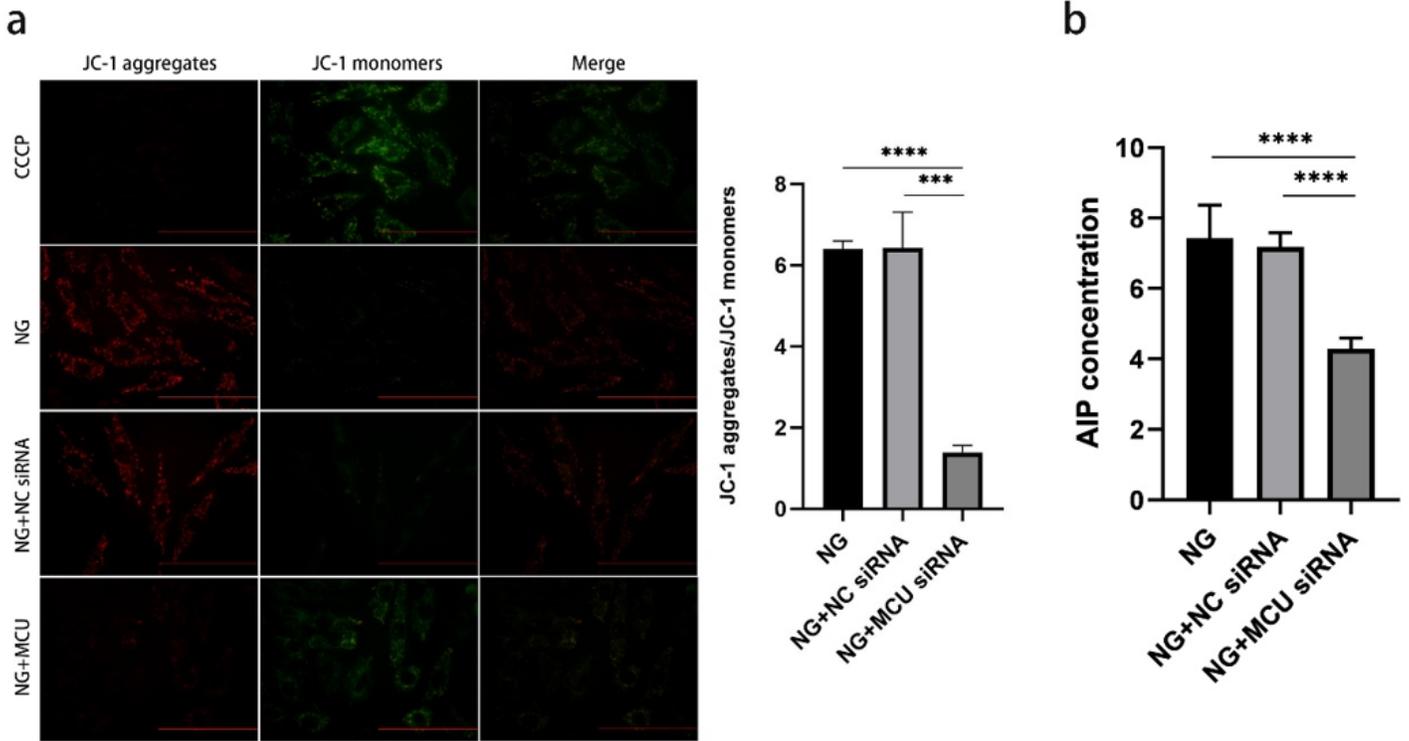
**Figure 3**

Rhod-2 reagent was used to detect the uptake of mitochondrial  $Ca^{2+}$  in H9C2 cardiomyocytes (compared with NG group,  $***P < 0.001$ , compared with NG+NC siRNA group,  $****P < 0.0001$ ).



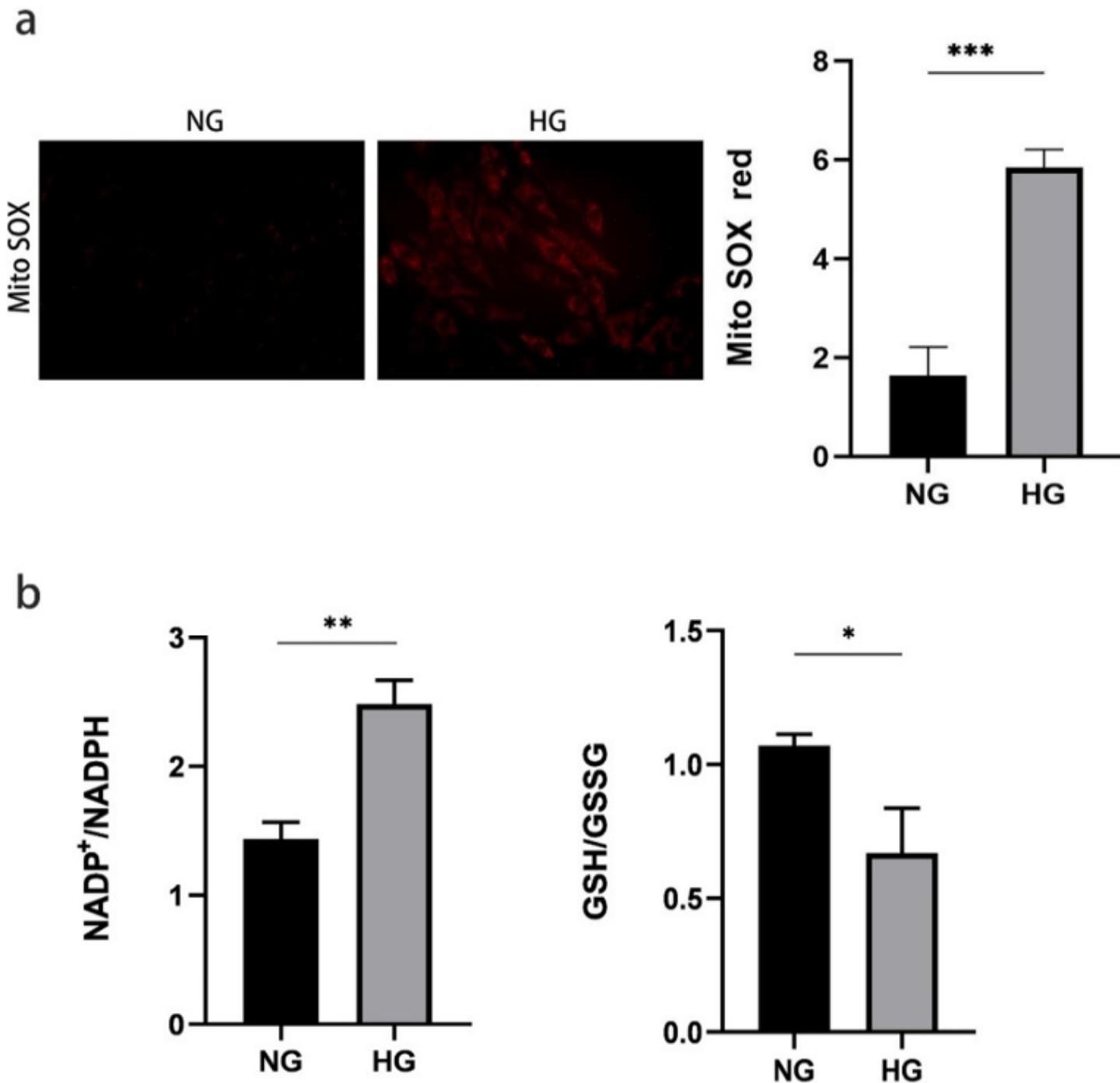
**Figure 4**

Detection of mitochondrial function of H9C2 cardiomyocytes in high glucose environment. a: JC-1 mitochondrial membrane potential reagent was used to detect the mitochondrial membrane potential of H9C2 cardiomyocytes (compared with NG group,  $****P < 0.0001$ ). b: ATP reagent was used to detect the ATP production concentration of H9C2 cardiomyocytes (compared with NG group,  $***P < 0.001$ ).



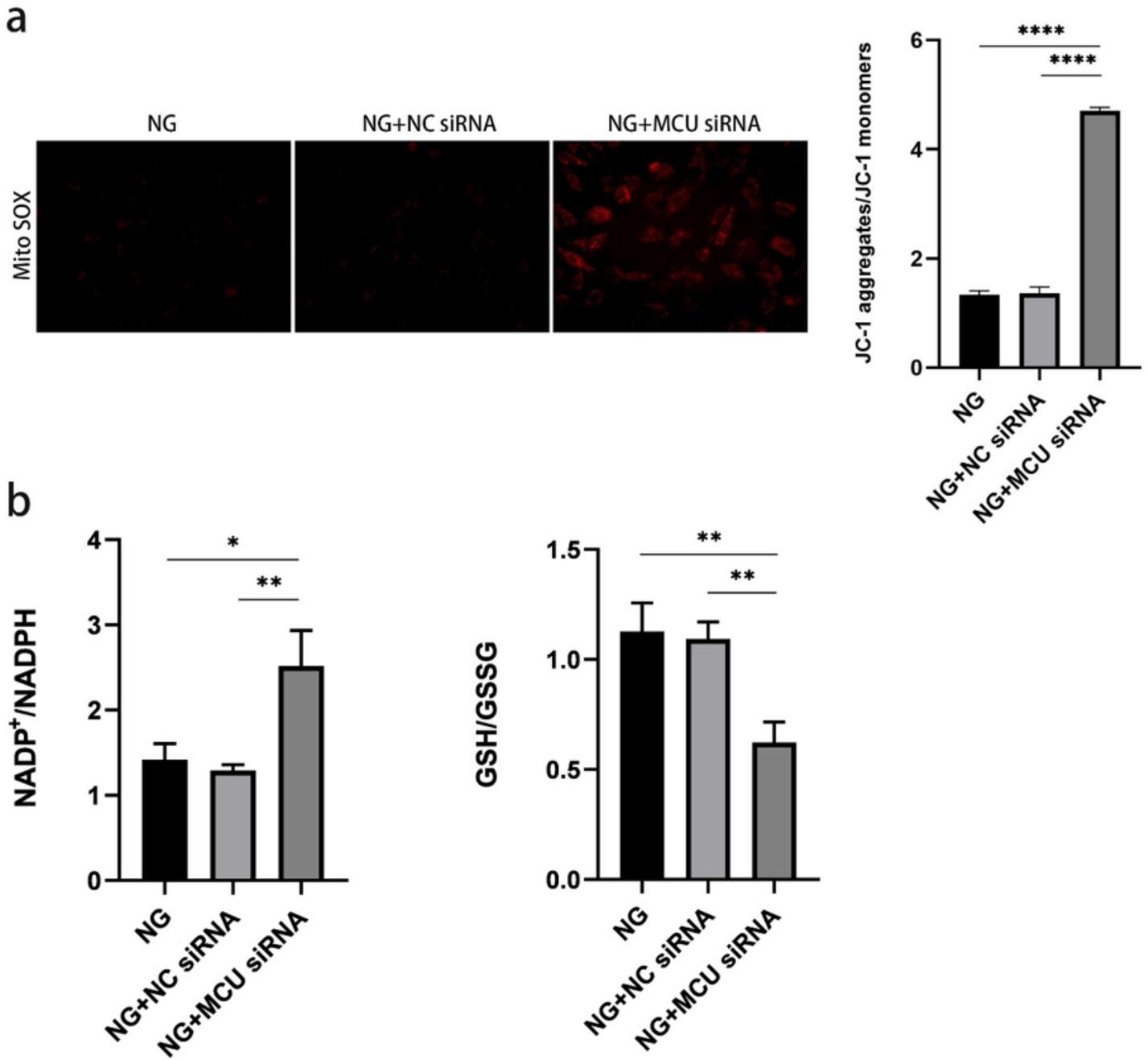
**Figure 5**

Detection of mitochondrial function of H9C2 cardiomyocytes. a: JC-1 mitochondrial membrane potential detection reagent detects the mitochondrial membrane potential of H9C2 cardiomyocytes (compared to the NG group, \*\*\*\* $P < 0.0001$ ; compared to the NG+NC siRNA group, \*\*\* $P < 0.001$ ). b: ATP detection kit to detect ATP production concentration of H9C2 cardiomyocytes (compared with NG group, \*\*\*\* $P < 0.0001$ ; compared with NG+NC siRNA group, \*\*\*\* $P < 0.0001$ ).



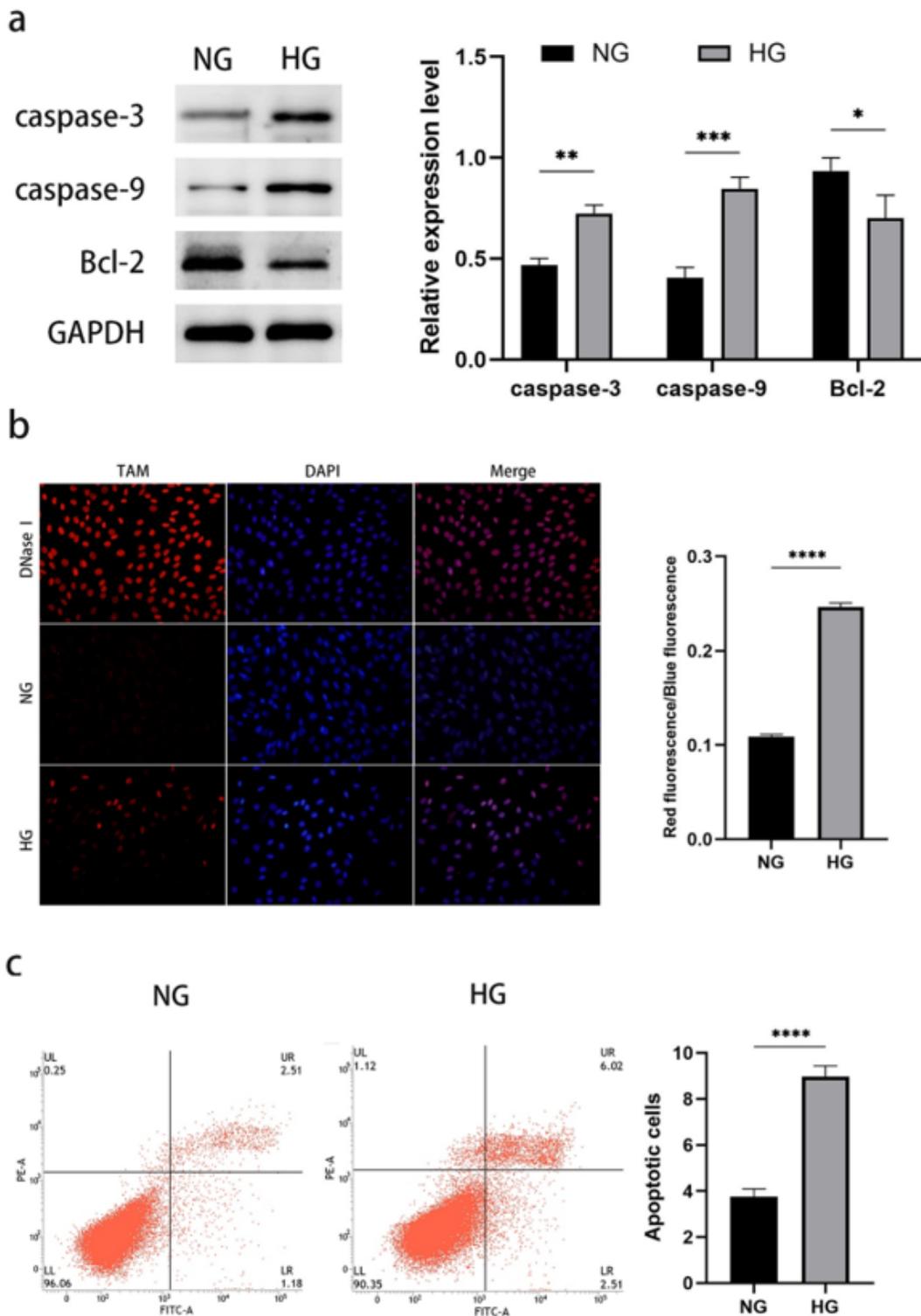
**Figure 6**

Detection of oxidative stress in H9C2 cardiomyocytes under high glucose conditions. a: Mito-SOX™ Red reagent detects the level of ROS (compared with NG group, \*\*\* $P < 0.001$ ). b: NADP<sup>+</sup>/NADPH detection kit detects the value of NADP<sup>+</sup>/NADPH in H9C2 cardiomyocytes (compared to NG group, \*\* $P < 0.01$ ) The GSH/GSSG detection kit was used to detect the GSH / GSSG value of H9C2 cardiomyocytes (compared to the NG group, \* $P < 0.05$ ).



**Figure 7**

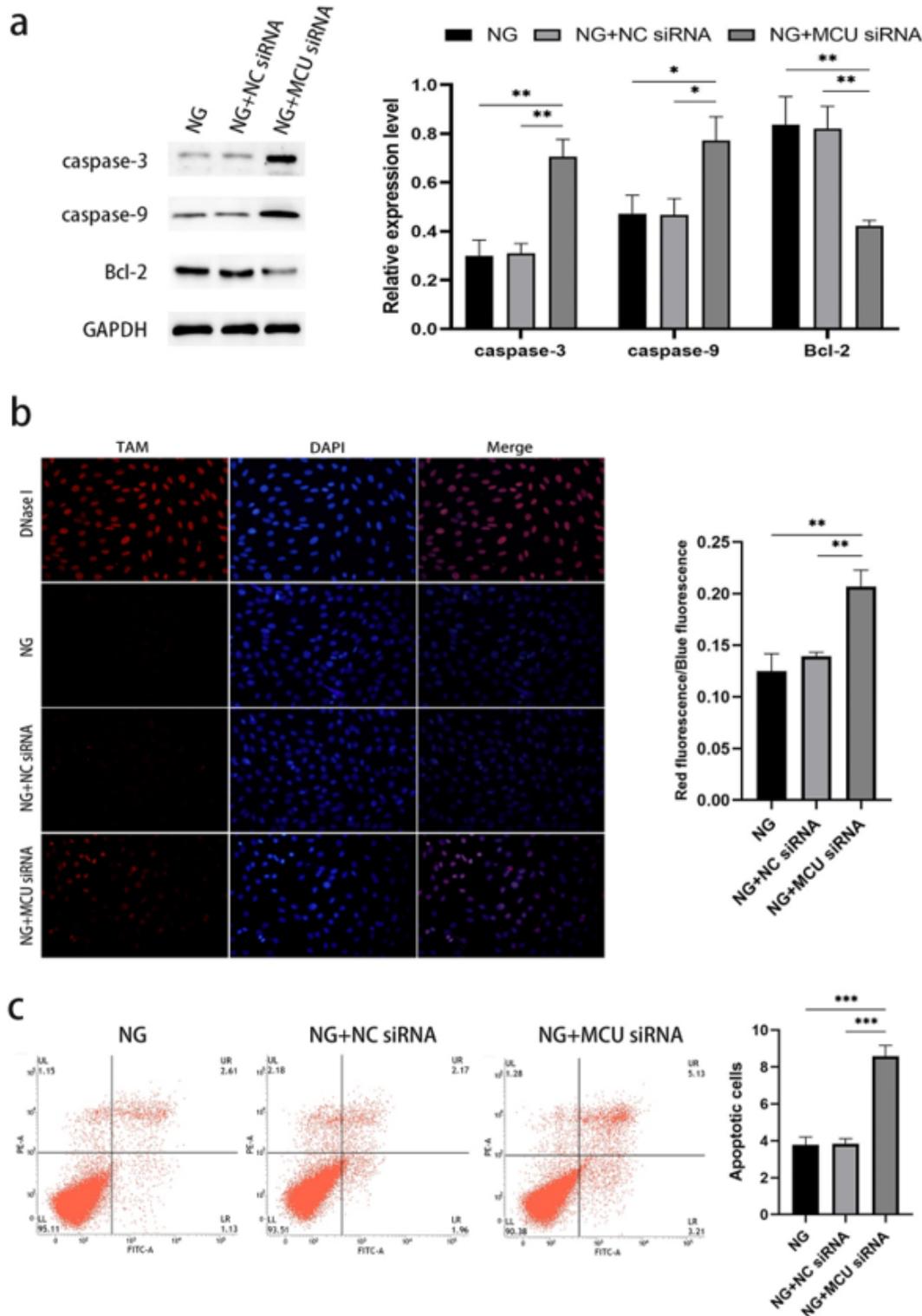
Detection of oxidative stress in H9C2 cardiomyocytes. a: The level of ROS detected by Mito SOX™-Red reagent (compared with NG group, \*\*\*\* $P < 0.0001$ ; compared with NG+NC siRNA group, \*\*\*\* $P < 0.0001$ ). b: NADP<sup>+</sup>/NADPH detection kit detects the value of NADP<sup>+</sup>/NADPH in H9C2 cardiomyocytes (compared to the NG group, \* $P < 0.05$ ; compared to the NG+NC siRNA group, \*\* $P < 0.01$ ). The GSH/GSSG detection kit was used to detect the GSH/GSSG value of H9C2 cardiomyocytes (compared to the NG group, \*\* $P < 0.01$ ; compared to the NG+NC siRNA group, \*\* $P < 0.01$ ).



**Figure 8**

Detection of apoptosis of H9C2 cardiomyocytes in a high glucose environment. a: Western Blot detected the expression of caspase-3, caspase-9 and Bcl-2 in H9C2 cardiomyocytes of each group (compared with NG group, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \* $P < 0.05$ ) b: TUNEL apoptosis detection kit detects the apoptosis of H9C2 cardiomyocytes in each group (compared to the NG group, \*\*\*\* $P < 0.0001$ ). c: Flow cytometry

combined with Annexin-V-FITC/PI dye to detect apoptosis of H9C2 cardiomyocytes (compared to NG group, \*\*\*\*P<0.0001).



**Figure 9**

Detection of H9C2 cardiomyocyte apoptosis. a: Western Blot detection of caspase-3 expression in H9C2 cardiomyocytes (compared with NG group, \*\*P<0.01; compared with NG+NC siRNA group, \*\*P<0.01), caspase-9 expression (compared with NG group, \*P<0.05; compared with NG+NC siRNA group, \*P<0.05),

Bcl-2 expression (compared with NG group,  $**P<0.01$ ; compared with NG+NC siRNA group,  $**P<0.01$  ). b: TUNEL apoptosis detection kit detects the apoptosis of H9C2 cardiomyocytes in each group (compared with NG group,  $**P<0.01$ ; compared with NG+NC siRNA group,  $**P<0.01$ ). c: Flow cytometry combined with Annexin-V-FITC/PI dye to detect the apoptosis of H9C2 cardiomyocytes (compared with NG group,  $***P<0.001$ ; compared with NG+NC siRNA group,  $***P<0.001$ ).