ORIGINAL ARTICLE

Naringenin Inhibit the Hydrogen Peroxide-Induced SH-SY5Y Cells Injury Through Nrf2/HO-1 Pathway

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Abstract

Naringenin (NGN), a flavonoid, abundantly present in citrus fruits, has been established as a neuroprotective agent. However, the precise protective mechanisms remain worthy of further investigation. The present study was designed to explore the protective effects of NGN against hydrogen peroxide (H_2O_2)-induced neurotoxicity in human neuroblastoma SH-SY5Y cells and the possible mechanisms involved. Exposure of cells to 400 μM H_2O_2 for 2 h caused viability loss, apoptotic increase, and reactive oxygen species (ROS) increase, pre-treatment with NGN for 12 h significantly reduced the viability loss, apoptotic rate, and attenuated H_2O_2-mediated ROS production. In addition, NGN inhibited H_2O_2-induced mitochondrial dysfunctions, including lowered membrane potential, decreased Bcl-2/Bax ratio, cytochrome c release, and the cleavage of caspase-3. We also showed that NGN increased HO-1 expression. NGN treatment caused nuclear translocation of the transcription factor NF-E2-related factor 2 (Nrf2). NGN activated both ERK and PI3 K/Akt, and treatments with the specific ERK inhibitor PD98059, the PI3 K inhibitor LY294002, and the specific Nrf2 shRNA suppressed the NGN-induced HO-1 expression. The HO-1 inhibitor ZnPP abolished the neuroprotective effect of NGN against H_2O_2-induced neurotoxicity. Taken together, the present study demonstrates that regulation of Nrf2/HO-1 pathway through activation of ERK and PI3 K/Akt, and the inhibition of mitochondria-dependent apoptosis together may render NGN protect SH-SY5Y cells from H_2O_2-induced neurotoxicity.

Keywords Naringenin · Nrf2 · Heme oxygenase-1 · Oxidative stress · Neuroprotection

Introduction

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), are generated during normal cellular metabolism, playing important roles in signaling pathways (Dringen et al. 2005; Forman 2007). However, H_2O_2 also presents toxicological effects, which can produce new radicals and induces damage to major cellular biomolecules (Droge 2002; Halliwell 2006). Moreover, it has been demonstrated that exogenous H_2O_2 promotes an imbalance between production and removal of ROS towards the pro-oxidative state, often referred to as oxidative stress (OS). OS leads to the generation of ROS and electrophiles. When ROS production is greater than the cellular detoxification capacity, excessive ROS can stimulate free radical chain reactions, which subsequently damage cellular

biomolecules such as proteins, lipids, and DNA and finally lead to disease conditions. It has been reported that ROS participate in causing human diseases such as diabetes, tumors, atherosclerosis, stroke, and neurodegenerative diseases (Melo et al. 2011; Gandhi and Abramov 2012). Therefore, cells must constantly control the levels of ROS and prevent them from accumulating. With the help of antioxidants, these diseases can be reversed by the regulation of antioxidants with considerable success. There is a lot of research being conducted to develop effective and safe antioxidants that would be useful in the therapy or prevention of neurodegenerative diseases (Karpinska and Gromadzka 2013).

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is the most important transcription factors in regulating multiple antioxidants, which binds to the antioxidant response elements (AREs) in the antioxidant defense system (Loboda et al. 2016; Bellezza et al. 2018; Dinkova-Kostova et al. 2018). It has been reported that Nrf2 is an essential transcription factor, encoding for antioxidative and phase 2 enzymes, which include heme oxygenase 1 (HO-1) and NAD(P)H-quinone oxidoreductase 1 (NQO1). Emerging evidence supports a role for HO-1

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enzymes as important components of the cellular antioxidant system (Bellezza et al. 2018). It has been demonstrated that upregulation of HO-1 expression and elevated heme oxygenase (HO) activity play a key role in protecting cells against the toxicity caused by a variety of oxidative insults. In the central nervous system (CNS), HO pathway has been reported to be active and to operate as a fundamental defensive mechanism for cells exposed to an oxidant challenge (Scapagnini et al. 2004). Increases in HO-1 protein levels are associated with protection against oxidative stress(Le et al. 1999; Ahmad et al. 2006).

Flavonoids and curcuminoids are naturally occurring polyphenolic compounds that display a variety of therapeutic importance against oxidative stress. A growing number of experimental evidence support the concept that flavonoids with their strong antioxidant activities ameliorate oxidative stress. Naringenin (2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavanone, flavonoid, abundantly present in citrus fruits. Naringenin has different pharmacological activities including antidiabetic (Ortiz-Andrade et al. 2008), anti-inflammatory (Al-Rejaie et al. 2013), and also possesses neuroprotective activities in different experimental models of rodents (Raza et al. 2013; Wang et al. 2018). Most recent studies provided biological evidence supporting the usefulness of NGN for improvement of cognitive abilities (Khajevand-Khazaei et al. 2018; Liaquat et al. 2018). NGNameliorated learning and memory impairment following systemic lipopolysaccharide challenge in the rat. NGN dose dependently improved spatial recognition memory in Y maze, discrimination ratio in novel object discrimination task, and retention and recall capability in passive avoidance test. Furthermore, NGN lowered hippocampal malondialdehyde (MDA) as an index of lipid peroxidation and improved antioxidant defensive system comprising superoxide dismutase (SOD), catalase, and glutathione (GSH) in addition to decreasing acetylcholinesterase (AChE) activity. Additionally, NGN was able to upregulate Nrf2 (Khajevand-Khazaei et al. 2018).

Particularly, many studies highlighted NGN as potential candidates which can protect neurons against various toxic compounds-induced-oxidative stress and exert beneficial effects on neuronal cells (Al-Rejaie et al. 2015; Das et al. 2016; Roy et al. 2016; Al-Dosari et al. 2017). NGN could alleviate LPS-induced neuroinflammation, as was evident from attenuation of oxidative stress and modulation of Nrf2 (Khajevand-Khazaei et al. 2018). NGN-induced enhanced antioxidant defense system by enhancing antioxidant enzyme activities and increasing antioxidant compound concentration. Oxidative stress in terms of lipid peroxidation was significantly prevented in treated rats (Liaquat et al. 2018).

However, the precise protective mechanisms of NGN remains worthy of further investigation. In this study, we designed to explore the protective effects of NGN against hydrogen peroxide (H_2O_2)-induced neurotoxicity in human neuroblastoma SH-SY5Y cells and the possible mechanisms involved. These results demonstrate that NGN is an activator of Nrf2 and inducer of HO-1 expression. We also showed that NGN increased HO-1 expression through activation of ERK and PI3 K/Akt signal pathways. NGN attenuates the H_2O_2-induced oxidative stress via Nrf2/HO-1 activation in SH-SY5Y cells. Upregulation of HO-1 by NGN may involve in the neuroprotection against H_2O_2-induced neurotoxicity.

Results

Naringenin (NGN) Ameliorates H_2O_2-Induced Cell Death in SH-SY5Y Cells

MTT assay was used to test the toxicity of NGN to SH-SY5Y cells. As shown in Fig. 1a, NGN at each of these concentrations (10–160 μM) alone did not cause any apparent neurotoxicity. SH-SY5Y cells were incubated with 400 μM H_2O_2 for 2 h with/without different concentrations of NGN (5, 10,
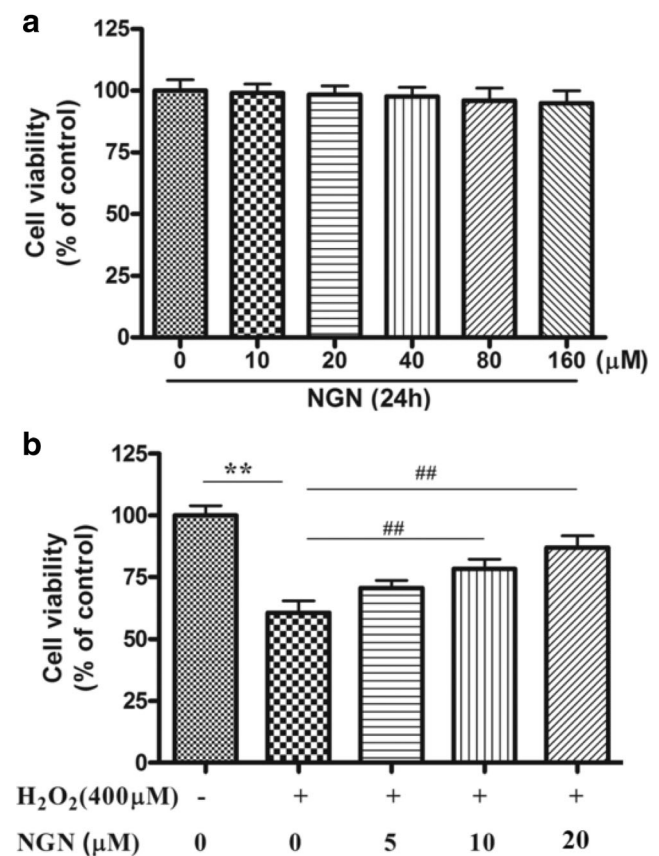

Fig. 1 Naringenin (NGN) prevents H_2O_2-induced cell death in SH-SY5Y cells. **a** SH-SY5Y cells were incubated with various concentrations of NGN for 24 h; **b** cells were incubated without or with NGN (5, 10, 20 μM) for 12 h, followed by incubation with H_2O_2 for another 2 h. After this incubation, cell viability was determined with the MTT assay. [**]$P < 0.01$; [##]$P < 0.01$

20 μM). The H_2O_2-induced cytotoxicity was first evaluated by the MTT reduction assay. As shown in Fig. 1c, H_2O_2 significantly decreased cell viability. However, the neurotoxic effects were attenuated by the pre-treatment with NGN (10 and 20 μM), which significantly blocked the cytotoxic effects of H_2O_2 on cell viability.

Naringenin (NGN) Decreases ROS Levels in SH-SY5Y Cells

To determine the generation of intracellular ROS induced by 400 μM H_2O_2 for 2 h, we performed flow cytometry analysis using the ROS-sensitive fluorescence probe, DCF. Cytometry assay showed that the exposure to H_2O_2 caused an elevation of the intracellular ROS levels which was about 1.5-fold relative to that of control cells (Fig. 2). Pretreatment with 20 μM NGN suppressed the intracellular ROS elevation. These results indicated that NGN has the ability to scavenge ROS induced by H_2O_2.

Naringenin (NGN) Inhibits H_2O_2-Induced Disruption of Mitochondrial Membrane

Change in mitochondrial membrane potential (MMP) is a key indicator in apoptotic cell death. Levels of MMP SH-SY5Y cells were determined using flow cytometry with the fluorescence probe JC-1. Fluorescence intensity of JC-1 revealed that NGN could attenuate H_2O_2-induced mitochondrial membrane potential disruption compared with the H_2O_2 treatment alone (Fig. 3a). At the same time, we detected the effect of NGN on the MMP (Fig. 3b). NGN significantly improved H_2O_2-induced impairments in MMP using Rh123. These results suggested that NGN attenuates H_2O_2-induced mitochondrial
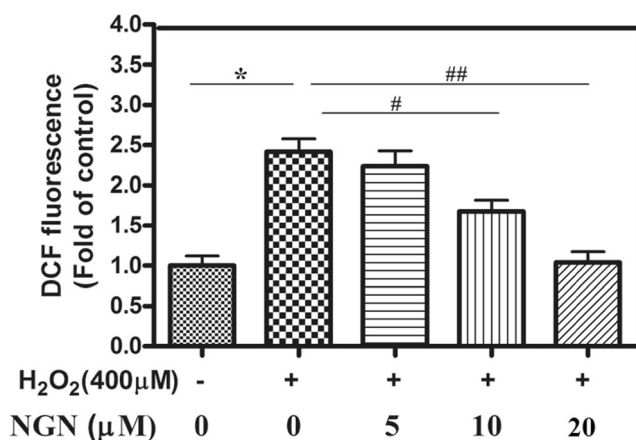

Fig. 2 Naringenin (NGN) decreases ROS levels in SH-SY5Y cells. Cells were pretreated with the indicated concentrations of NGN (20 μM) for 12 h or not, followed by H_2O_2 (400 μM) administration for 2 h. The fluorescence intensity of the cells stained with DCFH-DA and recorded by flow cytometer

dysfunction, by which play an important role in protecting SH-SY5Y cells against H_2O_2-induced neurotoxicity.

Effect of Naringenin (NGN) on H_2O_2-Induced Caused Apoptosis

Results from annexin V/PI double staining assay, Bcl-2/ Bax ratio, release of cytochrome c and caspase-3 activity detection (Fig. 4) indicated that the apoptotic rates were lower in SH-SY5Y cells pretreated with NGN and H_2O_2 comparing to the groups of H_2O_2 treatment alone. The annexin V/PI double staining also assessed cell death and DNA fragmentation in H_2O_2-treated cells. More apoptosis rate was observed in cells treated with H_2O_2, whereas apoptosis rate was lower in NGN-treated cells (Fig. 4a). NGN pretreatment (20 μM) significantly reduced apoptotic cells induced by H_2O_2.

Next, we also detected the expression of Bcl-2 and Bax in the mitochondria-dependent apoptosis. As shown in Fig. 4c, treatment of cells with H_2O_2-induced an increase in the protein level of Bax and robust decrease in the protein level of Bcl-2, and there was a significant decrease in the ratio of Bcl-2/Bax expression in H_2O_2 treatment compared with the control, while NGN pretreatment could prevent the H_2O_2-induced decrease of the Bcl-2/ Bax ratio. The protective effect of NGN against H_2O_2-induced apoptosis may be, at least in part, mediated by regulating Bcl-2 and Bax expression. The results of inhibition of caspase-3 activation confirmed apoptosis inhibiting of NGN. As shown in Fig. 4c, a marked increase of activated caspase-3 in H_2O_2 treatment was observed. However, H_2O_2-induced activation of caspase-3 was inhibited by NGN pretreatment in a dose-dependent manner. This result indicated that NGN can inhibit the induction of caspase-3.

After the disruption of MMP, mitochondrial cytochrome c was released, which ultimately cleave procaspase-3 to form active caspase-3. Next, we investigated the effect of NGN on H_2O_2-induced cytochrome c release. As shown in Fig. 4f, H_2O_2 significantly increased the release of cytochrome c from mitochondria to the cytosol, and NGN (20 μM) pretreatment inhibited the release of cytochrome c.

Collectively, these results indicated that NGN suppresses H_2O_2-induced apoptosis in SH-SY5Y cells.

NGN Upregulates HO-1 Protein Expression

We first investigated the possibility that NGN might alter the expression of the antioxidant enzyme HO-1. Pretreatments of the SHSY5Y cells with 20 μM NGN resulted in a time-dependent (Fig. 5a) and concentration-dependent (Fig. 5b) increase in HO-1 protein expression.
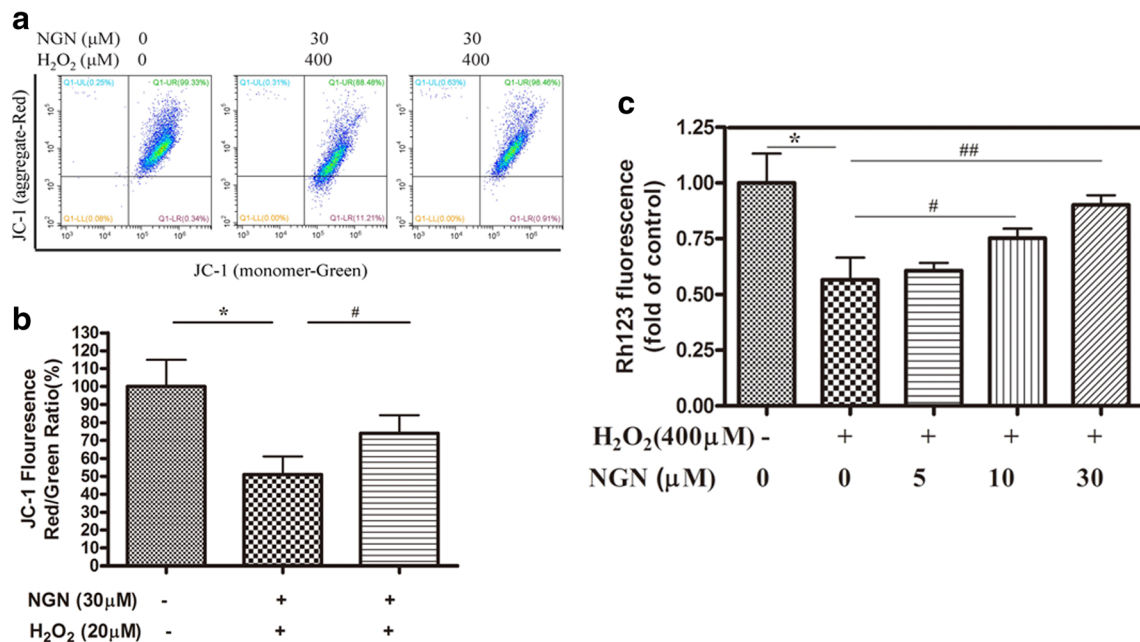
Fig. 3 Naringenin (NGN) inhibited H_2O_2-induced disruption of mitochondrial membrane potential (MMP) in SH-SY5Y cells. Cells were pretreated with the indicated concentrations of NGN (20 μM) for 12 h or not, followed by H_2O_2 (400 μM) administration for 2 h. Decrease of MMP was measured with JC-1 staining and qualified with flow cytometry analysis. **a** and **b** The transition of fluorescence density was shown. **c** MMP was evaluated by Rh123 staining by fluorescence microplate reader. Mean relative fluorescent density of Rh123 was calculated and normalized with that from control. Data were presented as mean ± SEM of three independent experiments. $^*p < 0.05$; $^#P < 0.05$; $^{##}P < 0.01$

Nrf2 Activation Involves in NGN-Induced Upregulation of HO-1

We analyzed to determine whether NGN is able to activate Nrf2 in association with HO-1 upregulation. We analyzed for the Nrf2 accumulation in the nuclei in the presence of NGN. The cells were pretreated with NGN, and the level of Nrf2 protein was then determined by Western blotting. NGN induced the accumulation of Nrf2 in the nuclei relative to untreated cells (Fig. 6a, b). This result suggests that NGN stimulates activation of Nrf2.

To further verify the requirement of Nrf2 for NGN-induced HO-1 expression, cells were transfected with Nrf2 shRNA for 24 h before NGN treatment. As shown in Fig. 6e, the NGN-induced expression of HO-1 was markedly suppressed by shRNA knockdown of Nrf2 gene. Taken together, these findings support that NGN activates Nrf2, which elicit the NGN-induced upregulation of HO-1.

ERK and the PI3 K/Akt Pathways Activation Result in NGN-Induced HO-1 Expression

To elucidate the plausible signal transduction pathways involved in the NGN-induced HO-1 expression, we examined the phosphorylation of several upstream kinases. We found that the activation of ERK and Akt, both of which are major signaling enzymes involved in cellular protection against oxidative stress. Exposure to NGN caused an increase in the phosphorylation of ERK, and Akt in a time-dependent manner (Fig. 7a, c).

To identify the signaling pathways used by NGN to induce HO-1 expression, we then analyzed to determine whether ERK and Akt pathways were involved in the increase of HO-1 expression. We examined the effects of the specific ERK inhibitor PD98059 and the PI3 K inhibitor LY294002 on NGN-induced HO-1 expression. Result showed that the HO-1 expression was blocked by PD98059 and LY294002 (Fig. 7I).

Upregulating HO-1 Expression Involve in the Neuroprotective of NGN H_2O_2-Induced Neurotoxicity

Previous findings support the importance of HO-1 in protecting neurons against H_2O_2-induced oxidative stress-dependant injury. In an attempt to determine whether the increased HO-1 expression induced by NGN is indeed responsible for the cytoprotective effects against the H_2O_2-derived oxidative cell death, we utilized the inhibitor of HO-1 activity, ZnPP. Pretreatment of SH-SY5Y cells with ZnPP for 1 h before H_2O_2 challenge attenuated the NGN-mediated cytoprotection, but ZnPP alone does not result in neurotoxicity (Fig. 8). These results suggest that the neuroprotective effect elicited by NGN is mediated through the induction of HO-1 expression.
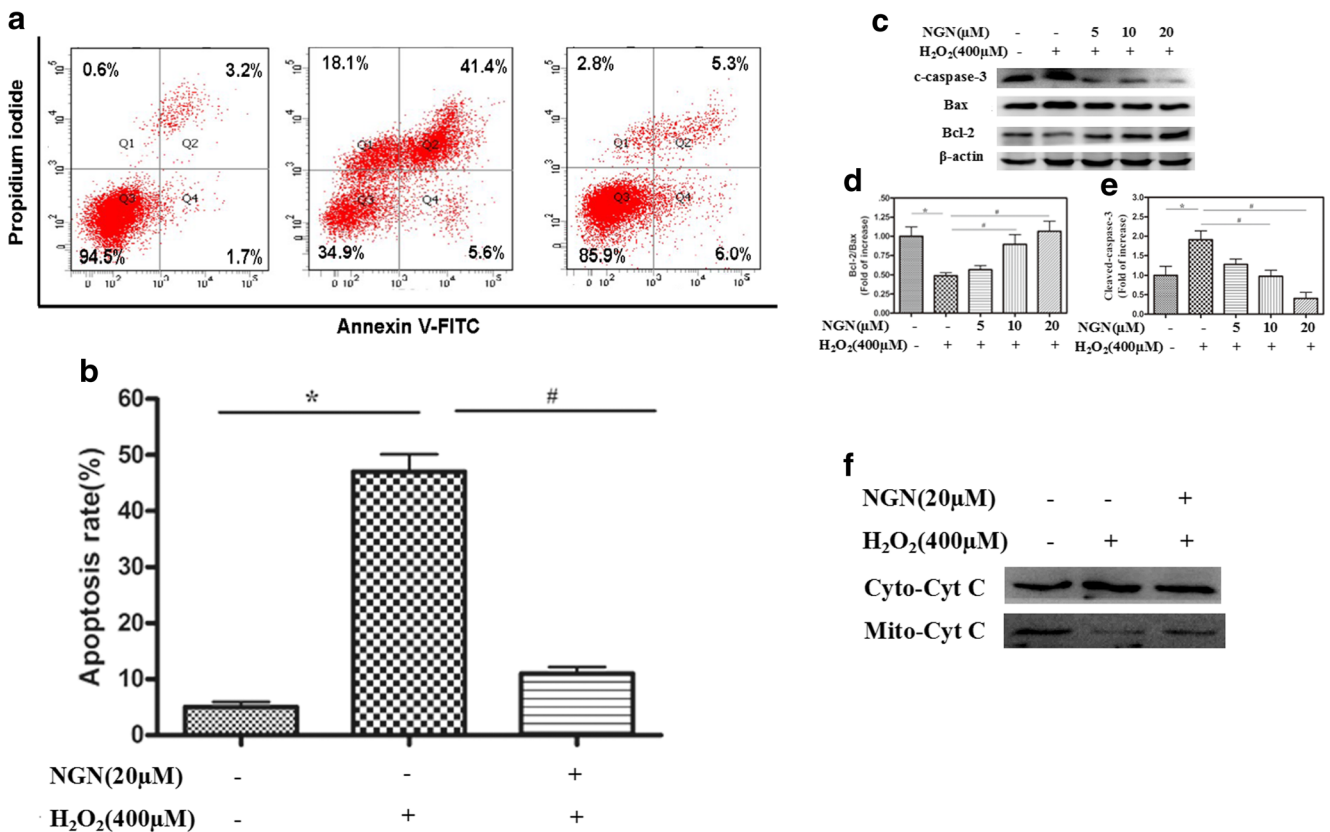
Fig. 4 Effect of naringenin (NGN) on H_2O_2-induced caused apoptosis. **a** The rate of apoptotic cells of different groups of cells was detected using flow cytometry with FITC-conjugated annexin V and propidium iodide (PI). **b** The apoptosis rate was calculated. **c** The cells were pretreated with NGN for 12 h prior to exposure to 400 μM H_2O_2 for 2 h. Assessment of Bcl-2, Bax, and cleaved caspase-3 protein levels by Western blotting. Densitometric analysis of changes in **d** the ratio of values of Bcl-2/Bax and in **e** levels of cleaved caspase-3. **f** The amount of cytosolic cytochrome c (Cyto Cyto C) and mitochondrial cytochrome c (Mito Cyto C) were determined by Western blot analyses. Data are expressed as mean ± SEM. $^*p < 0.05$; $^#P < 0.05$

Discussion

In the present study, we demonstrated that NGN possesses significant neuroprotective effects in SH-SY5Y cells. One of the most salient features of this study is that NGN cause Nrf2-mediated-HO-1 induction through ERK1/2 and PI3 K-Akt pathways, thereby protecting the SH-SY5Y cells against H_2O_2-induced oxidative neurotoxicity.

H_2O_2, one of the main reactive oxygen species (ROS), is produced during the redox process and is considered as a

Fig. 5 NGN upregulates HO-1 protein expression in SH-SY5Y cells. Cells were incubated with **a** 20 μM NGN for the indicated amounts of time and **b** various concentrations of NGN for 12 h. HO-1 protein expression was detected by Western blotting. **c** and **d** Densitometric analysis is mean ± SEM of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$
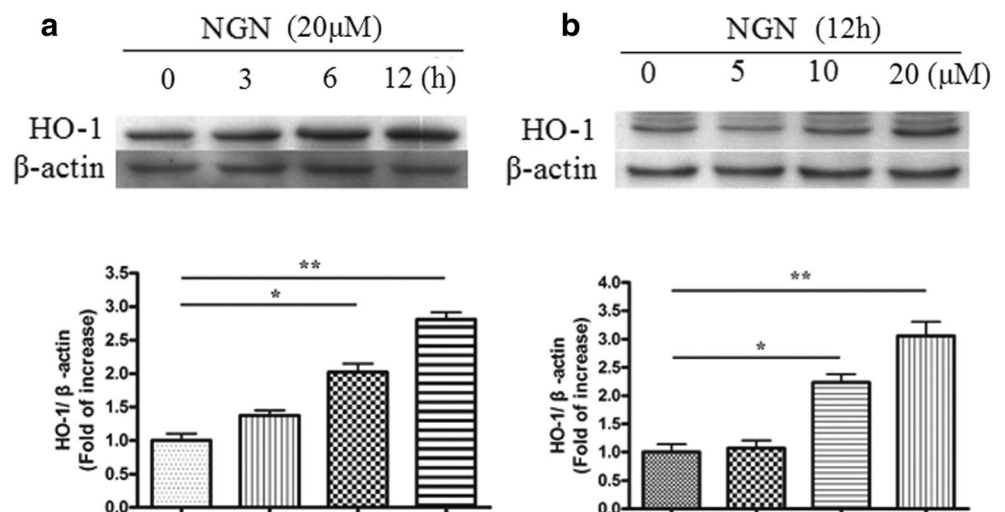
Fig. 6 Nrf2 activation involves in NGN-induced upregulation of HO-1 in SH-SY5Y cells. Cells were incubated with 20 μM NGN for the indicated time. Nrf2 protein in the **a** cytosolic and the **b** nuclei was detected by Western blotting. **d** Cells were transfected with control shRNA (Ctrl shRNA) or Nrf2 shRNA for 24 h, and then cells were harvested to detect Nrf2 protein levels by Western blot. **e** SH-SY5Y cells were transfected with Nrf2-negative Ctrl shRNA or Nrf2-shRNA for 24 h. The transfected cells were treated with NGN (20 μM) for 6 h, and expression HO-1 was measured by Western blot analysis
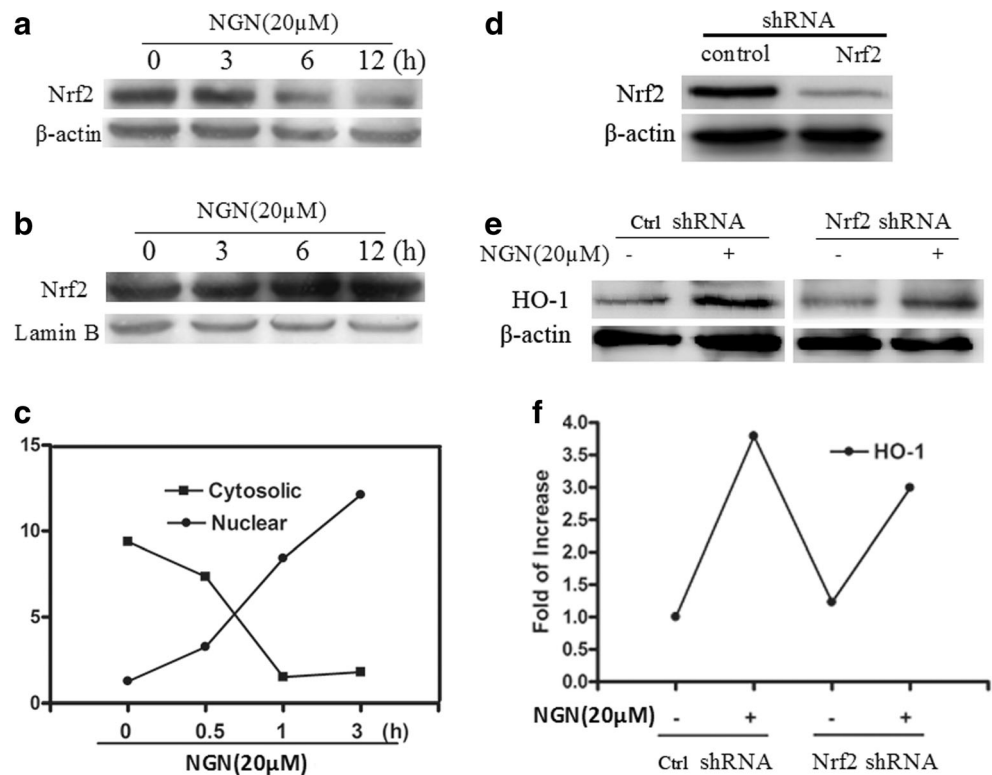
messenger in intracellular signaling cascades (Behl et al. 1994). H_2O_2 may induce the self-generation of free radicals known as ROS-induced ROS release at the mitochondrial level (Zorov et al. 2000), which has been widely used as a model of exogenous oxidative stress-induced apoptosis. It has been shown that H_2O_2 exposure can activate caspase-3, which as a final effector in apoptotic death in vitro (Matsura et al. 1999). Besides, ROS can activate many transcription factors including nuclear factor-erythroid-2-related factors (Nrf2) that regulate coordinated activation of a battery of genes in response to oxidative and/or electrophilic stress (Loboda et al. 2016). Nrf2 plays a crucial role in cellular defense and is the major regulator of antioxidant response elements present in the regulatory region of the majority of cytoprotective genes (Jaiswal 2004). Notably, the Nrf2/ARE signaling pathway is a common molecular target for natural products. Under normal conditions, Nrf2 forms covalent complex with Keap1 via intermolecular disulfide bonds. Upon specific cysteine residues of Keap1 protein are oxidized or modified by electrophile inducers, Nrf2 is no longer sequestered by Keap1 and subsequently translocated into the cell nucleus, binding to the promoter and activates the transcription of a number of phase 2 detoxifying enzymes including HO-1. Induction of HO-1 is highly recognized as an important therapeutic target for pharmacological intervention of oxidative disorders.

In recent years, great attention has been paid on natural dietary antioxidants especially flavonoids which are important for counteracting oxidative stress. NGN is a flavonoid, abundantly present in citrus fruits, which were highlighted by many studies can protect neurons against various toxic compounds-induced-oxidative stress and exert beneficial effects on neuronal cells (Al-Rejaie et al. 2015; Das et al. 2016; Roy et al. 2016; Al-Dosari et al. 2017). However, the precise protective mechanisms of NGN remains worthy of further investigation. In this study, we designed to explore the protective effects of NGN against hydrogen peroxide (H_2O_2)-induced neurotoxicity in human neuroblastoma SH-SY5Y cells and the possible mechanisms involved.

The present study has shown that H_2O_2 administration significantly increases intracellular ROS levels and causes SH-SY5Y cells damage as indicated by the inhibition of cell viability. We found that pretreatment of SH-SY5Y cells with NGN significantly reduced the H_2O_2-induced loss of cell viability, the generation of intracellular ROS, apoptotic rate, and the cleavage of caspase-3. NGN strikingly inhibited H_2O_2-induced mitochondrial dysfunctions, including lowered membrane potential, decreased Bcl-2/Bax ratio, and the release of cytochrome c. Overall, these results suggest that NGN protects SH-SY5Y cells against H_2O_2-induced neurotoxicity through the mitochondrial apoptotic pathway. Our results are consistent with previous reports, which suggested that enhancing NGN can alleviate oxidative stress-induced H9c2 cell damage by enhancing antioxidative enzymatic activity, thus reducing intracellular ROS levels (de Oliveira et al. 2017).

Other reports have demonstrated that NGN treatment can inhibit apoptosis through the activation of HO-1 in vitro. NGN
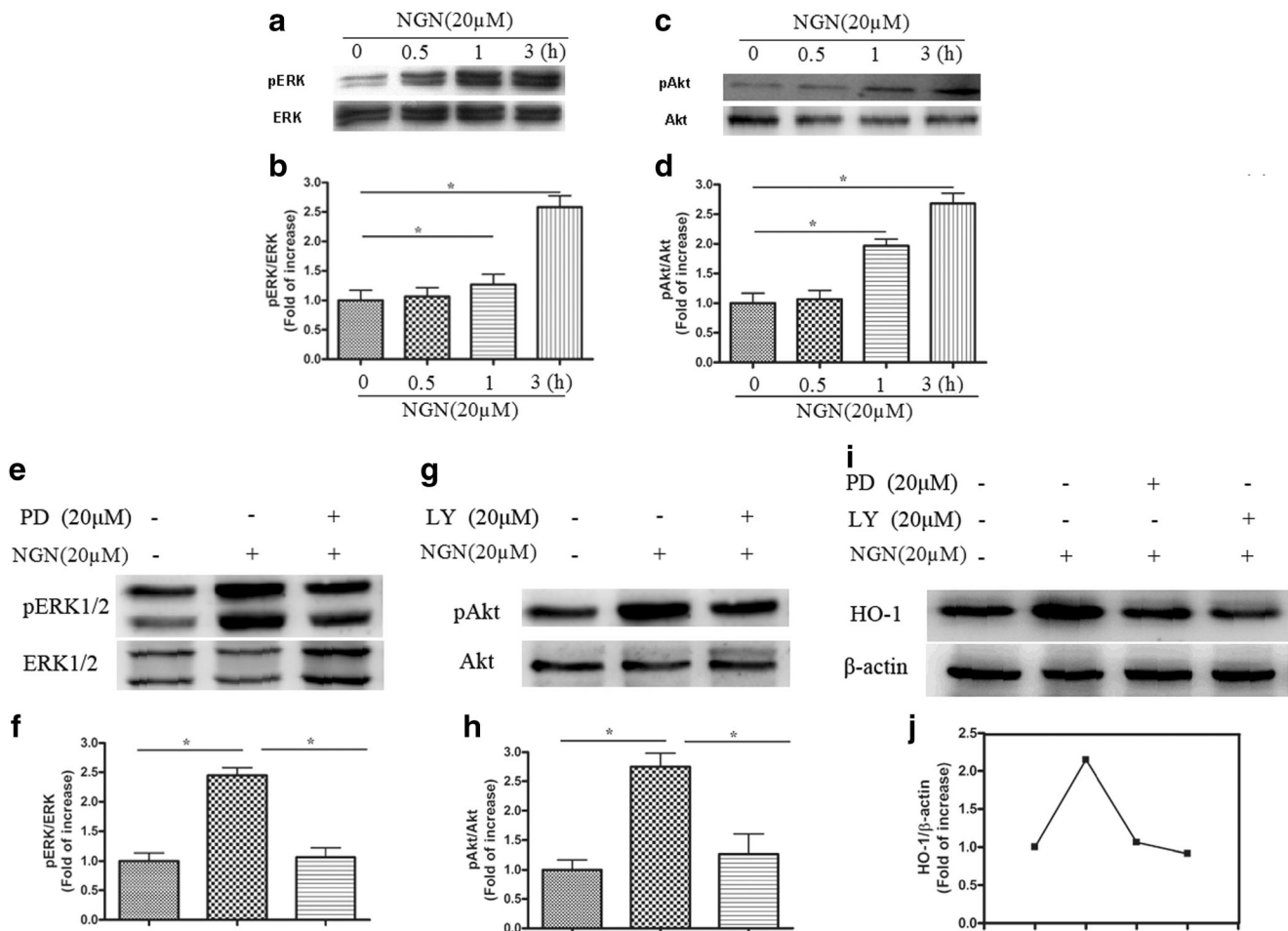
Fig. 7 Naringenin (NGN) activates the ERK and the PI3 K/Akt pathways, which activation result in NGN-induced HO-1 expression. SH-SY5Y cells were stimulated with 20 μM NGN for the indicated times. Specific protein was immunoblotted with antibodies that recognize a phospho-ERK (pERK1/2), then the blots for **a** pERK1/2 were stripped and reprobed with total ERK antibodies, and **c** phospho-Akt (pAkt) then the blots for pAkt were stripped and reprobed with antibodies against total Akt. SH-SY5Y cells were preincubated with PD98059 (PD) or/and LY294002 (LY) for the indicated dose for 1 h and further incubated for 6 h after the addition of NGN (20 μM). Cell lysates were electrophoresed and then immunoblotted with activation-specific antibodies that recognize **e** phospho-ERK (pERK1/2) and total ERK, and **g** phospho-Akt (pAkt) and total Akt, or **i** HO-1 and β-actin. $^{*}P < 0.05$; $^{**}P < 0.01$

has been reported to induce HO-1 expression in several cell types (Podder et al. 2014), so understanding the molecular mechanisms of NGN-induced HO-1 activation is very important for the therapeutic application of NGN. We investigated whether the antioxidant activities of NGN could be related to its ability to induce HO-1 expression. Pretreatments of the SHSY5Y cells with 20 μM NGN resulted in a time-dependent and concentration-dependent increase in HO-1 protein expression. NGN induced the nuclear translocation of Nrf2. Thus, the results from this study demonstrated that the activation of the Nrf2/ARE pathway is a key mechanism underlying the induction of HO-1 expression. To identify the signaling pathways used by NGN to activate Nrf2 and induce HO-1 expression, we tested to determine whether NGN-induced expression of HO-1 occurs through a specific MAPK and PI3 K/Akt pathway. We found that NGN activated the ERK1/2 cascade and PI3 K/Akt. In addition, the use of

specific inhibitors for ERK1/2 and PI3 K/Akt pathways confirmed the involvement of these two pathways in NGN-induced HO-1 expression. We analyzed to determine whether NGN is able to activate Nrf2 in association with HO-1 upregulation. We analyzed for the Nrf2 accumulation in the nuclei in the presence of NGN. The cells were pretreated with NGN, and the level of Nrf2 protein was then determined by Western blotting. NGN induced the accumulation of Nrf2 in the nuclei relative to untreated cells. We found that the HO-1 inhibitor ZnPP markedly blocked the neuroprotective effect of NGN. Thus, we postulate that the antioxidant activity of NGN is highly dependent on the induction of HO-1.

In summary, NGN induces HO-1 expression to protect against oxidative stress through inhibition the production of ROS and sequential activation of the MAPK pathway and Akt in SH-SY5Y cells. The upregulation of HO-1 is a novel pleiotropic effect of NGN on neuroprotective effect. These results
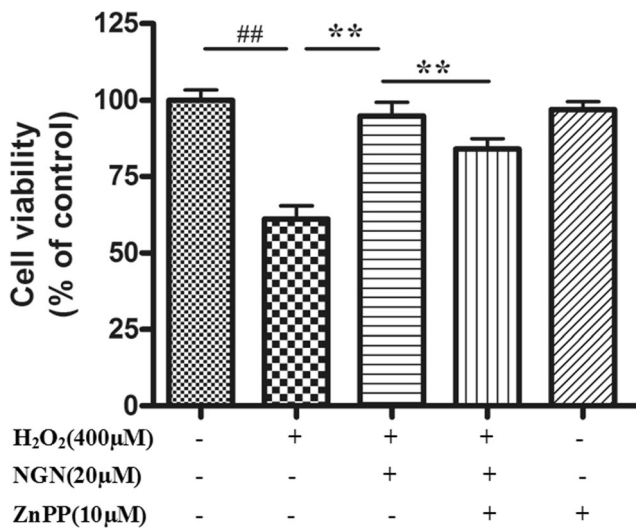
Fig. 8 Naringenin (NGN) protects against H_2O_2-induced neurotoxicity by upregulating HO-1 expression. HO-1 enzyme inhibitor ZnPP reversed the protective effect of NGN against H_2O_2-induced cell death. Cells were pretreated with ZnPP for 1 h in the absence or presence of NGN and were exposed to H_2O_2 for 2 h. Cell viability was measured by MTT assay. Data represent the means ± SE of three independent experiments. $^{##}P < 0.01$; $^{**}P < 0.01$

demonstrate that NGN is an activator of Nrf2 and inducer of HO-1 expression. NGN attenuates H_2O_2-induced oxidative stress-mediated neurotoxicity by induction of HO-1 via ERK and PI3 K/Akt signaling. These results contribute to shed some light on the mechanisms whereby NGN protects against H_2O_2-induced neurotoxicity.

Materials and Methods

Materials

Naringenin was obtained from Sigma-Aldrich. The stock solution of naringenin was made with modified Dulbecco's Eagle's medium (DMEM) and stored at 4 °C. The stock solution was diluted to working concentrations before use. Rhodamine 123 (Rh123) and 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Inc. The fluorescent dyes 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were purchased from Invitrogen. 4′,6-diamino-2′-phenylindole dihydrochloride (DAPI), and complete protease inhibitor was from Roche Diagnostics GmbH (Penzberg, Germany). DMEM supplement was obtained from Gibco Invitrogen Corporation. PD98059 (SC-3532) and ZnPP (SC-200329) were from Santa Cruz. HO-1 antibody was from Stressgen (#SPA895, Victoria, BC, Canada). Antibodies against cleaved caspase-3 were from Calbiochem. Rabbit anti-Bcl-2 (#2876), anti-Bax (#2772), rabbit anti-phospho-p38 (Thr180/Tyr182) (#9211), anti-p38 (#9212), rabbit anti-phospho-ERK1/2

(Thr202/Tyr204) (#9101) and ERK (#9102), rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) (#9251), rabbit anti-SAPKJNK (#9252), rabbit antiphospho-Akt (Ser473) (#9271), and rabbit anti-Akt (#9272) antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Horseradish peroxidase-linked IgG antibodies were obtained from Invitrogen. All the other chemicals used were of the high grade available commercially.

Cell Culture and Treatment

Human neuroblastoma SH-SY5Y cells were cultured in DMEM medium that was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 5% CO_2 at 37 °C. SH-SY5Y cells were stably transfected with control shRNA or NRF2 shRNA using a transfection reagent. In brief, cells were seeded in antibiotic-free normal growth medium and incubated at 37 °C in a CO_2 incubator until the cells reached 60~80% confluency. For each transfection, 6 μl of shRNA duplex and shRNA transfection reagent was diluted in 100 μl of shRNA transfection medium. The shRNA duplex and shRNA transfection reagent were gently mixed by pipetting and then incubated for 30 min at room temperature. In the meantime, cells were washed once with 2 ml of transfection medium. The mixture of shRNA duplex and shRNA transfection reagent was then added to the cells with 800 μl of transfection medium and then overlaid onto the cells. The cells were incubated at 37 °C in a CO_2 incubator for 6 h. After incubation, the transfection medium was aspirated, and normal growth medium was added for an additional 24 h of incubation under normal cell culture conditions. The transfected SH-SY5Y cells were treated with NGN for the indicated time periods.

Cell Viability Assessment by MTT Assay

Cell viability was measured by the MTT assay. Briefly, 1×10^5 SH-SY5Y cells/ml were plated in a 96-well plate and incubated for 24 h to allow the cells to reach 80% confluency. The cells were pre-treated with different concentrations of NGN, and viability was measured at different time periods, and then were co-treated with 400 μM H_2O_2 for 2 h in the continued presence of vehicle and NGN. After incubation, the cells were treated with 5 mg/ml MTT for 4 h at 37 °C, and then the medium was removed, and the formazan crystals were dissolved in 150 μl of DMSO. The plate was then incubated for 30 min, and the absorbance was measured at 590 nm on a plate reader (Thermo Scientific Varioskan Flash).

Reactive Oxygen Species Determination

Reactive oxygen species (ROS) was detected by the 2′,7′-dichlorodihydro-fluorescein diacetate (DCFH-DA)

fluorescence assay. Briefly, cells were seeded in 96-well plates at the density of 5×10^3 per well for overnight incubation. After treatment with various concentrations of test samples, cells were incubated in serum-free medium containing 25 μM DCFH-DA at 37 °C for 30 min. After incubation, the cells were washed with $1 \times$ HBSS buffer, and then the cells were co-treated with NGN in a 96-well black cell culture plate for 2 h. ROS generation was measured by the fluorescence intensity of DCF at 525 nm after excitation at 488 nm on a fluorescence plate reader (Thermo Scientific Varioskan Flash).

Assay of the Mitochondrial Membrane Potential

The mitochondrial membrane potential (MMP) was monitored by the JC-1 staining in SH-SY5Y cells. Briefly, cells were digested by 0.25% trypsin, resuspended, and stained in culture medium containing 10 μg/ml JC-1 (Enzo Bioscience) at 37 °C for 20 min. After washing once with PBS, the cells were analyzed by a flow cytometer (FACSAria II). At the excitation wavelength of 488 nm, the fluorescence intensity of JC-1 at the emission wavelength at 575 nm (PE-A) and then at the emission wavelength at 518 nm (FITC-A) were detected. MMP was also monitored using the Rh123. Rh123 was added to media after the cells exposed to H_2O_2 with or without NGN pretreatment. After incubation at 37 °C for 30 min, cells were washed and then measured by fluorescence microplate reader.

Detection of Apoptotic Cells by Flow Cytometry

The cell apoptotic rate was detected by flow cytometry using the annexin V-FITC/PI double-labelling method. The cells (1×10^5 cells/ml) were seeded in 60-mm dishes and treated with NGN and H_2O_2. The annexin V-FITC apoptosis detection kit (Komabiotech) was used to double-stain the cells according to the manufacturer's instructions. After digestion with 0.25% trypsin and washing once with cold PBS, cells were resuspended in 100 μl cold $1 \times$ binding buffer. 5 μl of FITC-phycoerythrin-labeled annexin V was added to the cell suspension, which was incubated on ice for 15 min. Two hundred microliter $1 \times$ binding buffer and 5 μl PI staining solution were then added to the cell suspension. The samples were analyzed using a flow cytometer (BD Biosciences).

Cytochrome c Assay

For measurement of cytochrome c release, the cytosol fractions were prepared as previously reported (Wang et al. 2009).

Preparation of Nuclear Extract

The extraction and isolation of nuclear and cytoplasmic protein were performed according to the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China) as described previously (Wang et al. 2012).

Western Blot Analysis

After treatment, cells were washed in cold $1 \times$ PBS and lysed on ice in RIPA lysis buffer containing proteases inhibitors. Supernatants were collected by centrifugation at 10,000 rpm for 10 min at 4 °C. Denatured proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an 8 or 10% polyacrylamide gel and then transferred onto PVDF (Millipore, Billerica, MA, USA). After blocking overnight at 4 °C in 5%, BSA in Tris-buffered saline/Tween (with 0.05% (v/v) Tween 20), the membranes were first incubated with each antibody at dilutions of 1:2000. The second incubation was performed with horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody. To monitor potential artifacts in loading and transfer among samples in different lanes, the blots for phospho-MAPK were stripped and reprobed with antibodies against total MAPK. The blots were developed using an ECL Western blotting detection reagent (Santa Cruz).

Statistical Analysis

All data were presented as the mean ± SEM. Data were subjected to statistical analysis via one-way ANOVA followed by Student's t test with GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). Mean values were considered to be statistically significant at $P < 0.05$.

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