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Simvastatin inhibits the proliferation of A549 lung cancer cells through oxidative stress and up-regulation of SOD2

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Beyond lipid-lowering effect, statins, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase competitively inhibitors, have recently attracted wide concern on their anticarcinogenic properties in various cancer cells. However, the molecular mechanisms by which statins inhibit cancer cell proliferation remain unclear. In this study, we investigated the effect of simvastatin on cells proliferation and oxidative stress in the A549 lung cancer cells and the mechanisms underlying. MTT assay revealed that simvastatin inhibited the proliferation of A549 cells in a concentration- and time-dependent manner. Treatment with 50 μM simvastatin for 48 h significantly increased reactive oxygen species (ROS) production and malondialdehyde (MDA), a lipid peroxidation production, and augmented the activity of total superoxide dismutase (SOD) and manganese SOD (SOD2). Moreover, western blotting analysis showed that simvastatin effectively up-regulated the SOD2 relative protein level. These findings suggested that simvastatin could inhibit the proliferation of A549 lung cells through oxidative stress and up-regulation of SOD2.

1. Introduction

Statins are first line medications for the treatment of hypercholesterolemia in the clinic, due to their efficacy in reducing serum cholesterol and low-density lipoprotein with low side effects (Endo 1976; Hu et al. 2010). In addition to their lipid-lowering effect, studies have shown that statins also had non-lipid-lowering effects including inhibit proliferation, invasiveness and apoptosis of carcinomas cells (Wang and Hu 2010; Xing et al. 2010). As potential anticancer drugs, statins' anticarcinogenic properties in various cancer cells and the related mechanisms have attracted wide concern. It has been shown that in Caco-2 colon cancer cells, statins could induce the cell cycle arrest in the G0/G1 phase and G2/M phase, accompanied by decreased expression of the cyclin-dependent kinase (cdk) 2, cdk 4 and cdk 6. In addition, statins could increase expression of the cell cycle inhibitors p21 and p27 (Wächtershäuser et al. 2001). It had been reported that statins induced apoptosis in HCT116, SW480, LoVo, and HT29 colon cancer cells through down-regulating the expression of the antiapoptotic protein bcl-2 and up-regulating the proapoptotic protein bax expression (Agarwal et al. 1999). In 2008, it was reported that statins could significantly inhibit the proliferation of human Michigan cancer foundation-7 (MCF-7), in which the oxidative stress is involved (Sánchez et al. 2008).

Oxidative stress is associated with increased generation of ROS which is a second messenger and participates in the intracellular oxidative stress signal pathways (Valko et al. 2006). MDA is a natural production of lipid oxidation under oxidative stress conditions. Detection the degree of MDA has been used to evaluate the level of lipid oxidation and oxidative stress (McGrath et al. 2001). Organism has built-up an effective defense system in the long evolutionary process, such as SOD, glutathione peroxidase (GSH-Px), catalase (CAT) and antioxidants (such as vitamin C, vitamin E) (Dalle-Donne et al. 2003; Uchida et al. 1998). The metal-containing antioxidant enzyme SOD is an important oxidative damage defensive biological enzyme. So far, three isoforms of SOD have been identified: Cu/ZnSOD (SOD1), FeSOD and MnSOD (SOD2). MnSOD plays an important role in keeping the intracellular redox balance. It is the major enzyme for free radical scavenger in mitochondria. Recent studies demonstrated that the MnSOD activity is usually at a low level in most types of cancer, therefore, the reduction of MnSOD activity is always considered as characteristic of cancer (Church et al. 1993; Zhao et al. 2000). Moreover, recent evidence showed that *sod2* also expresses at a lower level in many types of transformed and neoplastic cells, suggesting that loss of SOD2 activity may also be a general characteristic of tumorigenesis (Bravard et al. 1992). Generally, cancer cells mainly survive in a low oxygen concentration environment and they often have a much vulnerable antioxidant defense system. Although numerous studies have revealed that statins could significantly inhibit the proliferation and induce apoptosis of cancer cells, the mechanisms underlying have not been fully studied (Goldstein and Brown 1990; Demierre et al. 2005; Hoque et al. 2008). To further investigate the mechanisms of statins on cancer cells whether are associated with oxidative stress and tumor suppressor gene *sod2*, A549 lung cancer cells were chosen as research model. Vitamin C (Vc), a common reductant, is used to resist oxidation process caused by simvastatin.

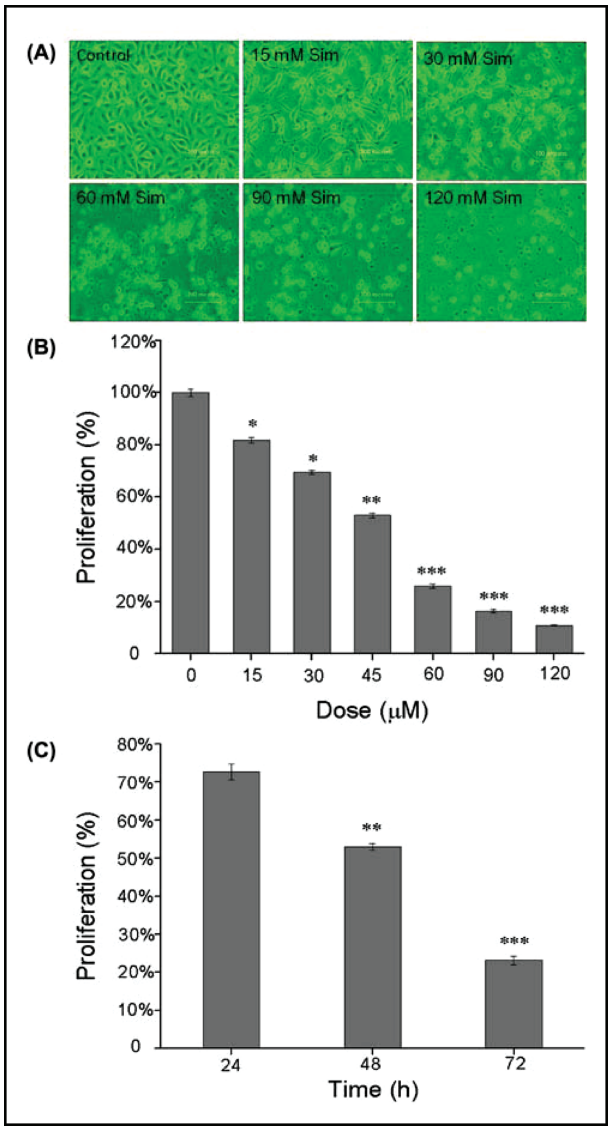

Fig. 1: The inhibitory effect of simvastatin on cell growth of A549 cells Sim is the abbreviation of simvastatin). (A) The morphology effect of simvastatin on A549 cells after 48 h treatment. (B) Cells were treated with the menstruum 0.01% ethanol (Et), 15 -120 μM simvastatin for 48 h, cell viability was then measured by the MTT assay. The viability of control cells was considered as 100%. The results are presented as the mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs*. control). (C) Cells were treated with 45 μM simvastatin for 24, 48 or 72 h. The results are presented as the mean ± SEM (*$p < 0.001$, **$p < 0.01$ *vs*. the group treated with Sim for 24 h).

2. Investigations and results

2.1. Simvastatin inhibits the proliferation of A549 human lung cancer cells

A549 cells cultures were exposed to simvastatin at a concentration from 15 to 120 μM for 48 h. Both the microscopic imaging and MTT assay results suggested that simvastatin at all of these concentrations led to a dose-dependent effect in cell morphology and number of A549cells. From the microscopic images, obvious toxicity was observed from a concentration of 30 μM (Olympus IX71 microscope, Japan) (Fig. 1A). As shown in Fig. 1B, the half inhibitory concentration (IC50) of simvastatin in A549 cells for 48 h was 45 μM which is in consistent with the microscope images. In this concentration, we investigated the inhibitory effect of simvastatin on cell proliferation after 24, 48 and 72 h simvastatin treatment. Fig. 1C shows that simvastatin time-dependently inhibited cell proliferation.

2.2. Simvastatin increases the production of ROS and MDA

When oxidative stress increases, there will be an increase in the activity of antioxidative enzymes and production of ROS to reduce the oxidative damage triggered by stress experienced (Rigas and Sun 2008). In order to examine whether simvastatin could enhance ROS generation, we measured the degree of ROS with fluorescent dye DCFH-DA. As shown in Fig. 2A - C, after treatment with 50 μM simvastatin for 48 h, the ROS level of A549 cells significantly increased compared to the control ($183 \pm 0.31\%$, $p < 0.001$). As Fig. 2D shows, the MDA degree exhibited a significant increase with the incubation of simvastatin ($817 \pm 41.67\%$ *vs*. control, $p < 0.01$). Moreover, the increased MDA production by simvastatin was abolished when cells were treated with simvastatin and Vc simultaneously. The results indicated that the proliferation inhibitory of simvastatin on A549 cells was associated with oxidative stress.

2.3. Simvastatin enhances the activity of total SOD and SOD2

SOD, a peroxidase enzyme, is an important antioxidant defense substance. MnSOD, one of the three SOD isoforms, plays an important role in keeping intracellular redox balance (Zhao et al. 2000). Therefore, we examined and compared the total SOD and SOD2 activities of A549 cells before and after treatment with simvastatin. As Fig. 3A shows, cells treated with 50 μM simvastatin had a higher total SOD activity ($139.8 \pm 5.23\%$ *vs*. control, $p < 0.01$). The SOD2 activity exhibited the same trend and increased to $173.7 \pm 10.11\%$ ($p < 0.01$). Comparing with the total SOD, there was a more obvious up-regulation of SOD2 activity, indicating that SOD2 may be more relevant to the oxidative stress. Vc, an antioxidant, was used to suppress the up-regulation of the total SOD ($23.18 \pm 3.35\%$ *vs*. cells were treated with simvastatin alone, $p < 0.05$) and SOD2 activities ($30.4 \pm 4.12\%$ *vs*. cells treated with simvastatin alone, $p < 0.05$). As expected, by simultaneously incubating with Vc when cells were treated with simvastatin, there was a certain degree of reduction in the activities of both total SOD and SOD2.

2.4. Simvastatin alters the SOD2 expression

In order to further investigate the association between SOD2 and simvastatin, western blotting was used to detect the protein expression of SOD2 in simvastatin-treated A549 cells. We found that the inhibitory effect on cell proliferation induced by simvastatin was accompanied by a 3-fold increase in the SOD2 protein expression ($p < 0.01$). Vc inhibited the up-regulation of SOD2 protein expression ($20.56 \pm 0.03\%$ *vs*. cells were treated with sim alone, $p < 0.05$) (Fig. 4).

3. Discussion

The present study indicated that simvastatin inhibited the proliferation of A549 lung cancer cells through up-regulating oxidative stress metabolites and the expression of SOD2.

Recent studies showed that cancer cells survived mostly in an environment with low ambient oxygen, and their antioxidant defense system was more fragile than that of normal cells. Therefore, most anticancer agents may play a role in cancer inhibition mainly through modulating the generation of reactive oxygen and free radicals with mitochondrial dysfunction, ROS production and the autophagic destruction of mitochondria (Martinez-Outschoorn et al. 2010; Jorgenson et al. 2013). Several studies have reported that the stromal tumor

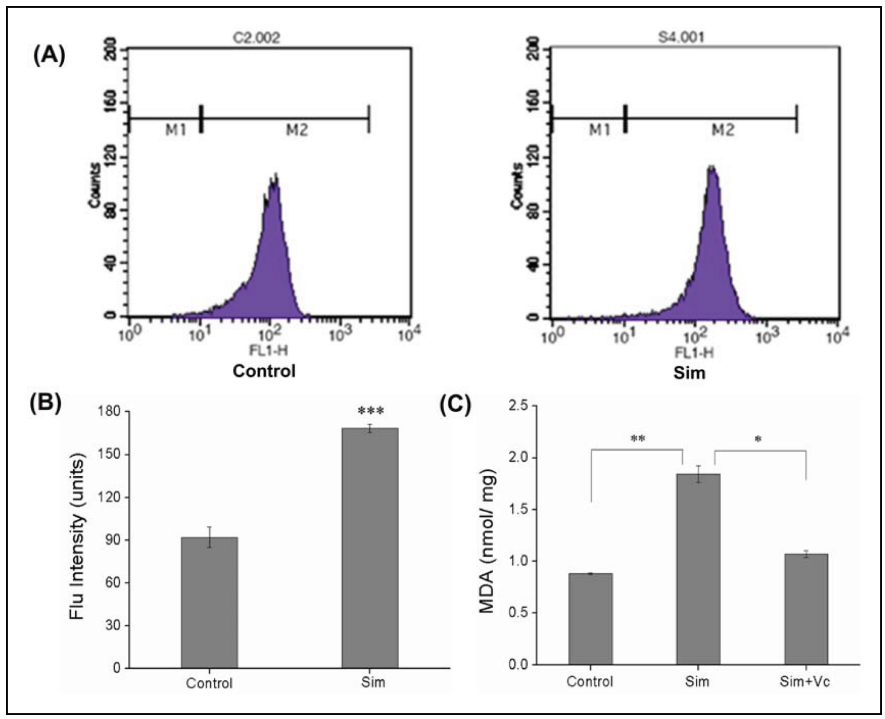

Fig. 2: Effect of simvastatin and Vc on ROS and MDA prodution. (A) The fluorescence intensity of the control group which A549 cells were treated with the menstruum 0.01% Et and the Sim group which was treated with 50 μM Sim for 48 h was measured by flow cytometer. (B) The flu intensity ratio of the group treated with Sim compared to the control. The results are presented as the mean ± SEM (***$p < 0.001$ *vs*. control). (C) The ratio of MDA level compared to the control. After treated with 0.01% Et, 50 μM Sim with the presence of 60 μM Vc or not in A549 cells for 48 h, measuring the level of MDA. The results are presented as the mean ± SEM (**$p < 0.01$ *vs*. control, *$p < 0.05$ *vs*. the group treated with Sim alone).

suppressor gene *sod2* had a potential regulation effect on cancer cells through lowering oxidative stress in the tumor microenvironment (Bravard et al. 1992; Trimmer et al. 2011). Recent studies also showed that *sod2* gene polymorphisms was associated with gastric cancer, prostate cancer, lung cancer, tongue squamous cells carcinoma in clinical (Xu et al. 2012; Han et al. 2013). Moreover, statins could inhibit cancer cell proliferation via influencing the isoprenylation of small G proteins Ras or Rho (Khanzada et al. 2005). Another study reported that statins inhibited tumor growth and angiogenesis through inhibiting tumor necrosis factor (TNF-α) (Feleszko et al. 1999). Therefore, investigation of the exact mechanisms that statins can show some anticancer effects and the related SOD2 expression manner is an essential work.

The present study demonstrated a dose- and time-dependent inhibitory effect of simvastatin on A549 cell proliferation. This was consistent with Hitchler's study which found that fluvastatin had similar inhibitory effect on renal carcinoma cells (Hitchler and Domann 2009). Under the conditions of oxidative stress, the degree of ROS and MDA in the body will increase. Simultaneously, the activity of SOD which is an important peroxidase and *in vivo* antioxidant defense substances will increase (Dalle-Donne et al. 2003). Our study suggests an important association between cancer inhibition of simvastatin and oxidative stress. After treated with 50 μM simvastatin, the ROS and MDA degrees and SOD activity of A549 was significantly upregulated. SOD2, a main enzymatic free radical scavenger in mitochondria, played an important role in keeping the balance of intracellular redox (Jorgenson et al. 2013). Our results showed that the effects of simvastatin on the activity and the expression of SOD2 were similar compared to its effect on the total SOD activity. Therefore, the inhibitory effect of simvastatin on cell proliferation was not only related to oxidative stress but also involved in transcriptional regulation of tumor suppressor gene *sod2*.

In order to further investigate the relationship between inhibition of A549 cells proliferation and oxidative stress, antioxidant Vc was chosen to relieve the up-regulation mentioned above. Vc significantly attenuated the up-regulation of SOD2 expression induced by simvastain treatment. The results further indicated that the inhibition of simvastatin on A549 cells proliferation was related to oxidative stress.

To our knowledge, this is a prior report that provides definite evidence for the association between statins-regulated the cell proliferation and SOD2 expression in A549 cells. The latest study showed a significant association between the epigenetics and *sod2* alteration in human cancer (Cyr et al. 2013). As a potential anticancer drug, simvastatin inhibits the proliferation of A549 lung cancer cells through oxidative stress and upregulation of SOD2, but the specific mechanisms with epigenetic modification and signaling pathways need to be further clarified. The inhibitory effect on cancer cell proliferation through changing cancer cell survival micro-environment is an effective way for anti-cancer drug screening.

4. Experimental

4.1. Materials

A549 human lung cancer cells were preserved in our lab. RPMI-1640 and fetal bovine serum (FBS) were obtained from Hyclone Co., Ltd (North America). Simvastatin was purchased from Dalian Meilun Biotech Co., Ltd (China). Dimethyl sulfoxide (DMSO), 2',7'-dichlorofluorescein diacetate fluorescent yellow (DCFH-DA) were purchased from Sigma-Aldrich Chemie GmbH (America). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), RIPA Lysates and 1% 0.5 mM PMSF were purchased from Beijing Dingguo Changsheng Biotech Co., Ltd (China). Protein Assay Kit was purchased from Beijing Transgen Biotech Co., Ltd (China). Commercially available SOD and MDA assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (China). Primary antibody against SOD2 was obtained from Epitomic, Inc. (America). β-Actin was obtained from Beijing Bioss Biotech Co., Ltd (China). Anti-rabbit

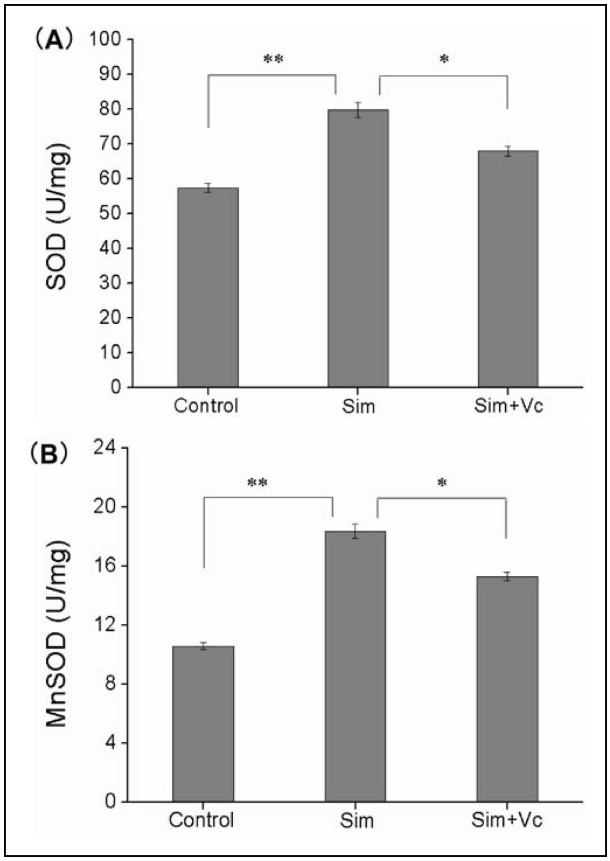

Fig. 3: The effect of simvastatin and Vc on total SOD and MnSOD activity in A549 cells. (A) After cells were treated with Sim and Vc, alone and in combination, for 48 h, the activity of total SOD and MnSOD was measured. The ratio of total SOD activity compared to the control. The results are presented as the mean ± SEM (**$p < 0.01$ *vs*. control, *$p < 0.05$ *vs*. the group treated with Sim alone). (B) The ratio of MnSOD activity compared to the control. The results are presented as the mean ± SEM (**$p < 0.01$ *vs*. control, *$p < 0.05$ *vs*. the group treated with Sim alone).

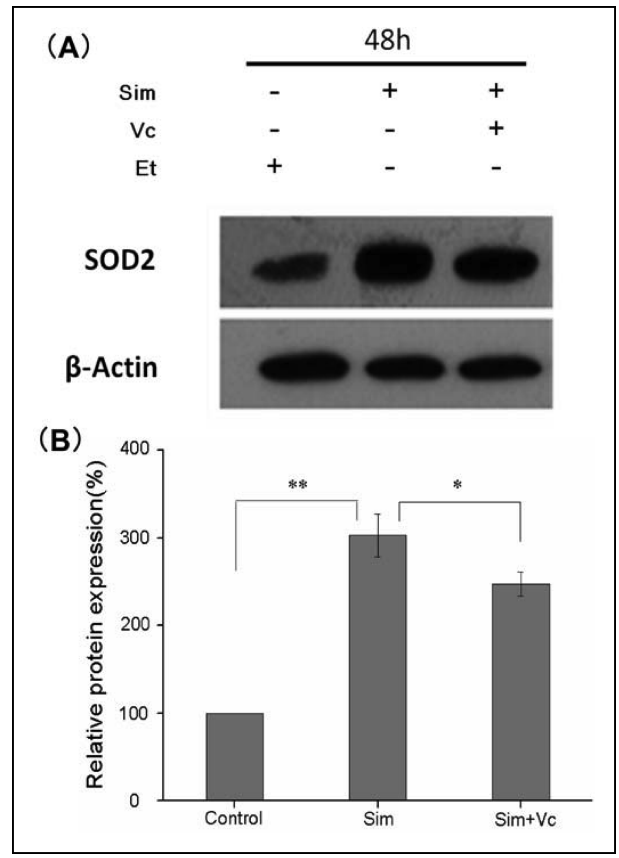

Fig. 4: Western blotting for SOD2 protein expression in A549 cells. (A) Cells were pretreated with the menstruum 0.01% Et, 50 μM Sim without or with 60 μM Vc for 48 h, then the cell lysate was subjected to 12% SDS-PAGE to measure the expression of SOD2. The β-Actin was used as a loading control. (B) The relatively SOD2 protein expression level. The results are presented as the mean ± SEM (**$p < 0.01$ vs. control, *$p < 0.05$ vs. the group treated with Sim alone).

IgG-conjugated horseradish peroxidase (HRP) antibody was purchased from Cell Signaling Technology, Inc. (America). Enhanced chemiluminescent (ECL) kit was purchased from Gen-view Scientific, Inc. (America).

4.2. Cell culture and viability test

A549 lung cancer cells were grown in 5% CO_2 incubator at 37 °C and maintained in RPMI-1640-contataining FBS. Culture medium was changed twice weekly. Cell viability was determined using MTT assay. Cells were grown in 96-well plates and treated with simvastatin at 15, 30, 45, 60, 90 and 120 μM respectively, culture medium as a control. After 48 h, 5 mg/mL of MTT was added to each well, and the plates were incubated at 37 °C for 4 h. After the medium was removed, the insoluble formazan product was dissolved in 150 μL DMSO. The absorbance at 490 nm of each well was then measured using a microplate reader (Bio-Rad Model 680, America). The percentage of the absorbance of the treated cells compared to the control cells was used to express the viability.

4.3. Measurement of MDA and activity of SOD

A549 cells in log phase were incubated with 50 μM simvastatin for 48 h followed by collected by 0.25% Trypsin-EDTA solution and washed three times in ice-cold PBS (pH 7.4). Cells were cleaved by sonication (power, 300w; crushed 25 s; paused 25 s). The supernatant was collected after centrifugation at 12,000 *g for 15 min, and the protein concentration was determined using Protein Assay Kit. The estimation of SOD and MDA was performed according to manufacturers' protocol.

4.4. Measurement of ROS by flow cytometry

A549 cells in log phase were treated with 50 μM simvastatin in 12-well plates for 48 h, then cells were collected, and washed gently in serum-free 1640 medium for three times. The supernatant was removed after centrifugation at 1,000 *g for 10 min, then added 1640 medium to obtain cell suspension. Cells were analyzed on a flow cytometer FACSVantage SE (BD Biosciences, America) after stained with DCFH-DA at 37°C and light-sealed environment for 30 min.

4.5. SDS-PAGE and Western blotting analysis

A549 cells were washed with PBS twice and then 0.25% Trypsin-EDTA solution was added followed by centrifugation at 1000 *g for 5 min. 300 μL RIPA Lysates and 1% 0.5 mM PMSF were added into the remaining precipitation. All samples were gently shaked at 4 °C for 30 min. The lysate was centrifuged at 12,000 *g at 4 °C for 10 min and supernatant was stored at − 80 °C.

Protein concentrations were determined using Protein Assay Kit. Protein samples were separated by SDS-PAGE and then transfered to PVDF membrane at 4 °C for 30 min. PVDF membrane was incubated with 5% non-fat milk in TBST (0.1% Tween 20 in Tris-buffered saline) for 1 h. After washed with TBST for three times, the membrane was incubated with the primary antibody at 4°C overnight. Then the membrane was washed three times with TBST and incubated with secondary antibody at room temperature for 1 h. The ECL kit standard protocol was used for detection the protein expression of SOD2 and the loading control β-actin. The band intensities were analyzed and quantitated with Quantity One software (Bio-Rad, USA).

4.6. Statistical analysis

The statistics were performed by the SPSS 19.0 program (Chicago, IL, USA). Each independent experiment had been repeated at least three times, and the data were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test for multiple comparisons. All results were presented as the mean ± SEM (x ± s), and the criterion for statistical significance was $P < 0.05$.

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