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Research

Keywords: arsenic trioxide, sec-o-glucosylhamaudol, cardiotoxicity, H9c2, detoxify

Posted Date: March 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-177594/v1>

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A study on the attenuation of ATO cardiotoxicity by SOG though Nrf2/ARE pathway

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Received: ; Accepted: ; Published:

Abstract: Arsenic trioxide (ATO, Trisenox) is an ancient Chinese medicine which have good clinical effects for acute promyelocytic leukemia (APL). However, ATO has serious cardiotoxicity which has restricted its widely applied in clinic. *Saposhnikovia divaricata* (Trucz.) Schischk was an antidote to ATO in Chinese medical book. Sec-O-Glucosylhamaudol (SOG) is one of its four main active ingredients. So we explore if SOG can detoxify the ATO's cardiotoxicity through the Nuclear factor erythroid 2-related factor 2(Nrf2) signal pathway and apoptosis pathway. We added 10 μ M ATO and 10, 50, 100 μ M SOG in Rat cardiac myocyte-like H9c2 cells. The results indicate SOG can active Nrf2 signal pathway, improve Nrf2 phosphorylation levels, promote the expression of Nrf2, HO-1, NQO1 gene and protein, increase the contents of antioxidant enzyme SOD, CAT and GSH-PX, reduce the ROS levels, increase cell activity. By testing the apoptosis-related protein Bax, Bcl-2 and caspase-3, also proved that 10 μ M SOG can improve ATO-induced cell apoptosis. So SOG can plays a role in detoxifying ATO cardiac toxicity.

Key words: arsenic trioxide; sec-o-glucosylhamaudol; cardiotoxicity; H9c2; detoxify

1. Introduction

ATO is an effective clinic drugs against APL [1-7]. And it was quickly approved by the Food and Drug Administration. But Arsenic is a concerned hazardous substance of global that is present in soil, drinking water, and food [8,9], it has systemic toxicity, causing death by poisoning or suffering from tumors, such as human skin derangements, lung and kidney diseases, or even cancer[10-13]. However, its clinical application is limited by its side effects that include hepatotoxicity, cardiotoxicity, and nephrotoxicity, as well as sudden death[14-17], especially its severe cardiotoxicity. The mechanisms underlying ATO-induced cardiotoxicity are complex and include mainly oxidative stress and calcium overload [18,19]. Several studies have shown that these two mechanisms affect each other, as excessive production of ROS can inhibit the uptake of Ca^{2+} by the release of Ca^{2+} from the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR), and oxidative stress is widely considered as one of the main mechanisms underlying the toxicity of ATO [20,21]. Researchers have made many efforts to reduce the cardiotoxicity of ATO, such as using intermittent administration, developing new dosage forms and adding cardiac protectants. A better method is the cardiac

protectants, and drugs to improve cardiac toxicity will enable to take advantages of the full therapeutic potential of ATO, and expand its clinical application. In addition, ATO has also study on the treatment of liver cancer, breast cancer, SIV infection, stomach cancer and many other cancers[22-44]. Therefore, it is important to develop a cardiac protectant to reduce the cardiotoxicity of ATO.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that plays a critical role in protecting tissues and cells from oxidative stress and toxic damage[47-50]. It can activate antioxidant reaction elements (ARE). In resting cells, the activity of Nrf2 is tightly controlled by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm [51]. Under oxidative stress Keap1 separate with Nrf2, then Nrf2 was phosphorylated and transferred to the nucleus, when Nrf2 was activated in could protect against arsenic trioxide-induced injury[52], and promote protective genes expression, such as heme oxygenase 1(HO-1)[53,54], recombinant Human Heme Oxygenase 1(Hmox-1), quinone oxidoreductase(NQO1), glutathione transferase(GST) and glutathione(GSH). Bax, Bcl-2 and Caspase-3 are the key proteins in apoptosis pathway.

Studies have shown that Saposhnikovia divaricata (Trucz.) Schischk has many functions. Saposhnikovia divaricata (Trucz.) Schischk can detoxify the toxicity of ATO. And it can effectively detoxify the cardiotoxicity caused by ATO. Sec-O-Glucosylhamaudol(SOG) is One of the four active ingredients. So we investigate if SOG can ease the cardiotoxicity of ATO through Nrf2 signal pathway and apoptosis pathway.

2. Results

3.1.SOG can increase H9c2 cells viability after ATO treatment

After incubated with various concentrations of SOG (0-256 μ M) for 48 h, H9C2 cells have no obviously cell death, so SOG has no obviously cytotoxic (Figure 1). Study has shown the IC₅₀ value of ATO is 15.56 μ M. After adding 10 μ M ATO and 10, 20, 50 μ M SOG in H9c2 cells, cultured for 24, 48 and 72 h. The results shown that the cell viability of 10 μ M and 50 μ M SOG and 10 μ M ATO co-incubated group was significantly higher than 10 μ M ATO group (Figure 2).

3.2.SOG can reduce the intracellular oxidation in H9c2 cells after ATO treatment

Here we measured ROS level of H9c2 cells with DCFH-DA after treatment with ATO and SOG. The fluorescence probe DCFH-DA enters H9c2 cells, and ROS rapidly oxidized the DCFH to DCF with high fluorescence intensity, and the fluorescence intensity is proportional to the content of ROS in cell. We respectively use fluorescence microscope and Flow Cytometer to determine whether SOG can reduce the ROS level in H9c2 cells. The result shown that ATO can increase the ROS level in H9c2 cells, indicated that ATO can case soxidative damage of H9c2 cell, after adding 10 μ M and 50 μ M SOG the ROS level in H9c2 cells were reduced (Figure 3,4).

3.3.SOG increase antioxidant enzyme SOD, GSH-Px and CAT enzyme contents in H9c2 cells after ATO treatment

We next investigated the effect of SOG and ATO on SOD, GSH-Px and CAT enzyme contents after ATO treatment in H9c2 cells (Figure 5). SOD is a main antioxidantase that

eliminate superoxide anion radical, protect cells from damage. CAT destroys oxygen free radicals and has an antioxidant effect. GSH-px specifically catalyzes the reduction reaction of reduced glutathione (GSH) to hydrogen peroxide, protect cell membrane structure and keep its function integrity. The SOD, GSH-Px and CAT enzyme contents in 10 μ M ATO group were significantly decreased, H9c2 cell occurred oxidative damage after ATO treatment. But after adding 10 μ M and 50 μ M SOG, the SOD, GSH-Px and CAT enzyme contents were significantly increased than ATO group. These results indicate that SOG can increase SOD, GSH-Px and CAT enzyme contents specifically under oxidative stress.

3.4.SOG upregulate Nrf2, HO-1, NQO1 mRNA and protein expression, promote p-Nrf2 expression in H9c2 cells after ATO treatment

We examined the effect of SOG and ATO on Nrf2, HO-1, NQO1 mRNA expression using the RT-PCR method. We extract the RNA of H9c2 cells, then we can see the 5s, 18s, 28s three distinct bands by agarose gel electrophoresis, indicated the RNA were integrity (Figure 7), the expression of Nrf2, HO-1, NQO1 mRNA and protein, and the p-Nrf2 protein expression in 10 μ M ATO group were higher than the normal group, because the ATO caused oxidative damage and activated the Nrf2 pathway. But after adding 10 μ M and 50 μ M SOG the expression of Nrf2, HO-1, NQO1 mRNA and protein, and the p-Nrf2 protein expression were significantly increased than ATO group (Figure 8,9,10). The results of indirect immunofluorescence shown that the Nrf2, HO-1 protein expression in the H9c2 cells were significantly increased after ATO treatment (Figure 11,12,13). But after adding 10 μ M and 50 μ M SOG the expression of the Nrf2, HO-1 protein was significantly increased than ATO group. These results indicated that the Nrf2 signal path were activated after adding 10 μ M ATO, and 10 μ M and 50 μ M SOG can promote the Nrf2 signal path further activation.

3.5.SOG reduces the H9c2 cell apoptosis caused by ATO

We use flow cytometry to explore H9c2 cell apoptosis (Figure 6). The H9c2 cells apoptosis rate in 10 μ M ATO group was significantly increased than normal group, indicated that the H9c2 cells occurred oxidative damage and led to apoptosis after ATO treatment. But after adding 10 μ M and 50 μ M SOG the H9c2 cells apoptosis rates were significantly decreased than ATO group. We use WB to examine the effect of SOG and ATO on apoptosis-related protein Bax, Bcl-2, caspase-3 (Figure 9,10), the results shown that the Bax, and caspase-3 protein expression in the H9c2 cells were significantly increased, and the Bcl-2 protein expression was significantly reduced after ATO treatment. But after adding 10 μ M SOG, the expression of the Bax and caspase-3 protein was reduced, and the Bcl-2 protein expression was increased than ATO group. These results indicate that SOG can decrease the H9c2 cell apoptosis that is caused by ATO.

3. Discussion

Despite the well-known toxicity of arsenic (As), arsenic trioxide (ATO) has been used for centuries. It is a effective Chinese medicine in the treatment of APL. But ATO is also a potent carcinogen and environmental toxin, has the ability to induce apoptosis in many tumors/leukemias[55-57]. Nrf2 is a master regulator of numerous cytoprotective genes[58], such as antioxidant enzymes HO-1, NQO-1, glutathione S-transferase and superoxide dismutase. ROS plays a crucial role in mediating both the toxic and therapeutic effects of

ATO. A moderate level of ROS facilitates cell proliferation, whereas a high level of ROS leads to cell death[59,60]. A lot of studies have researched the cardiotoxicity and hepatotoxicity of ATO. And the researchers started to search for cardiac protectors which can reduce the cardiotoxicity of ATO, and help expand its clinical application. Researchers have used slow intervenous drop infusion and combination medication to detoxify its cardiac toxicity. These two methods can reduce ATO concentrations in the blood, relieve cardiac toxicity. This also reduced the drug effectiveness. Studies have shown that some antioxidants, such as oxymatrine[61], melatonin[62] can detoxify the hepatotoxicity that is caused by ATO. The resveratrol[63] can detoxify the cardiotoxicity that is caused by ATO. In addition, selenium and zinc can also detoxify the toxicity of ATO[64-66]. Therefore, some antioxidants are currently used to detoxify the toxicity caused by ATO, such as polyphenols, flavonoids, anthocyanins, vitamins, resveratrol [67], rice bran extract[68], ginsenosides[69] and so on. More and more chemical compounds have the protective myocardium function and detoxify the cardiotoxicity of ATO-induced. Salvianolic acid B can significantly inhibit ATO-induced H9c2 cells apoptosis, the mice cardiac dysfunction, reduce myocardial structural damage, enhance the activity ATO against cancer cells HepG2 and Hela[70,71]. Linseed oil can effectively reduce ATO-induced cardiac toxicity in Wistar male rats. This may related to improvement of cardiac tissue antioxidant capacity. The ω -3 fatty acid can improve the antioxidant enzyme activities to reduce oxidative stress and the intracellular Ca^{2+} concentration, to improve mitochondrial membrane potential(MMP), to protect ATO induced cardiotoxicity[72]. Morphine can reduce the toxicity of ATO on H9c2 cells, reduce ROS production, resist the effect that ATO inhibit DNA synthesis, resist Caspase-3 activate, and reverse NF- κ B inhibiting effect mediated by ATO[73]. Flavonoids compounds in *Sorbus pohuashanensis* can alleviate ATO-induced myocardial injury in BALB/c mice, reduce the toxicity of H9c2 cells, ROS, alleviate apoptosis, and improve the Akt phosphorylation level to increase Nrf2 expression[74]. Genistin flavone can reduce intracellular Ca^{2+} overload, to reduce cardiomyocytes apoptosis[75], Artemisinin and tetrandrine can significantly reduce the proliferative effect of A549, HepG2 and Hela cells, at the same time reduced ATO induced cardiotoxicity in mice[76]. Salvianolic acid a protects H9c2 cells from arsenic trioxide-induced injury via inhibition of the mapk signaling pathway[77]. According to the results and these studies indicate SOG can detoxify the cardiotoxicity of ATO, and have the capacity to the heart protectant of ATO. Our study has shown that SOG can relieve arsenic-induced cardiotoxicity by activating Nrf2 pathway to against oxidative damage. The H9c2 cell will cause oxidative damage after ATO treatment. The results have shown that ATO can decrease the cell viability, SOD, GSH-Px, CAT contents, the mRNA expression of Nrf2 and its downstream factors, increase the apoptosis rate, ROS level, active the Nrf2 signal path, promote the expression of Nrf2 and its downstream protein and its phosphorylation then the normal group($P<0.05$). After adding SOG in the H9c2 cell that treatment by ATO, the cell viability, SOD, GSH-Px, CAT contents, the mRNA expression of Nrf2 and its downstream factors, the expression of Nrf2 and its downstream protein and its phosphorylation were higher than the ATO group($P<0.05$). And the apoptosis rate, ROS level was higher than the ATO group($P<0.05$). These datas indicate the Nrf2 pathway was activated after treating with ATO, and SOG can increase or prolong Nrf2 activation to protect the H9c2 cell against the ATO-induced oxidative damage and cell apoptosis .

4. Materials and methods

4.1. Regents and Antibodies

Rat cardiac myocyte-like H9c2 cells were purchased from National Infrastructure of Cell Line Resource (Beijing China). The primary antibodies against Nrf2 (proteintech, 16396-1-ap), HO-1 (abcam, ab80588), NQO1 (abcam, ab68477), phospho-Nrf2 (Biorbyt, orb336606), β -actin (proteintech, 66009-1-ig) and the secondary antibody anti-rabbit IgG, HRP-linked Antibody (CST, 7074s) and HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L) (proteintech, SA00001-1) used in Western blot analyses.

4.2. Cell culture and treatment

H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, SH30022.01) supplemented with 56 °C heat inactivated 10%(v/v) fetal bovine serum (FBS, Zhejiang tianhang Biotechnology CO., LED., 11011-8611), and 1% (v/v) penicillin/streptomycin (solarbio, P1400), and grown in a 95% air and 5% CO₂ humidified atmosphere at 37 °C.

4.3. Establishment of Oxidative Injury Model of ATO in H9c2 Cells

H9c2 cells were seeded in 6-well plates (1×10^5 /well) and cultured overnight in DMEM with 10% FBS. Then H9c2 cells treated with 10 μ M ATO for 24 h, in the meantime add respectively 10, 20, 50 SOG (dissolve in dimethyl sulfoxide, DMSO; Shanghai Pure One Biotechnology Co., Ltd.) in different wells. And the only added 10 μ M ATO as positive control, without ATO and SOG as normal control.

4.4. Analysis of cell viability

The H9c2 cell viability was measured by Thiazolyl Blue Tetrazolium Bromide (MTT) method. The H9c2 cells were seeded on 96-well plates at a density of 1.5×10^4 cells/well and cultured overnight. The cells were then subjected to the SOG and/or ATO treatments. After co-culture specific time, the H9c2 cells were incubated with 20 μ l MTT solution (5 mg/ml in phosphate buffer, solarbio, M8180) at 37 °C for 4 h, and the purple formazan crystals were then solubilized with SDS joint solution (10% SDS(v/v), 0.01mol/L HCl) at 37 °C for 4 h. The optical density at 570 nm was measured using a microplate reader (Thermo Fisher Scientific, USA). The cell inhibition rate (%) = (OD value of control group - OD value of dosing group) / OD value of control group $\times 100\%$.

4.5. Measurement of intracellular oxidation

The intracellular oxidation was measured using a fluorescence probe 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (sigma, D6883, USA). The H9c2 cells were seeded in 6-well plates (1×10^5 /well) and cultured overnight in DMEM with 10% FBS. After treatment 24 h with ATO and SOG, the cells were washed 3 times with PBS, and then incubated with 10 μ M DCFH-DA (diluted in serum-free DMEM medium) at 37 °C for 30 min. Then the fluorescence intensity was monitored after excitation at 488 nm and emission at 525 nm using a fluorescence microscope (Becton-Dickinson, San Diego, CA, USA) and Flow Cytometer for quantitative analysis.

4.6. Apoptosis analysis

The H9c2 cells were seeded in 6-well plates (1×10^5 /well) and cultured overnight in

DMEM with 10% FBS. After treatment 24 h with ATO and SOG, use the Annexin V FITC Apoptosis Kit (BD, 556547) to test H9c2 cell apoptosis according to the instruction book. And it must have been detected by flow cytometry within 1 hour.

4.7. Analysis of SOD, GSH-PX, CAT

The H9c2 cells were seeded in 6-well plates (1×10^5 /well) and cultured overnight in DMEM with 10% FBS. After treatment 24 h with ATO and SOG, collect H9c2 cells after washing 3 times with PBS, according to the Superoxide dismutase (SOD) kit (Nanjing jiancheng bioengineering institute, HY-60001), glutathione peroxidase (GSH-PX) kit (Nanjing jiancheng bioengineering institute, HY-60005) and catalase (CAT) kit (Nanjing jiancheng bioengineering institute, HY-60015) instructions to test the activities of SOD, GSH-PX and CAT.

4.8. Real time PCR assay

Total RNA was extracted by TRNzol total RNA extraction reagent (TIANGEN, DP405-02, China). RNA contents measured by Nanodrop 2000 (Thermo Fisher scientific, USA). The RNA integrity was confirmed by Agarose gel electrophoresis. Total RNA (1 μ g) from each sample was submitted to synthesis cDNA with PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, RR047B, China). β -actin was used as the invariant control. Real-time PCR was performed with SYBR® Premix Ex Taq™ II (TliRNaseH Plus) ROX plus (TaKaRa, RR82LR, China) on fluorescence ration PCR instrument (Applied Biosystems, QuantStudio5, USA). The primers of genes (Invitrogen company, China) were as follows: Nrf2: (forward) 5' -AAGATGCCTTGTACTTTGAAGACTGT-3' and (reverse) 3'-GGAAAATAGCTCCTGCCA AACTT-5' ; HO-1: (forward) 5' -AGCACAGGGTGACAGAAGAG-3' and (reverse) 3' -AACTCTGTCTGTGAGGGACT-5'; NQO1 (forward) 5'-GAAGAAGCGTCTGGAGACTGTC -3' and (reverse) 3' -CGGCTGGAATGGACTTGC-5' ; β -actin (forward) 5' -CCTAAGGCCAACCGTGAAAA-3' and (reverse) 3'-GACCAGAGGCATACAGGGACA-5'.

4.9. Western Blot Analyses

Protein expressions of Nrf2, HO-1, NQO1 and p-Nrf2 were determined by western blot (WB). Total protein was isolated from H9c2 cells using RIPA buffer (Solarbio, R0020, China) with PMSF (1:100) and phosphatase inhibitors (1:100). The protein concentration was detected with BCA protein assay (Solarbio, PC0020, China). Then add equal amounts of protein samples (50 μ g/lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis (100 V for 1 h) proteins were transferred to PVDF membrane using the Mini Blotting System (Biorad, USA), then blocked at room temperature (RT) in 5% nonfat-dried milk in TBST for 1 h before overnight incubation at 4 °C with primary antibodies Nrf2 (1:1000), HO-1 (1:2000), NQO1 (1:10000), phospho-Nrf2 (1:2000), β -actin (1:20000). Then incubated secondary antibody anti-rabbit IgG, HRP-linked Antibody (1:2000) and HRP-conjugated Affinipure Goat Anti-Mouse IgG (H+L) (1:8000) 1 h at RT. The PVDF membrane was processed using an electrochemiluminescence (ECL) reagent (proteintech, B500023) and use visualizer (Bio-rad, USA) to develop. Then subjected to a densitometric analysis use Image Lab software.

4.10. Indirect immunofluorescence assays

The cellular localization of Nrf2, HO-1 was determined by indirect immunofluorescence

assays. The H9c2 cells were seeded at 1 x10⁵ cells/well in 6 well cell culture plates and treatment with ATO and SOG, then washed twice with PBS and then fixed in 4% paraformaldehyde for 15 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100(Beyotime Biotechnology, ST795), and incubated with blocking buffer (3% BSA, solarbio, A8020) for 1 h at room temperature. The cells were then incubated overnight with primary antibodies against Nrf2(1:100), HO-1(1:50) in a humidified chamber at 4 °C. Subsequently, the cells were washed with PBS and incubated with secondary antibodies conjugated to IgG H&L Cy3 at a dilution of 1:500 for 1 h at room temperature in the dark, and were then counterstained with DAPI (Beyotime Biotechnology, C1002) dye to show the nuclear morphology. After the slides were rinsed with PBS, coverslips were mounted on slides, and images of the labeled cells were visualized and photographed using a fluorescent microscope (Olympus BX53, Japan).

4.11.IP

Use the Immunoprecipitation kit(proteintech, KIP-2) to Purify the nrf2 protein, and test the interaction between nrf2 and p-Nrf2.

4.12.Statistical analysis

Results were repeated at least 3 times, and presented as sample mean $\pm$ SEM. Statistical analysis was performed using Graphpad Prism software 8 and tested by one-way ANOVA (nonparametric) using Tukey 's posthoc test. All results are means of three independent experiments and P< (0.05) * was considered as significantly different from controls, and P< (0.05) # was considered as significantly different from ATO group.

5.Conclusions

ATO can produce overmuch ROS, lead H9c2 cell apoptosis, result in severe cardiotoxicity, at the moment Nrf2 signaling pathway was activated already, but SOG can further activated Nrf2 phosphorylation, activated Nrf2 signaling pathway promote the expression of downstream factors: HO-1, NQO1 mRNA and protein, improve the antioxidant SOD, CAT and GSG-Px, reduced the ROS level, apoptosis rates of H9C2 cell, improve cell survival rates. And after adding SOG, the expression of the Bax and caspase-3 protein were reduced , and the expression of Bcl-2 protein was increased, so the SOG reduce the cell apoptosis that caused by ATO. So SOG can resist ATO-induced oxidative damage and cell apoptosis to relieve the cardiotoxicity.

Author Contributions: Conceptualization, Yang Wang and Xiaoqiang Li; methodology, Yang Wang; software, Yang Wang; validation, Yang Wang; formal analysis, Yang Wang; investigation, Yang Wang; resources, Yang Wang; data curation, Yang Wang; writing—original draft preparation, Yang Wang,Jianying Shen; writing—review and editing, Yang Wang, Feng Sui; visualization, Yang Wang; supervision, Yang Wang; project administration, Yang Wang; funding acquisition, Yuqing Tan.

Acknowledgments:

The authors gratefully acknowledge Jianying Shen, Feng Sui for his assistance with article writing of the manuscript. The authors gratefully acknowledge Xiaoqiang Li for his donations in cells of the experiment. The authors gratefully acknowledge Tan Yuqing for his assistance with financial support.

In this section you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The authors declare no conflict of interest.

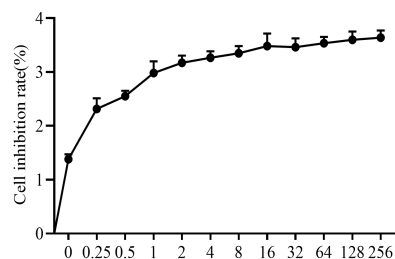


Figure 1. The 0~256 μM SOG has no toxic effect on H9c2 cells. Use MTT method to test the cell viability after treat 48 h with 0 μM , 0.25 μM , 0.5 μM , 1 μM , 2 μM , 4 μM , 8 μM , 16 μM , 32 μM , 64 μM , 128 μM , 256 μM SOG. The cell inhibition rate did not exceed 5%, showed the SOG has no obvious cytotoxicity. The IC₅₀ value of SOG on H9c2 cell = 26278.28 μM .

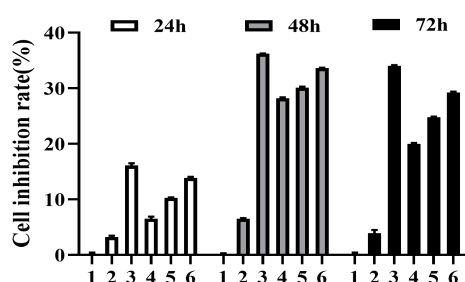


Figure 2. After adding 10, 50 μM SOG, the cell inhibition rates of H9c2 cell were significantly decreased than the ATO group. Using MTT method to test the cell viability after treating 24, 48, 72 h with 10 μM ATO and 10 μM , 50 μM , 100 μM SOG. The cell inhibition rate of 10 μM SOG group is lower than the other groups.

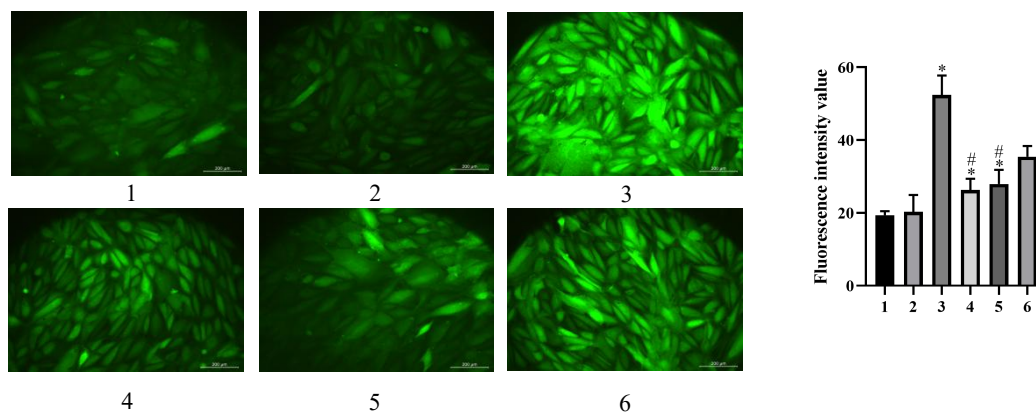


Figure 3. 10, 50 μM SOG significantly decreased the ROS levels of H9c2 cells compared with the ATO group. Using the DCFH-DA probe to test the ROS accumulation (200 $\times$) of H9c2 cells. The more ROS accumulation, the stronger the fluorescence intensity. Using the imageJ software analysed the fluorescence intensity. The results showed that the fluorescence intensity of ATO, 10 and μM SOG groups were significantly higher than the control group, but the ATO group is the strongest. After adding 10 and 50 μM SOG the fluorescence intensity were significantly decreased compared with the ATO group. $P < (0.05)^*$ was considered as significantly different from controls, $P < (0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μM SOG, 3. 10 μM ATO, 4. 10 μM ATO+10 μM SOG, 5. 10 μM ATO+50 μM SOG, 6. 10 μM ATO+100 μM SOG.

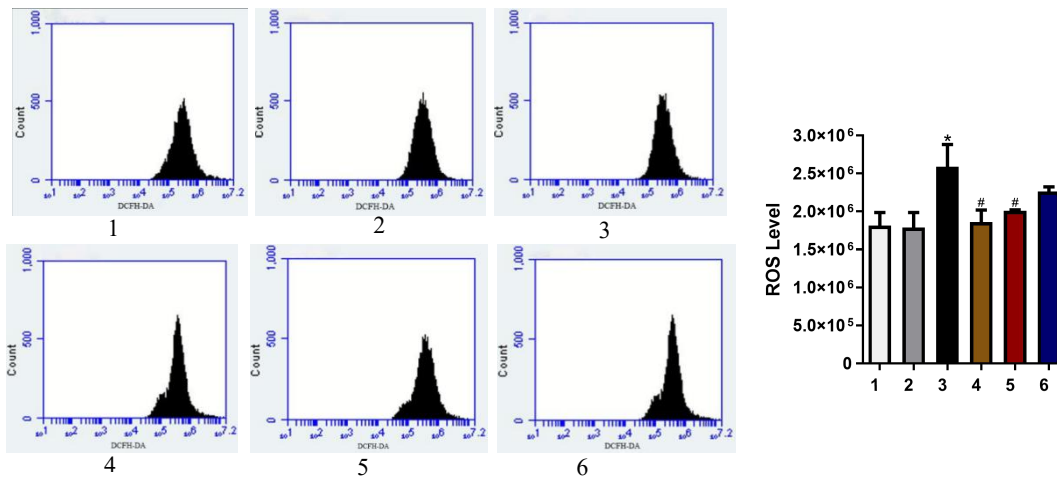


Figure 4. 10, 50 μM SOG significantly decreased the ROS levels of H9C2 cells compared with the ATO group. Use the flow cytometry to test the ROS accumulation. Y-axis represents the number of cells, X-axis represents relative fluorescence intensity. The fluorescence intensity in ATO group is the strongest. After add 10 and 50 μM SOG the fluorescence intensity were significantly decreased compared with the ATO group. $P < (0.05)^*$ was considered as significantly different from controls, $P < (0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μM SOG, 3. 10 μM ATO, 4. 10 μM ATO + 10 μM SOG, 5. 10 μM ATO + 50 μM SOG, 6. 10 μM ATO + 100 μM SOG.

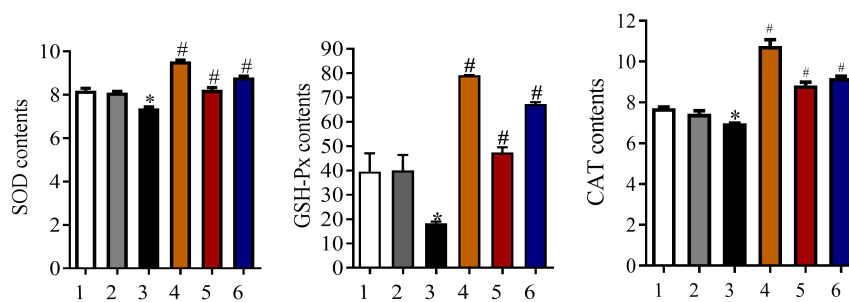


Figure 5. 10, 50 μM SOG significantly increased the SOD, CAT and GSH-Px contents in H9C2 cells compared with the ATO group. Use colorimetric method to test the SOD, CAT and GSH-Px contents. The SOD, GSH-Px and CAT enzyme contents of ATO group were significantly decreased, but after adding 10, 50, 100 μM SOG were significantly increased. $P < (0.05)^*$ was considered as significantly different from controls, $P < (0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μM SOG, 3. 10 μM ATO, 4. 10 μM ATO + 10 μM SOG, 5. 10 μM ATO + 50 μM SOG, 6. 10 μM ATO + 100 μM SOG.

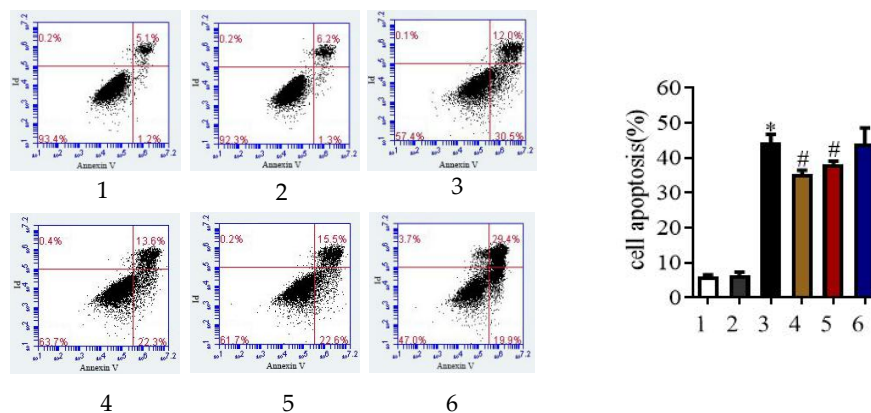


Figure 6. 10, 50 μM SOG significantly decreased the H9C2 cell apoptosis rates compared with the ATO group. Use flow cytometry to test the H9C2 apoptosis. The cell apoptosis rates in ATO group is the highest. After add 10 and 50 μM SOG the apoptosis rates were significantly lower than the ATO

group. $P<(0.05)^*$ was considered as significantly different from controls, $P<(0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μM SOG, 3. 10 μM ATO, 4. 10 μM ATO+10 μM SOG, 5. 10 μM ATO+50 μM SOG, 6. 10 μM ATO+100 μM SOG.

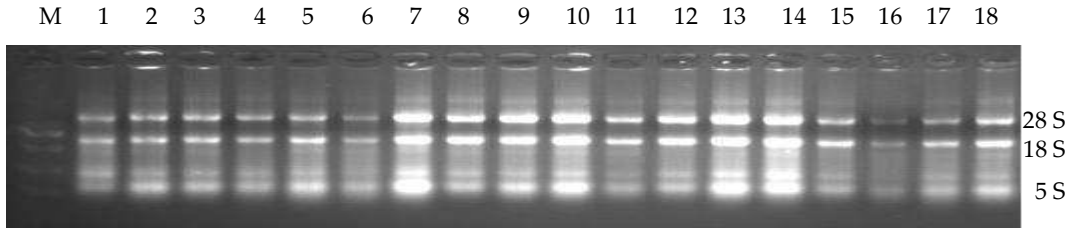


Figure 7. The RNA integrity was confirmed by agarose gel electrophoresis. The results show that the RNA was complete, because they all have three bands, respectively were 5s, 18s and 28s. M:Marker, 1-6, 7-12, 13-18 respectively show as Control, 100 μM SOG, 10 μM ATO, 10, 50, 100 μM SOG+10 μM ATO in Nrf2, HO-1, NQO1 mRNA.

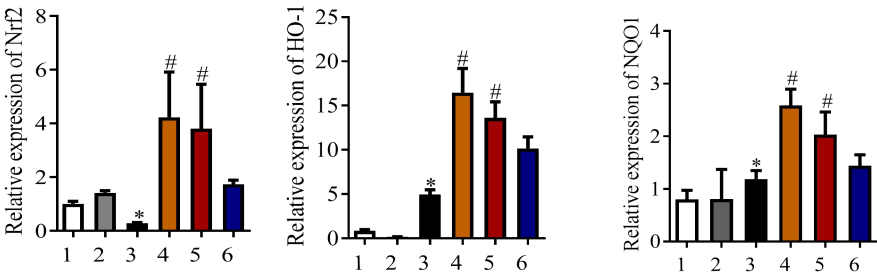
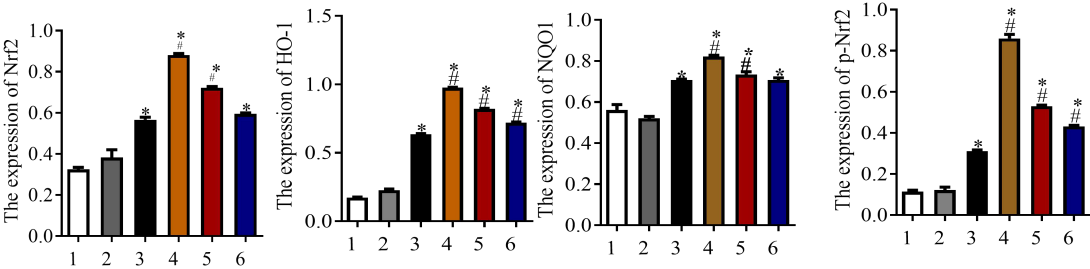
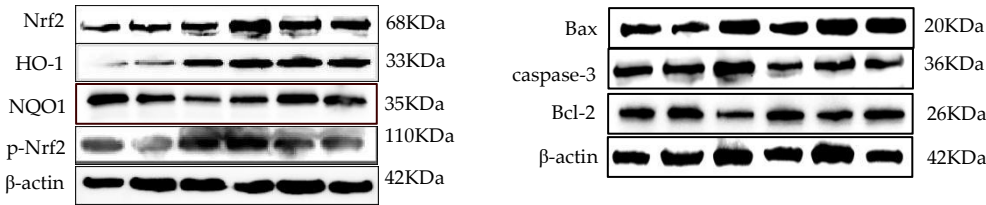


Figure 8. 10,50 μM SOG significantly increased the Nrf2, HO-1 and NQO1 mRNA expression levels compared with the ATO group. Use RT-PCR test the effects of SOG and ATO on the expression of HO-1, Nrf2 and NQO1 mRNA in H9c2 cells. The HO-1 and NQO1 mRNA expression levels of ATO group were significantly increased than control group, and the Nrf2, HO-1 and NQO1 mRNA expression levels of the 10, 50 μM SOG groups were significantly higher than the ATO group. $P<(0.05)^*$ was considered as significantly different from controls, $P<(0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μM SOG, 3. 10 μM ATO, 4. 10 μM ATO+10 μM SOG, 5. 10 μM ATO+50 μM SOG, 6. 10 μM ATO+100 μM SOG.



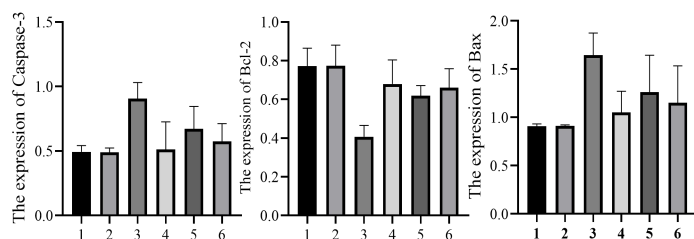


Figure 10. After adding 10,50 μ M SOG the Nrf2, HO-1, NQO1 and p-Nrf2 protein expression levels were significantly increased compared with the ATO group. Using the imageJ software to analyse the results of WB. The Nrf2, HO-1, NQO1 and p-Nrf2 protein expression levels of ATO group were significantly increased than control group, and after adding 10, 50 μ M SOG, the Nrf2, HO-1 and NQO1 protein expression levels were significantly higher than the ATO group. $P<(0.05)^*$ was considered as significantly different from controls, $P<(0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μ M SOG, 3. 10 μ M ATO, 4. 10 μ M ATO+10 μ M SOG, 5. 10 μ M ATO+50 μ M SOG, 6. 10 μ M ATO+100 μ M SOG.

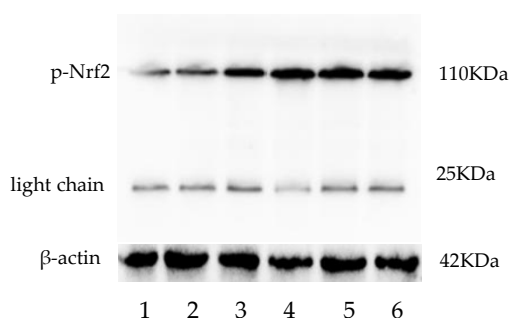


Figure 11. Use IP to test the effects of SOG and ATO on the expression levels of p-Nrf2 protein in H9c2 cells. Test group: 1. Control, 2. 100 μ M SOG, 3. 10 μ M ATO, 4. 10 μ M ATO+10 μ M SOG, 5. 10 μ M ATO+50 μ M SOG, 6. 10 μ M ATO+100 μ M SOG. When antibodies degrade, they produce light chains of 25KDa.

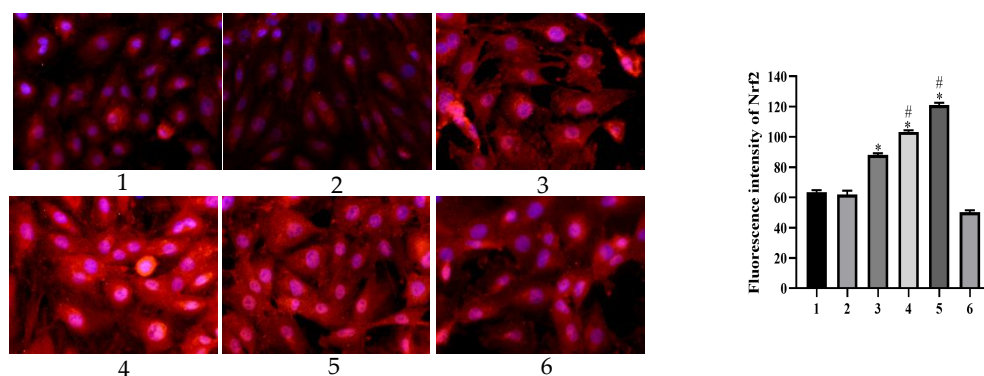


Figure 12. 10,50 μ M SOG significantly increased the Nrf2 protein expression levels compared to the ATO group. Using indirect immunofluorescence(400 $\times$) method to test the Nrf2 fluorescence intensity. The red colour is Nrf2 protein, the blue colour is the cell nucleus(Hoechst33258). The Nrf2 protein expression of ATO group were significantly increased than control group, and after adding 10, 50 μ M SOG, the Nrf2 protein expression were significantly higher than the ATO group. 1,Control, 2 100 μ M SOG, 3 10 μ M ATO, 4 10 μ M ATO+10 μ M SOG, 5 10 μ M ATO+50 μ M SOG, 6 10 μ M ATO+100 μ M SOG.

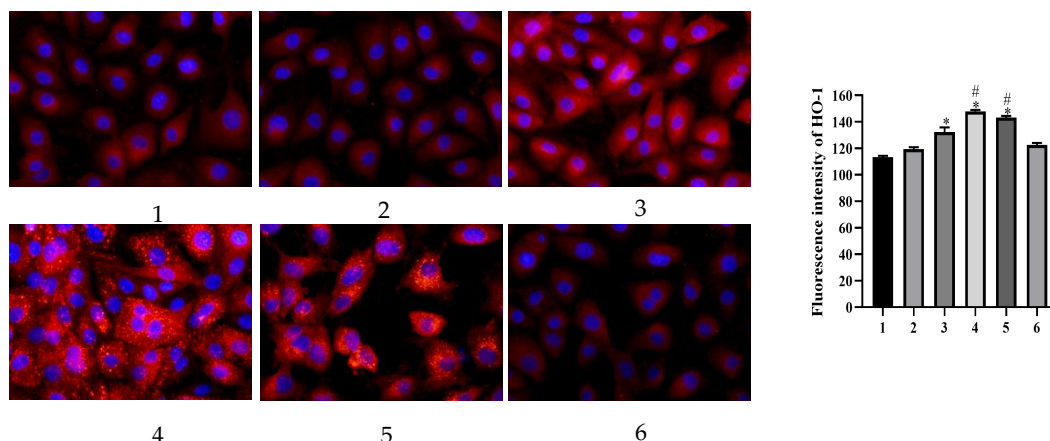


Figure 13. 10,50 μ M SOG significantly increased the HO-1 protein expression levels were compared to the ATO group. Using indirect immunofluorescence(400 $\times$) method to test the HO-1 fluorescence intensity. The red colour is Nrf2 protein, the blue colour is the cell nucleus(Hoechst33258). The HO-1 protein expression of ATO group were significantly increased than control group, and after adding 10, 50 μ M SOG, the HO-1 protein expression were significantly higher than the ATO group. 1. Control, 2 100 μ M SOG, 3. 10 μ M ATO, 4 ATO+10 μ M SOG, 5 ATO+50 μ M SOG, 6 ATO+100 μ M SOG.

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Figures

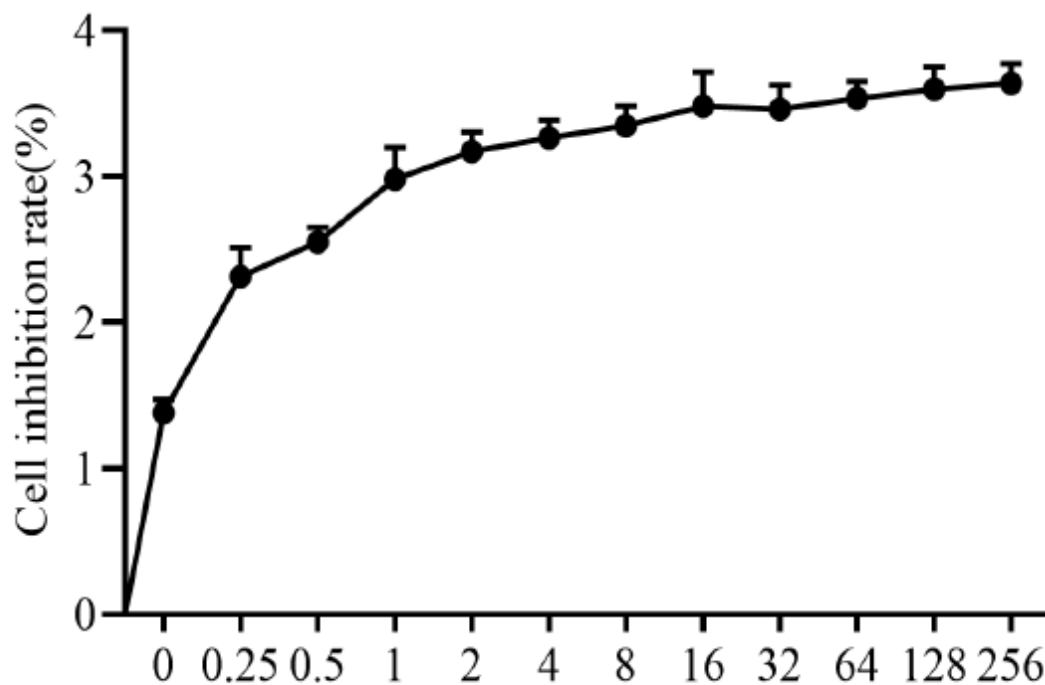


Figure 1

The 0~256 μM SOG has no toxic effect on H9C2 cells. Use MTT method to test the cell viability after treat 48 h with 0 μM , 0.25 μM , 0.5 μM , 1 μM , 2 μM , 4 μM , 8 μM , 16 μM , 32 μM , 64 μM , 128 μM , 256 μM SOG. The cell inhibition rate did not exceed 5%, showed the SOG has no obvious cytotoxicity. The IC_{50} value of SOG on H9c2 cell = 26278.28 μM .

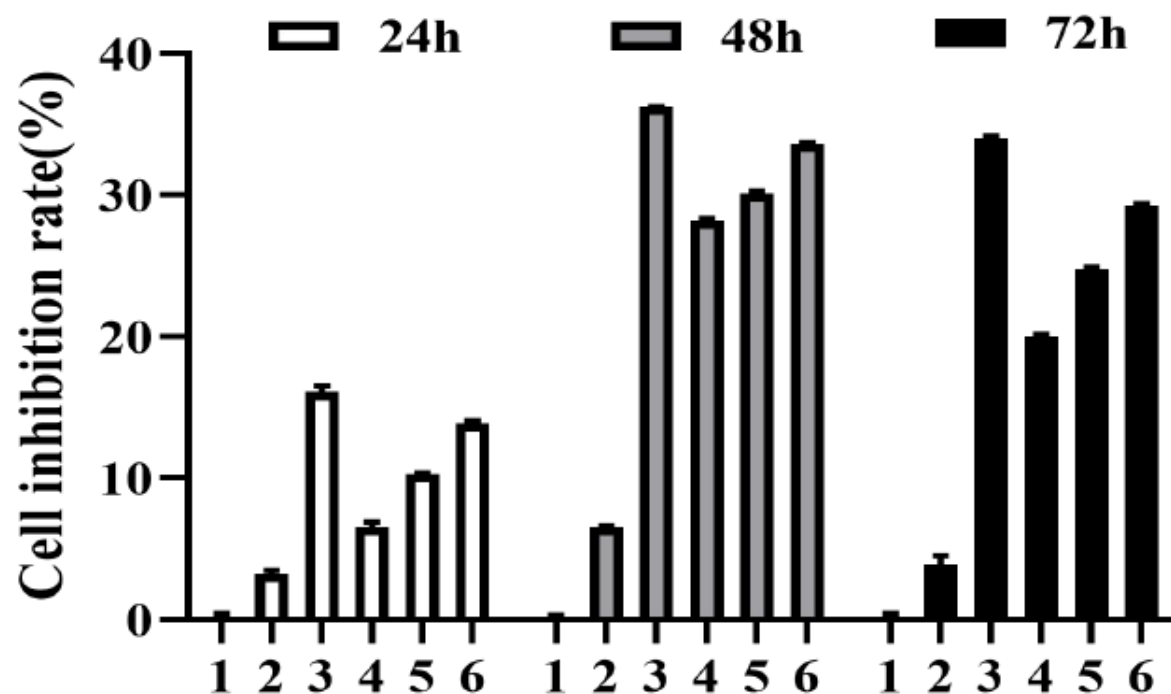


Figure 2

After adding 10,50 μM SOG, the cell inhibition rates of H9c2 cell were significantly decreased than the ATO group. Using MTT method to test the cell viability after treating 24, 48, 72 h with 10 μM ATO and 10 μM , 50 μM , 100 μM SOG. The cell inhibition rate of 10 μM SOG group is lower than the other groups.

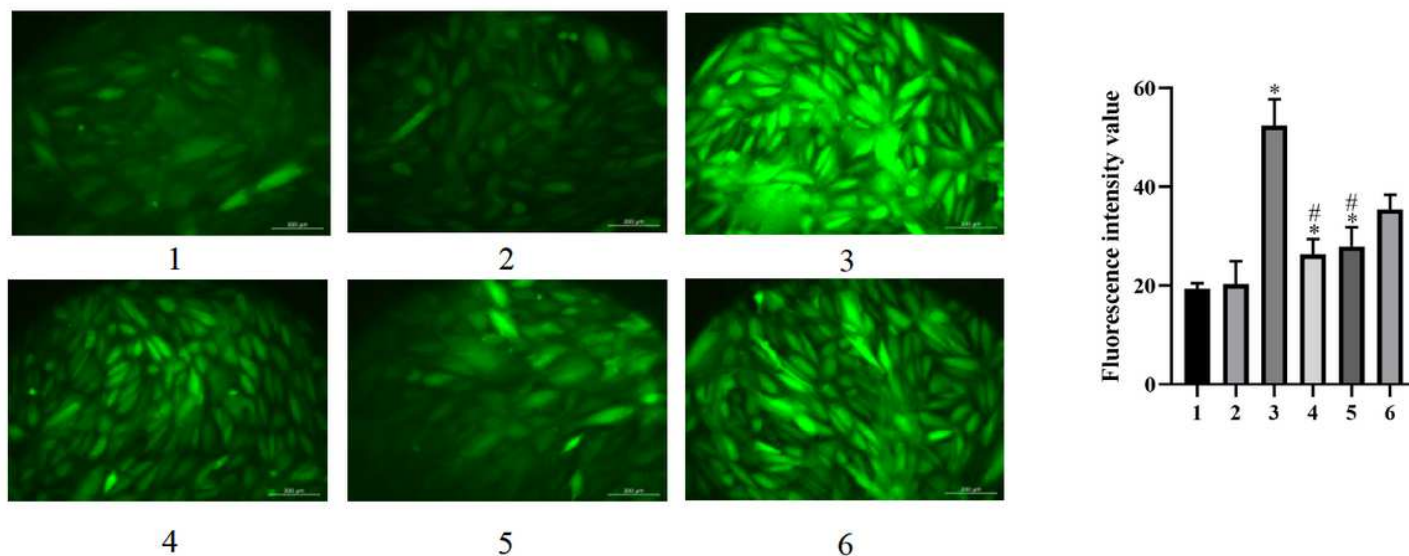


Figure 3

10, 50 μM SOG significantly decreased the ROS levels of H9c2 cells compared with the ATO group. Using the DCFH-DA probe to test the ROS accumulation(200 $\times$) of H9c2 cells. The more ROS accumulation, the stronger the fluorescence intensity. Using the imageJ software analysed the fluorescence intensity. The results showed that the fluorescence intensity of ATO, 10 and μM SOG groups were significantly higher than the control group, but the ATO group is the strongest. After adding 10 and 50 μM SOG the fluorescence intensity were significantly decreased compared with the ATO group. $P<(0.05)^*$ was considered as significantly different from controls, $P<(0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μM SOG, 3. 10 μM ATO, 4. 10 μM ATO+10 μM SOG, 5. 10 μM ATO+50 μM SOG, 6. 10 μM ATO+100 μM SOG.

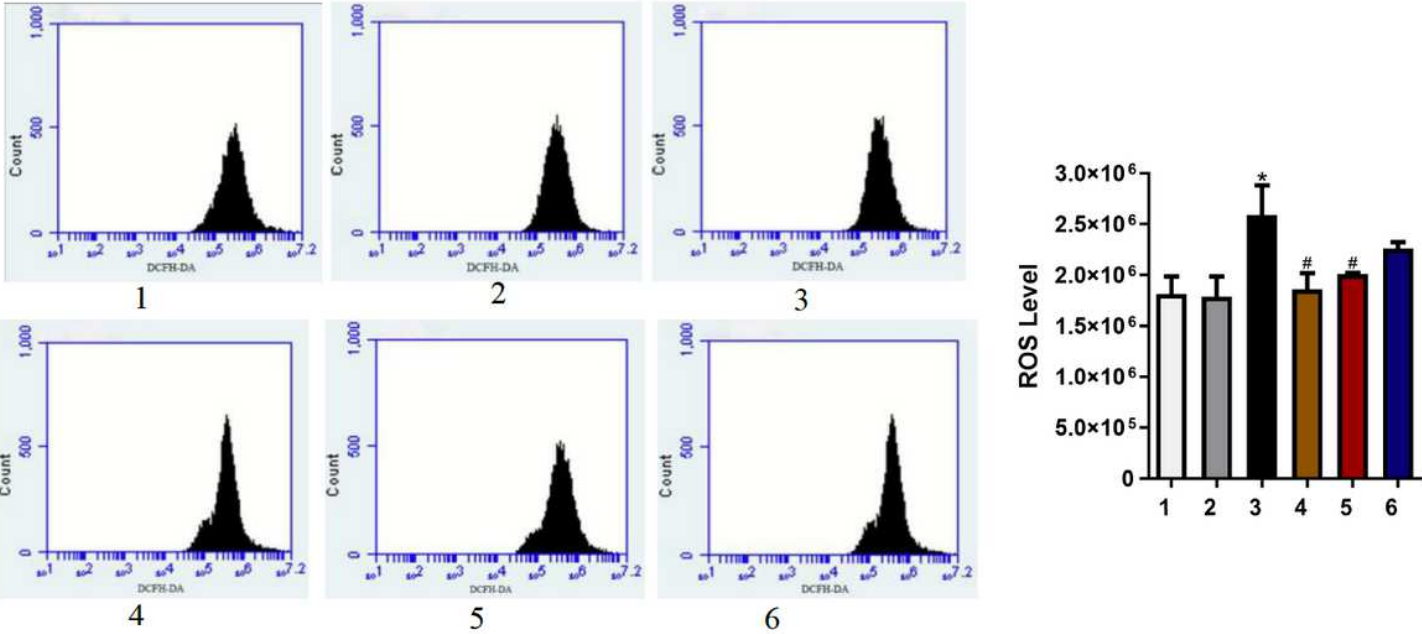


Figure 4

10,50 μM SOG significantly decreased the ROS levels of H9C2 cells compared with the ATO group. Use the flow cytometry to test the ROS accumulation. Y-axis represents the number of cells, X-axis represents relative fluorescence intensity. The fluorescence intensity in ATO group is the strongest. After add 10 and 50 μM SOG the fluorescence intensity were significantly decreased compared with the ATO group. $P<(0.05)^*$ was considered as significantly different from controls, $P<(0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μM SOG, 3. 10 μM ATO, 4. 10 μM ATO+10 μM SOG, 5. 10 μM ATO+50 μM SOG, 6. 10 μM ATO+100 μM SOG.

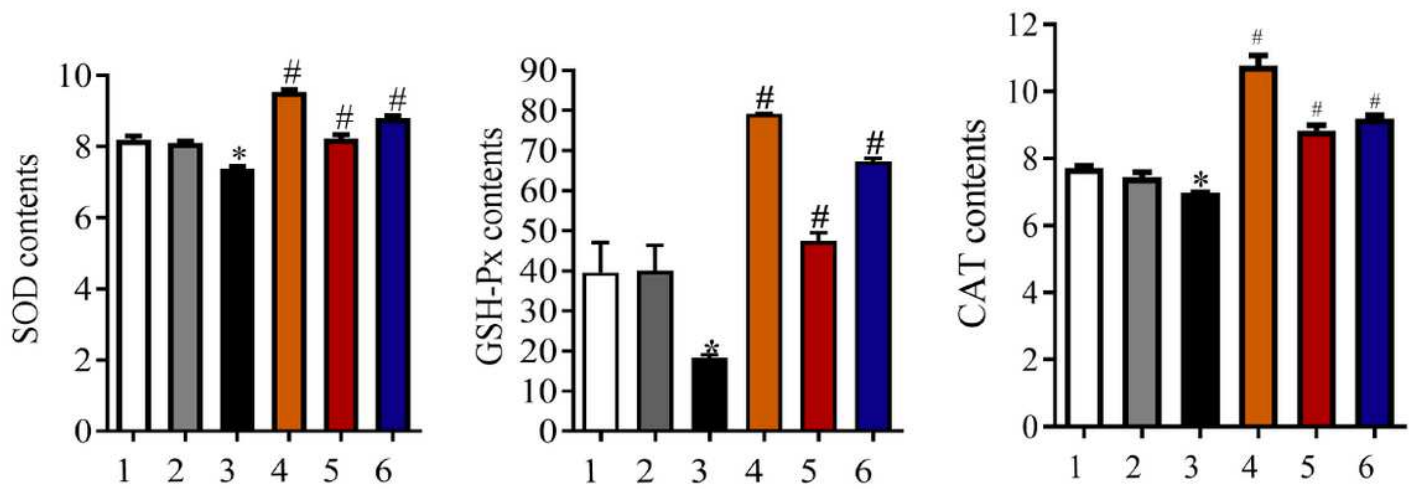


Figure 5

10,50 μ M SOG significantly increased the SOD, CAT and GSH-Px contents in H9c2 cells compared with the ATO group. Use colorimetric method to test the SOD, CAT and GSH-Px contents. The SOD,GSH-Px and CAT enzyme contents of ATO group were significantly decreased, but after adding 10, 50, 100 μ M SOG were significantly increased. $P<(0.05)^*$ was considered as significantly different from controls, $P<(0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μ M SOG, 3. 10 μ M ATO, 4. 10 μ M ATO+10 μ M SOG, 5. 10 μ M ATO+50 μ M SOG, 6. 10 μ M ATO+100 μ M SOG.

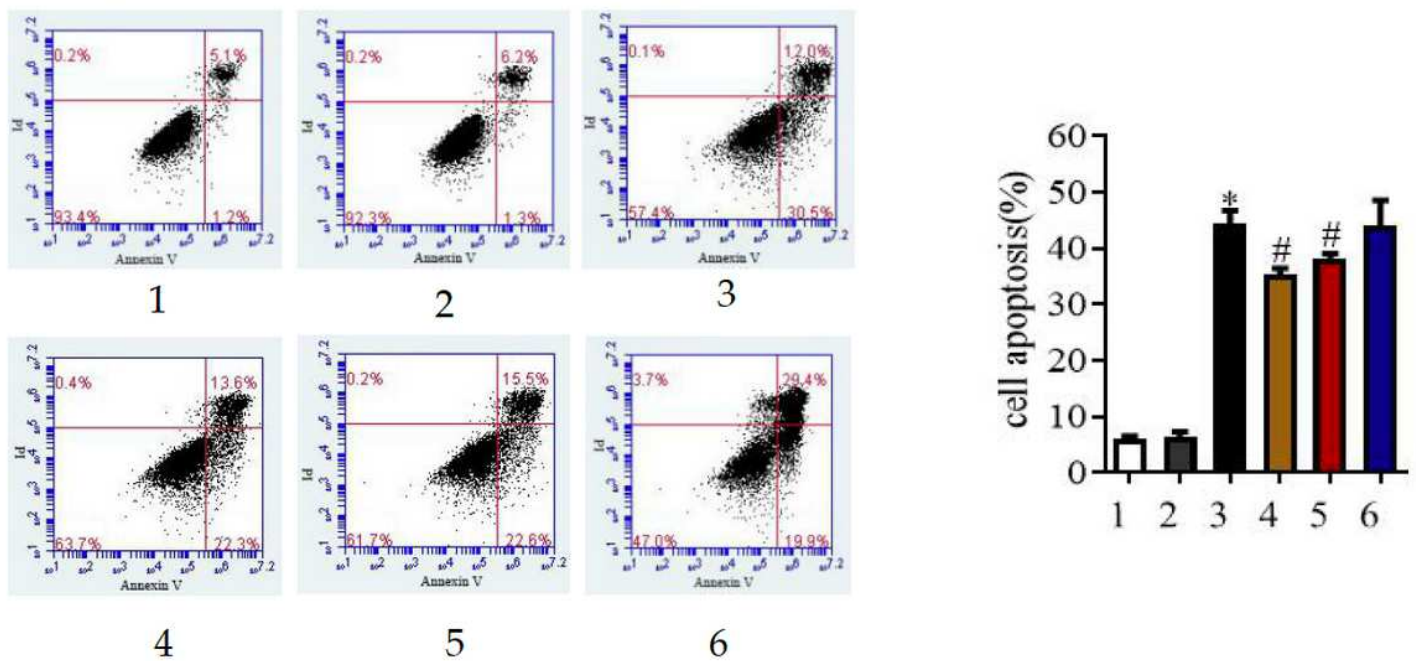


Figure 6

10, 50 μ M SOG significantly decreased the H9c2 cell apoptosis rates compared with the ATO group. Use flow cytometry to test the H9c2 apoptosis. The cell apoptosis rates in ATO group is the highest. After add 10 and 50 μ M SOG the apoptosis rates were significantly lower than the ATO group. $P<(0.05)^*$ was considered as significantly different from controls, $P<(0.05)^{\#}$ was considered as significantly different from ATO group. Test group: 1. Control, 2. 100 μ M SOG, 3. 10 μ M ATO, 4. 10 μ M ATO+10 μ M SOG, 5. 10 μ M ATO+50 μ M SOG, 6. 10 μ M ATO+100 μ M SOG.

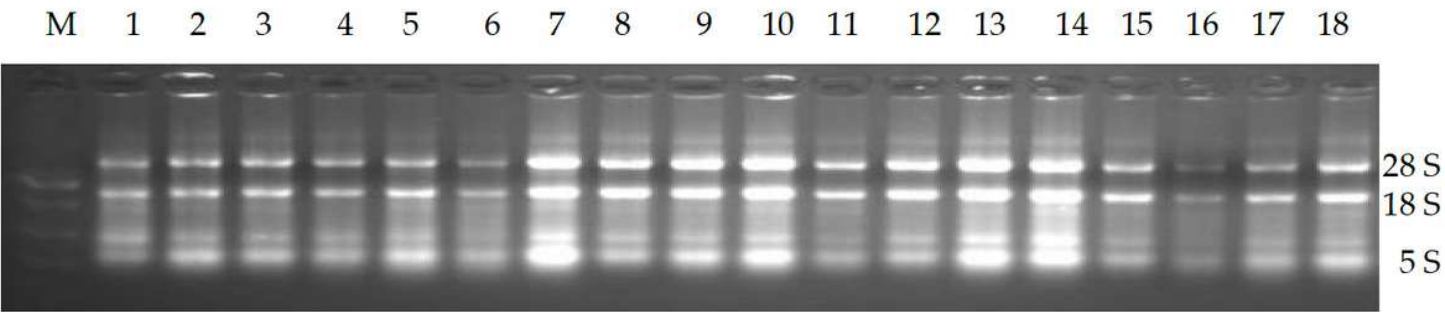


Figure 7

The RNA integrity was confirmed by agarose gel electrophoresis. The resluts show that the RNA was complete,because they all have three bands, respectively were 5s, 18s and 28s. M:Marker, 1-6, 7-12, 13-18 respectively show as Control, 100 μ M SOG, 10 μ M ATO, 10, 50, 100 μ M SOG+10 μ M ATO in Nrf2, HO-1, NQO1 mRNA.

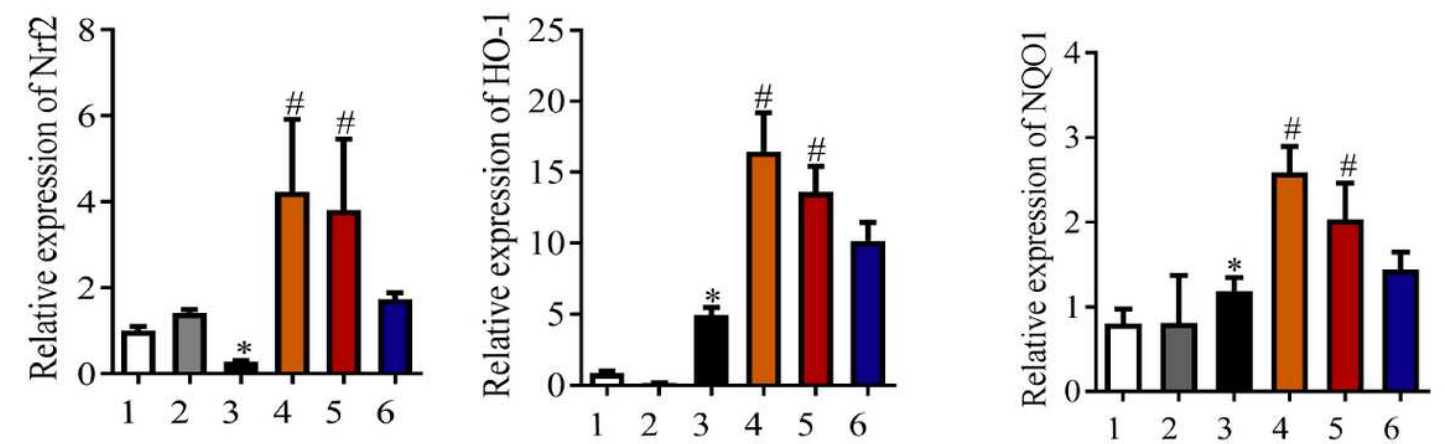


Figure 8

10,50 μ M SOG significantly increased the Nrf2, HO-1 and NQO1 mRNA expression levels compared with the ATO group. Use RT-PCR test the effects of SOG and ATO on the expression of HO-1, Nrf2 and NQO1 mRNA in H9c2 cells. The HO-1 and NQO1 mRNA expression levels of ATO group were significantly increased than control group, and the Nrf2, HO-1 and NQO1 mRNA expression levels of the 10, 50 μ M SOG groups were significantly higher than the ATO group. $P<(0.05)^*$ was considered as significantly different from controls, $P<(0.05)^{\#}$ was considered as significantly different from ATO group. Test group:

1. Control, 2. 100 μ M SOG, 3. 10 μ M ATO, 4. 10 μ M ATO+10 μ M SOG, 5. 10 μ M ATO+50 μ M SOG, 6. 10 μ M ATO+100 μ M SOG.

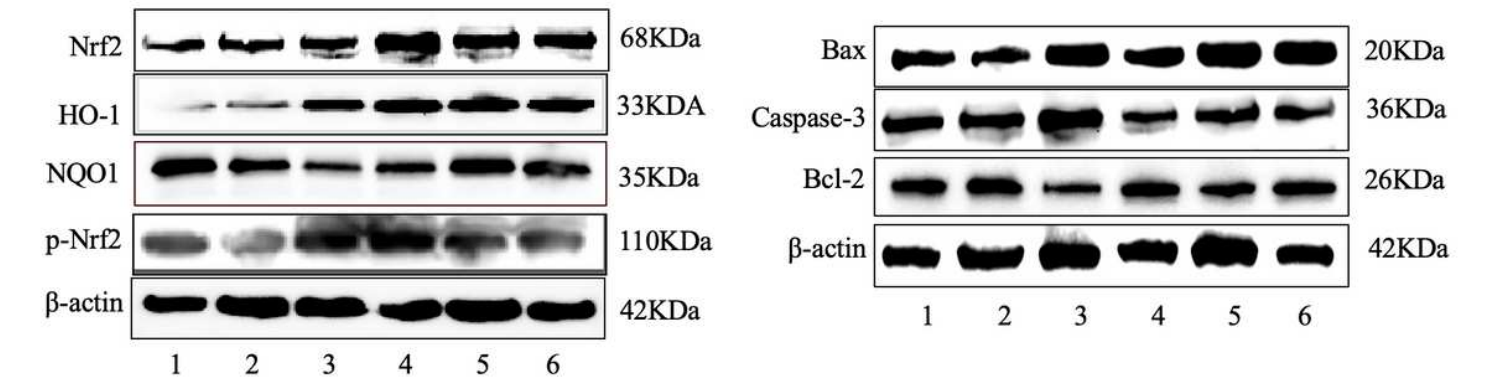


Figure 9

Use WB to test the effects of SOG and ATO on the expression levels of Nrf2, HO-1, NQO1, p-Nrf2, Bcl-2, Bax and Caspase-3 protein in H9c2 cells. Test group: 1. Control, 2. 100 μ M SOG, 3. 10 μ M ATO, 4. 10 μ M ATO+10 μ M SOG, 5. 10 μ M ATO+50 μ M SOG, 6. 10 μ M ATO+100 μ M SOG.

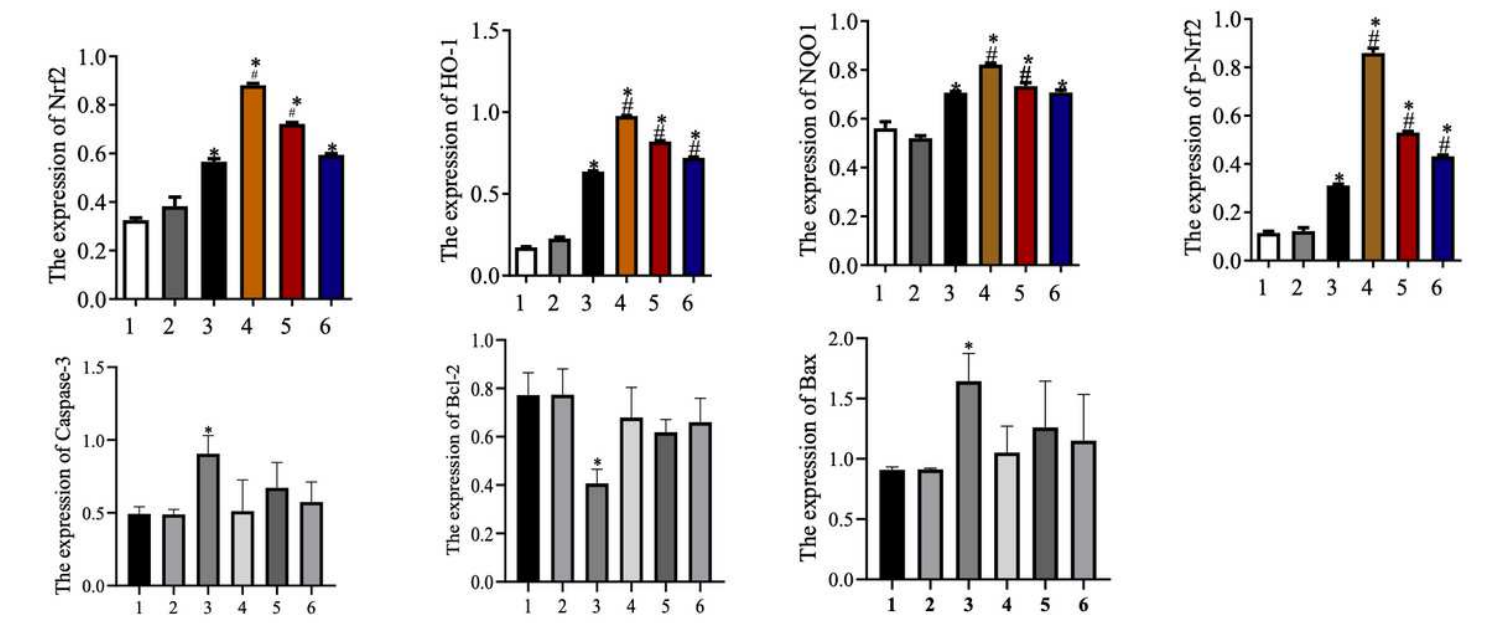


Figure 10

After adding 10,50 μ M SOG the Nrf2, HO-1, NQO1 and p-Nrf2 protein expression levels were significantly increased compared with the ATO group. Using the imageJ software to analyse the results of WB. The Nrf2, HO-1, NQO1 and p-Nrf2 protein expression levels of ATO group were significantly increased than control group, and after adding 10, 50 μ M SOG, the Nrf2, HO-1 and NQO1 protein expression levels were significantly higher than the ATO group. $P < (0.05)$ * was considered as significantly different from controls, $P < (0.05)$ # was considered as significantly different from ATO group. Test group: 1. Control, 2.

100 μ M SOG, 3. 10 μ M ATO, 4. 10 μ M ATO+10 μ M SOG, 5. 10 μ M ATO+50 μ M SOG, 6. 10 μ M ATO+100 μ M SOG.

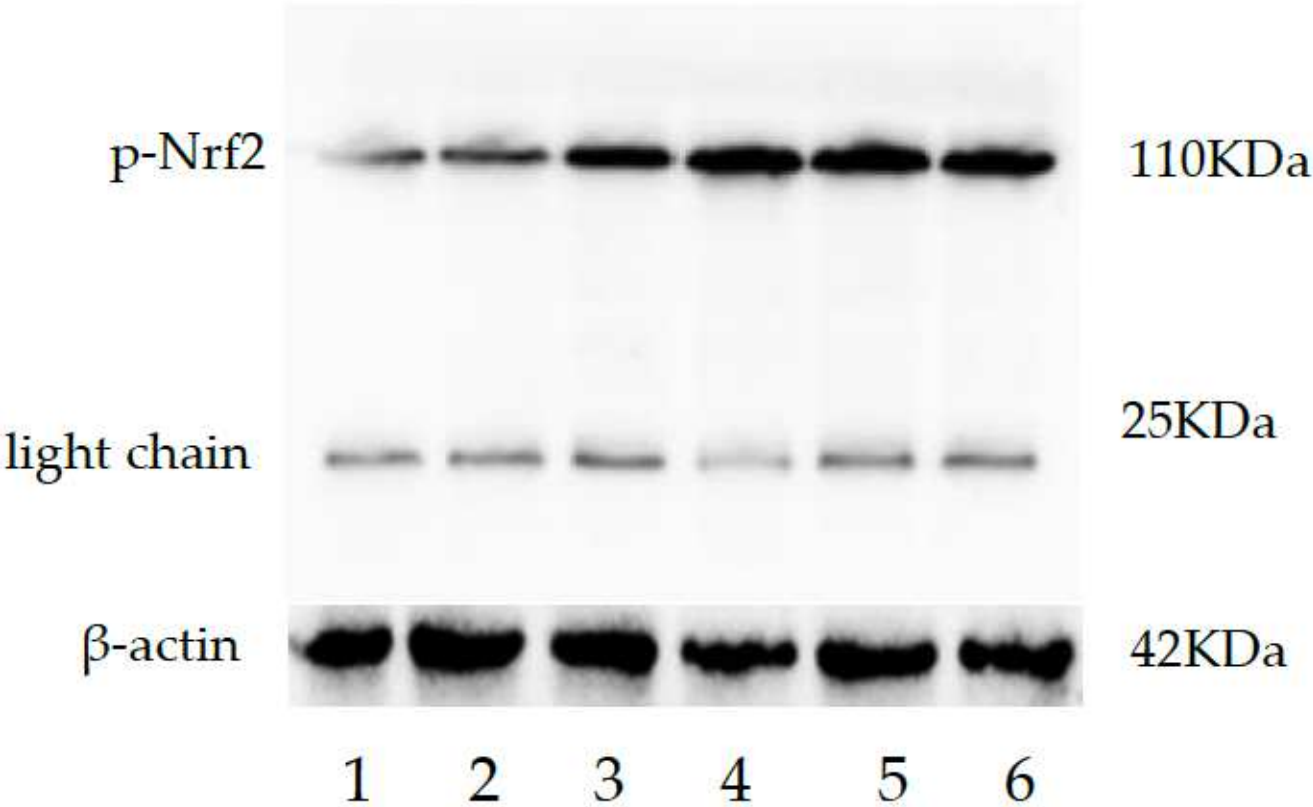


Figure 11

Use IP to test the effects of SOG and ATO on the expression levels of p-Nrf2 protein in H9c2 cells. Test group: 1. Control, 2. 100 μ M SOG, 3. 10 μ M ATO, 4. 10 μ M ATO+10 μ M SOG, 5. 10 μ M ATO+50 μ M SOG, 6. 10 μ M ATO+100 μ M SOG. When antibodies degrade, they produce light chains of 25KDa.

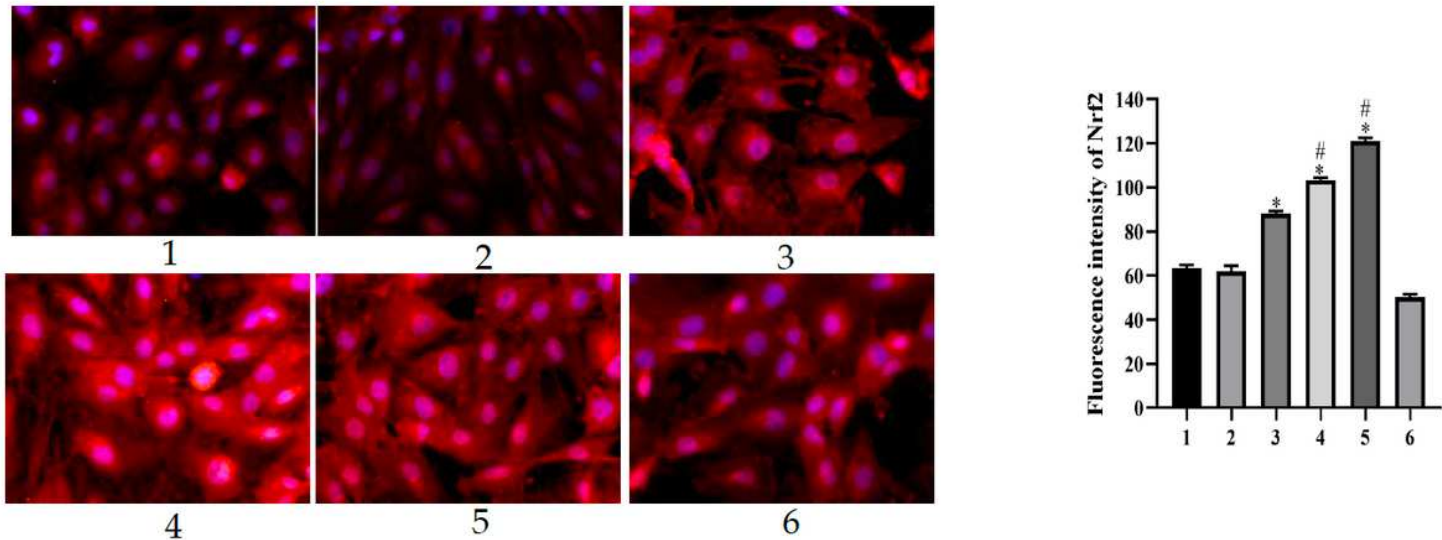


Figure 12

10,50 μM SOG significantly increased the Nrf2 protein expression levels were compared to the ATO group. Using indirect immunofluorescence(400 $\times$) method to test the Nrf2 fluorescence intensity. The red colour is Nrf2 protein, the blue colour is the cell nucleus(Hoechst33258). The Nrf2 protein expression of ATO group were significantly increased than control group, and after adding 10, 50 μM SOG, the Nrf2 protein expression were significantly higher than the ATO group. 1,Control, 2 100 μM SOG, 3 10 μM ATO, 4 10 μM ATO+10 μM SOG, 5 10 μM ATO+50 μM SOG, 6 10 μM ATO+100 μM SOG.

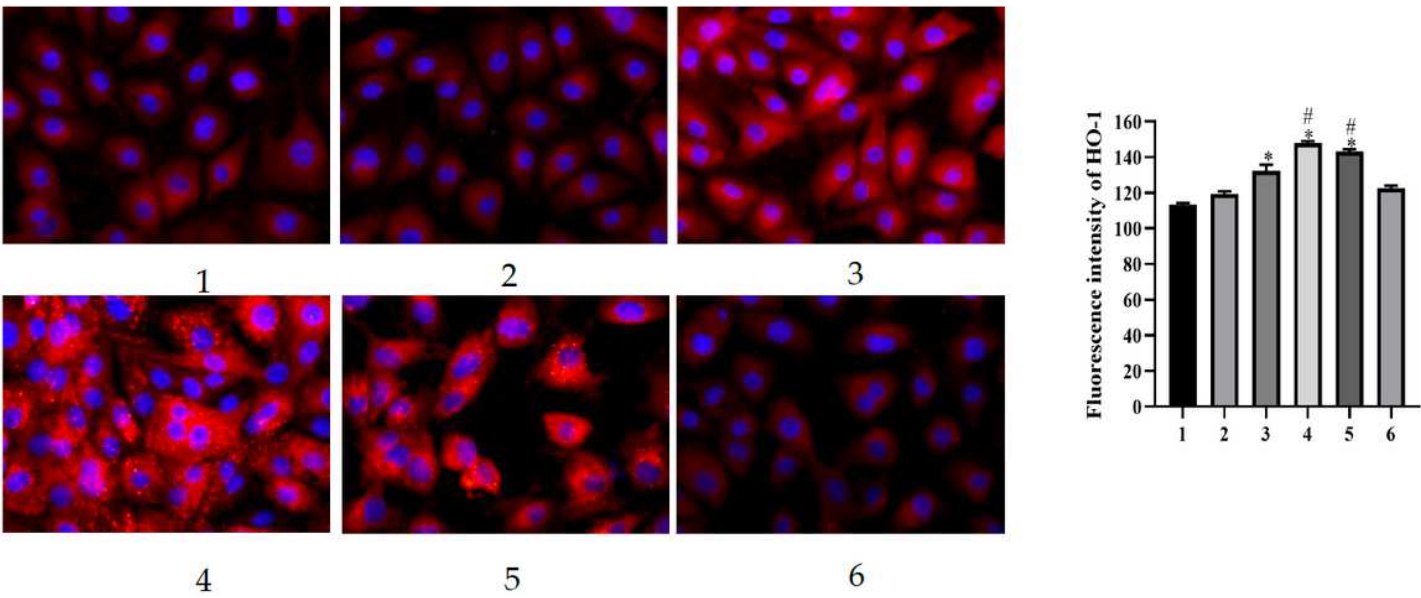


Figure 13

10,50 μM SOG significantly increased the HO-1 protein expression levels were compared to the ATO group. Using indirect immunofluorescence(400 $\times$) method to test the HO-1 fluorescence intensity. The red colour is Nrf2 protein, the blue colour is the cell nucleus(Hoechst33258). The HO-1 protein expression of ATO group were significantly increased than control group, and after adding 10, 50 μM SOG, the HO-1 protein expression were significantly higher than the ATO group. 1. Control, 2 100 μM SOG, 3. 10 μM ATO, 4 ATO+10 μM SOG, 5 ATO+50 μM SOG, 6 ATO+100 μM SOG.