Guangzhou Research Institute of Snake Venom, Guangzhou Medical University, Guangzhou, PR China

# High dose of epigallocatechin-3-gallate inhibits proliferation and induces apoptosis of H9C2 cardiomyocytes through down-regulation of SIRT1

YI CAI, LI ZHAO, YUAN QIN, YANHUAN HE

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*Yi Cai, Ph.D, Guangzhou Research Institute of Snake Venom, Guangzhou Medical University, 195 Dongfengxi Road, Guangzhou 510182, Guangdong Province, PR China 510180*
*yicaisysu@163.com*

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*Background:* Previous studies have suggested that high doses of (-)-epigallocatechin-3-gallate (EGCG) can induce toxicity in the liver, kidneys, and intestine. However, there have been no reports of myocardiotoxicity following treatment with EGCG. In this study, we investiged the proliferation and apoptosis of H9C2 cardiomyocytes treated with high dose of EGCG. *Methods:* Cell proliferation was measured by CCK8 assay, cell apoptosis rate was evaluated by TUNEL assay, and the expression alterations of Sirtuin 1 (SIRT1) protein was detected by Western blotting. *Results:* EGCG inhibits proliferation and induces apoptosis in time- and dose-dependent manner in H9C2 cardiomyocytes. SIRT1 participates in the inhibitory effect of EGCG on cell proliferation and apoptosis induction in H9C2 cardiomyocytes. *Conclusion:* This study demonstrates that high doses of EGCG inhibit proliferation and induce apoptosis in H9C2 cardiomyocytes. Down-regulation of SIRT 1 protein expression may be involved.

## 1. Introduction

(-)-Epigallocatechin-3-gallate (EGCG) is the major and most active catechin found in green tea (*Camellia sinensis, Theaceae*) (Krishnan et al. 2014; Zhang et al. 2014). The health benefits of green tea and EGCG have been extensively studied, and previous studies suggest that they might be useful in the prevention and treatment of several chronic diseases including cardiovascular diseases, cancer, and neurodegenerative diseases (Wolfram 2007; Zhou et al. 2014). Because of its wide-spread and long use, it is considered to be safe. Unfortunately, some reports of adverse effects associated to the consumption of high doses of green tea preparations have been published. Oral administration of Teavigo (a green tea polyphenol preparation containing 90% EGCG) to Beagle dogs resulted in dose-dependent toxicity and death. Moreover, oral administration of 2000 mg/kg, i.g. Teavigo to rats resulted in 80% mortality, and histological analysis revealed hemorrhagic lesions in the stomach and intestine (Isbrucker et al. 2006). In addition, high oral doses of EGCG would induce hepatotoxicity in mice, and this toxicity was related to EGCG-mediated pro-oxidant effects (Lambert et al. 2010). Taken together, these data suggest that high doses of EGCG can induce toxicity in the liver, kidneys, and intestine. However, to the best of our knowledge, there have been no reports of myocardiotoxicity following treatment with EGCG, and very little is known about potential targets for EGCG-induced myocardiotoxicity.

EGCG has been demonstrated to affect a number of cellular proliferation and survival molecules, such as Sirtuin 1 (SIRT1) (Ye et al. 2012). SIRT1, also known as NAD-dependent deacetylase, belongs to class III histone deacetylases and is a member of the silent information regulator (Sir2) family (Jiang et al. 2012). SIRT1 plays a pivotal role in a wide variety of cellular processes such as proliferation, apoptosis, and gene transcription (Du et al. 2014; Nihal et al. 2014). SIRT1 activation has been found to protect cell survival against oxidative stress in diverse cell populations (Alcendor et al. 2007; Di Emidio et al. 2014; Ou et al. 2014). However, whether EGCG regulates SIRT1 in H9C2 cardiomyocytes and whether those actions are related to EGCG-induced myocardiotoxicity has not been studied.

In the present study, we hypothesized that high doses of EGCG would inhibit proliferation and induce apoptosis in H9C2 cardiomyocytes, and this effect was related to EGCG-mediated down-regulation of SIRT1.

## 2. Investigations and results

### *2.1. EGCG inhibits the cell viability of H9C2 cardiomyocytes*

We first examined the effect of EGCG on the cell viability of H9C2 cardiomyocytes. H9C2 cardiomyocytes were incubated with increasing concentrations of EGCG for 24 h, and the cell viability was assessed by CCK8 assay. As shown in Fig. 1A, EGCG up to 5 μM did not alter the cell viability, but at higher concentrations, EGCG was cytotoxic to H9C2 cardiomyocytes. Figure 1B shows that 50 μM EGCG also inhibited cell viability in a time-dependent manner after 24 h of treatment.

### *2.2. EGCG induces the cell apoptosis of H9C2 cardiomyocytes*

We next investigated the effect of EGCG on cell apoptosis of H9C2 cardiomyocytes. To test the effects of EGCG on cell apoptosis, H9C2 cardiomyocytes were treated with EGCG for 24 h at concentrations of 1, 5, 10, 25 and 50 μM or with 50 μM EGCG for indicated time points. As shown in Fig. 2 and B, The results showed that EGCG induced the cell apoptosis in a dose- and time-dependent manner.

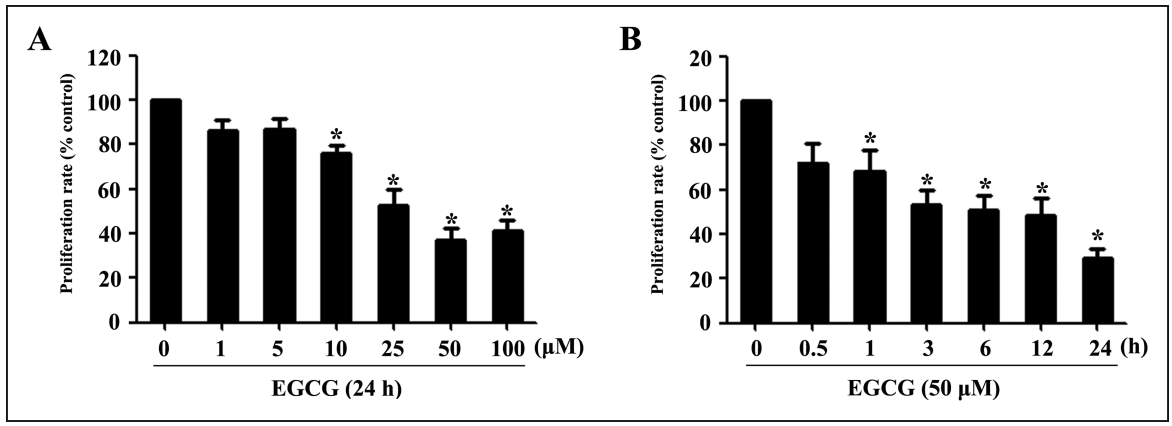


Fig. 1: EGCG inhibits the cell viability of H9C2 cardiomyocytes. (A and B) H9C2 cardiomyocytes were treated with various concentrations of EGCG for 24 h or with 50 μM EGCG for indicated time points. The cell viability was detected by CCK8 assay. *$P < 0.05$ vs. the group without treatment, n = 3.

### *2.3. SIRT1 participates in the inhibitory effect of EGCG on cell proliferation and apoptosis induction in H9C2 cardiomyocytes*

To evaluate a possible involvement of SIRT1, we assessed the protein expression of this key metabolic regulator. As shown in Fig. 3A and B, EGCG dose- and time-dependently decreased the protein expression of SIRT1. To further investigate the role of SIRT1 in the inhibitory effects of EGCG on cell proliferation and apoptosis induction in H9C2 cardiomyocytes, the activity of SIRT1 in H9C2 cardiomyocytes was activated by a selective activator (resveratrol) prior to the treatment with EGCG. Western blotting analysis showed that the expression of SIRT1 was significantly increased after treated with resveratrol as compared with control (Fig. 4A). Then cell viability and apoptosis were measured. As shown in Fig. 4B-D, when SIRT1 was activated by resveratrol, EGCG failed to inhibit proliferation and induce apoptosis in H9C2 cardiomyocytes, which suggested involvement of SIRT1 in the effects of EGCG on H9C2 cardiomyocytes.

## 3. Discussion

Previous studies had reported that EGCG caused toxicity in the liver, kidney and gastrointestinal tract of Beagle dogs when given in high oral doses (Mazzanti et al. 2009). However, to our knowledge, no information is available regarding the impact of EGCG on the cardiovascular system. In the present study, we

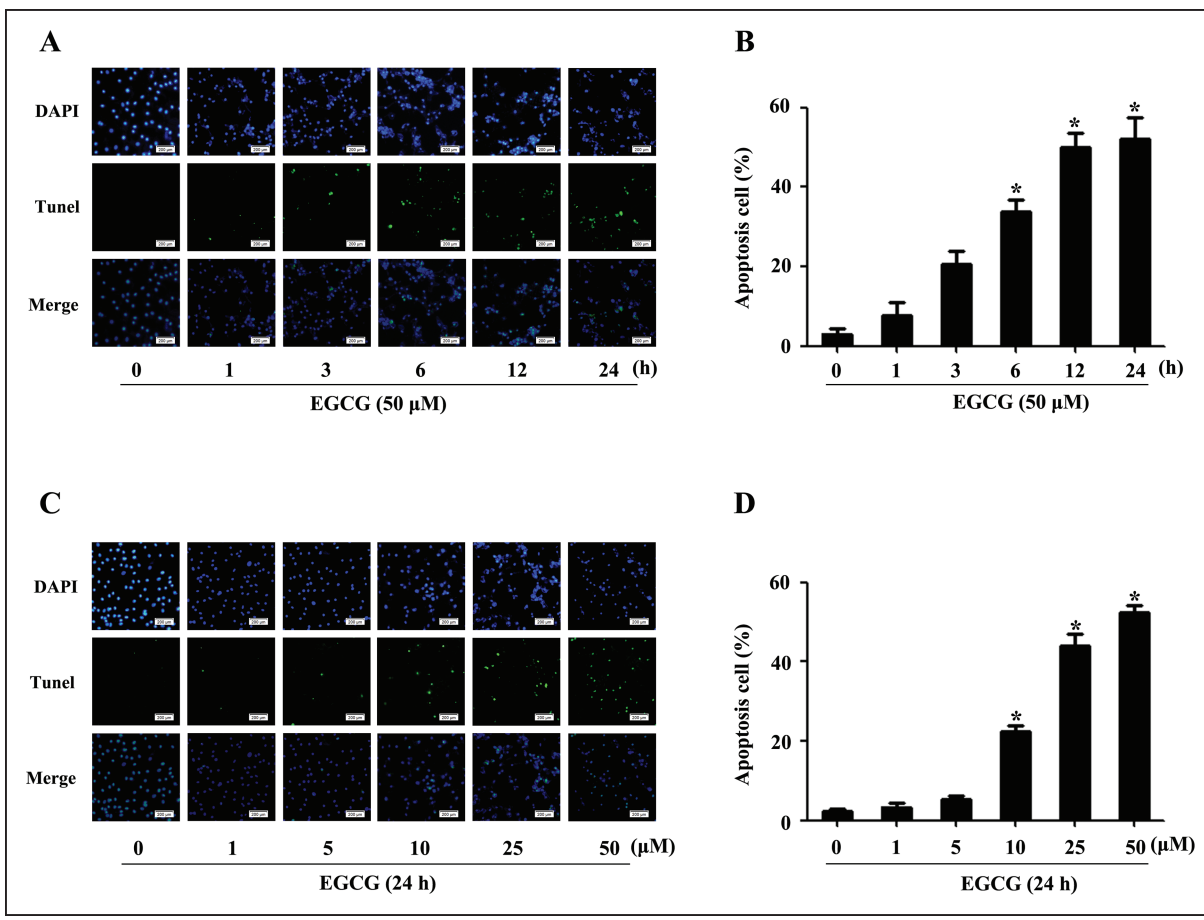


Fig. 2: EGCG induces the cell apoptosis of H9C2 cardiomyocytes. (A and B) H9C2 cardiomyocytes were treated with various concentrations of EGCG for 24 h or with 50 μM EGCG for indicated time points. The cell apoptosis was detected by tunnel assay. *$P < 0.05$ vs. the group without treatment, n = 3.

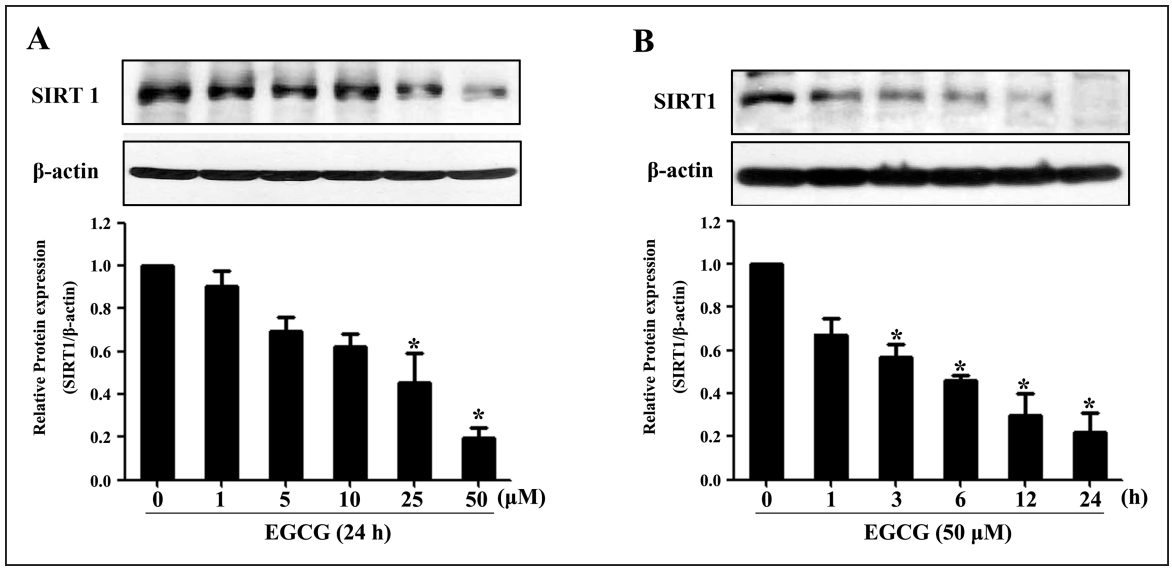


Fig. 3: EGCG inhibits the protein expression of SIRT1 in a time- and dose- dependent manner. (A and B) H9C2 cardiomyocytes were treated with various concentrations of EGCG for 24 h or with 50 μM EGCG for indicated time points. The protein expression of SIRT1 was detected. *$P < 0.05$ vs. the group without treatment, n = 3.

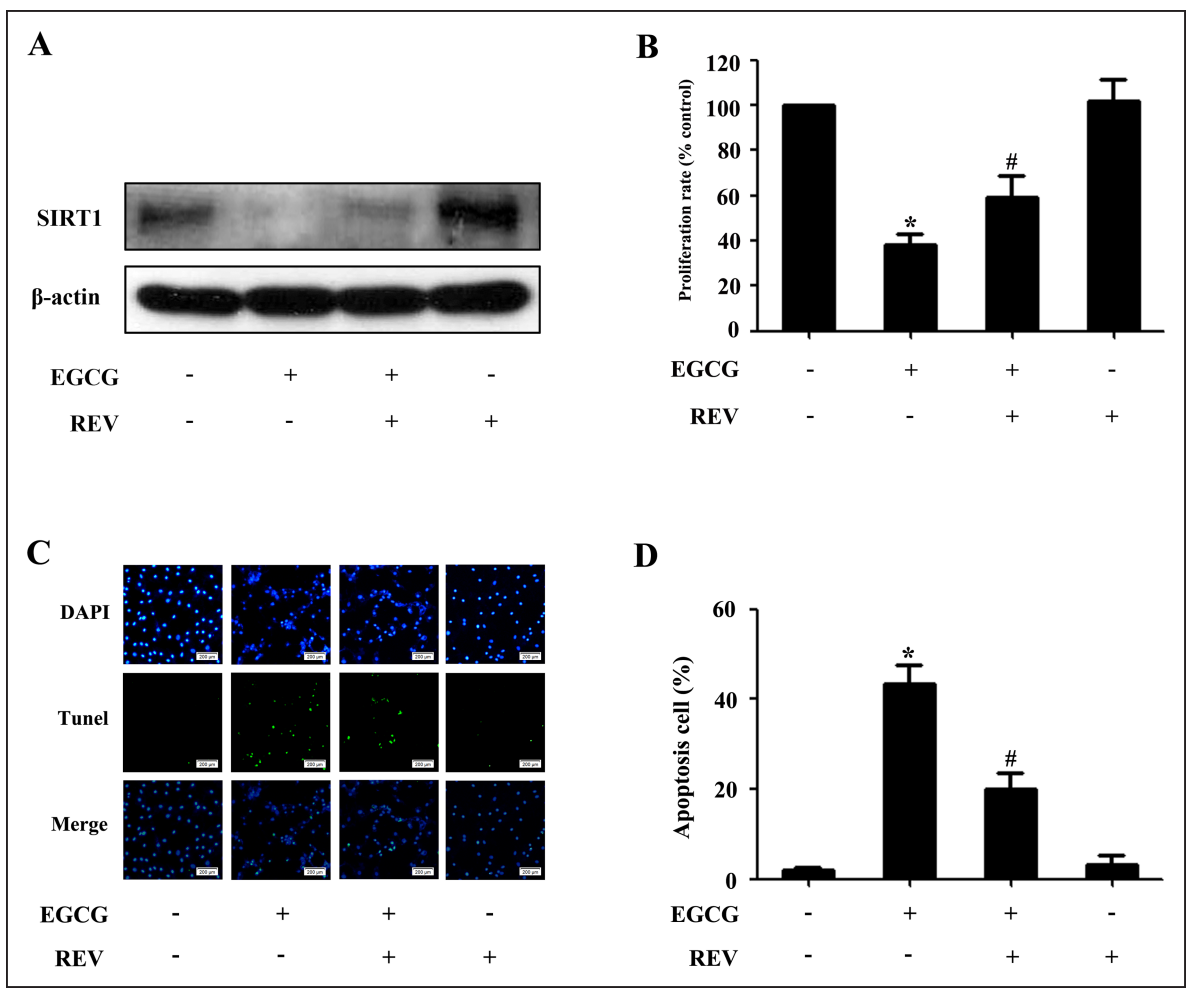


Fig. 4: SIRT1 participates in the inhibitory effect of EGCG on cell proliferation and apoptosis induction in H9C2 cardiomyocytes. (A) H9C2 cardiomyocytes were pretreated with 20 μM Resveratrol for 1 h followed by stimulation with EGCG for 24 h. The protein expression of SIRT1 was detected. The cell viability (B) and apoptosis (C and D) were assessed by CCK8 and tunnel assay respectively. *$P < 0.05$ vs. the group without treatment, #$P < 0.05$ vs. the group treated with EGCG, n = 3.

examined the potential toxicity of high doses of EGCG in H9C2 cardiomyocytes.

Tea is the most consumed beverage in the world, aside from water. Green tea have been found to exert protective effects on the cardiovascular system, including an anti-inflammatory effect, lowering serum cholesterol levels, increasing high-density lipoprotein, and reducing evolving atherosclerosis (Bhardwaj and Khanna 2013; Li et al. 2006; Santesso and Manheimer 2014). The effects of green tea are mainly attributed to its most abundant and biologically active catechin, EGCG.

Although numerous human intervention and bioavailability studies using low to moderate doses of green tea preparations or EGCG have reported no serious side effects. However, laboratory studies of green tea-derived preparations in rodents and dogs have revealed toxic effects at high doses (Isbrucker et al. 2006). Moreover, several cases of hepatotoxicity following the consumption of dietary supplements containing EGCG have been reported (Lambert et al. 2010). Since then, much attention has been given to the possible toxic effects of EGCG. However, little is known about the myocardiotoxicity of EGCG. In this study, we examined the effects of high doses of EGCG on H9C2 cardiomyocytes and found that EGCG could inhibit cell viability and induce apoptosis of H9C2 cardiomyocytes in a time- and dose-dependent manner, which suggested that EGCG exerts toxicity towards H9C2 cell line.

Whereas SIRT1 is an important regulator of energy homeostasis in response to nutrient availability and associated with increased longevity, recent studies have suggested that SIRT1 is an endogenous apoptosis inhibitor in cardiomyocytes (Alcendor et al. 2007). Zhang et al. (2011) found that resveratrol, a potent SIRT1 activator, inhibited doxorubicin-induced cardiomyocyte apoptosis in mice through SIRT1-mediated deacetylation of p53. Previous studies also showed that down-regulation of SIRT1 correlated with apoptosis in palmitate-induced cardiomyocytes and pharmacological activation of SIRT1 reduced apoptosis, whereas inhibition of SIRT1 enhanced apoptosis in response to palmitate (Zhu et al. 2011). These insights convergently suggest that SIRT1 may play a key role in regulation of cardiomyocytes apoptosis. Therefore, we hypothesized that the induction of EGCG on H9C2 cardiomyocytes apoptosis might involve the inactivation of SIRT1. In agreement with previous reports, in the present study, we demonstrated that exposure to EGCG is followed by a significant drop in the protein expression of SIRT1 in H9C2 cell line. Moreover, treatment of H9C2 cardiomyocytes with EGCG leads to a block of proliferation and induction of apoptosis, which is dependent on the down-regulation of SIRT1 expression. These findings suggest that the myocardiotoxic effect of EGCG on H9C2 cardiomyocytes may be at least partially attributed to SIRT1 inhibition.

In summary, we showed that high doses of the green tea polyphenol, EGCG, are toxic for H9C2 cardiomyocytes. This toxicity appears to be related to inhibition of SIRT1 protein expression in H9C2 cell line. However, the cardiomyocytes apoptosis induced by high doses of EGCG can only partially represent the pathogenesis of cardiotoxicity, and further investigations on healthy adult mice are necessary to confirm our conclusions.

## 4. Experimental

### *4.1. Reagents*

EGCG (purity > 95%) was purchased from Sigma Aldrich. Dulbecco's Modified Eagle Medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco (Logan, UT, USA) and Sijiqing Biological Engineering Materials Co Ltd. (Hangzhou, China), respectively. Antibodies against SIRT1 was purchased from Bioworlde (Guangzhou, China), anti-β-actin antibody was purchased from Santa Cruz Biotech (Santa Cruz Biotechnology). EGCG was dissolved in distilled water at 100 μM, and stored at −20 °C until dilution before use.

### *4.2. Cultures of H9C2 cardiomyocytes*

H9C2 rat cardiomyocytes were cultured in DMEM supplemented with 10% FBS. Cell culture media were changed every 2 days. Before EGCG stimulation, H9C2 cells were cultured in DMEM containing 1% FBS for serum starvation.

### *4.3. Cell viability analyses*

Cell viability was measured by Cell counting kit 8 (Dojindo Molecular Technologies, Inc.). H9C2 cells were seeded at $5 \times 10^3$ per well into 96 well microplate and incubated for 24 h peripheral wells of microplate were filled with sterile phosphate buffered saline (PBS). After addition of different concentrations of EGCG respectively, cells were incubated for another 24 h until testing. CCK8 solution (10 μl) was added to each well and the plate was incubated for one additional hour. Finally, the absorbance of each well was measured by BioTek Epoch multi-volume spectrophotometer system (BioTek Instruments Inc., USA) at 450 nm wavelength.

### *4.4. Tunel analyses*

Terminal deoxynucleotidyl transferase (TDT)-mediated dUTP nick end labeling (TUNEL) assay was performed to confirm DNA fragmentation of apoptosis, using a commercial kit according to the manufacturer's instructions (Nanjin KeyGen Biotech Co., Ltd., Nanjin, China). Cells were plated on glass cover slips and fixed in 4% formaldehyde for 30 min. After two washes in PBS, 0.2% TritonX-100 for 5 min was used to permeabilize the cells, and then the cells were washed again in PBS for 5 min. Biotinylated dUTP and TDT enzymes labeled the cells in a humidified box at 37 °C for 1 h. After three washes in PBS, cells were incubated in streptavidin-fluorescein for 30 min, followed by three washes in PBS. Finally, cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) at room temperature for 10 min before observation under a microscope. Cells were only labels as being TUNEL positive and expressed as percentage of the total nuclei.

### *4.5. Western blotting analysis*

Western blotting analyses were performed as previously described. Briefly, protein was separated by SDS-PAGE gel electrophoresis, and then transferred to PVDF membranes (Millipore). After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies, followed by incubation with appropriate horseradish peroxidase (HRP)-labeled second antibodies. Immunoreactive bands were detected with the Super-Signal West Pico Chemiluminescent Substrate (Pierce), and molecular band intensity was determined by densitometry.

### *4.6. Statistical analysis*

Data are presented as mean ± SE. Statistical analyses between two groups were performed by unpaired Student's *t*-test. Differences among groups were tested by one-way analysis of variance (ANOVA). In all cases, differences were considered statistically significant with $P < 0.05$.

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