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Targeting Nrf2-mediated heme oxygenase-1 enhances non-thermal plasma-induced cell death in non-small-cell lung cancer A549 cells

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ABSTRACT

Non-thermal plasma (NTP) treatment has been proposed as a potential approach for cancer therapy for killing cancer cells *via* generation of reactive oxygen species (ROS). As an antioxidant protein, Heme oxygenase-1 (HO-1) has been known to protect cells against oxidative stress. In this paper, we investigated the role of HO-1 activation in NTP-induced apoptosis in A549 cells. Distinctly increased ROS production and apoptosis were observed after NTP exposure. NTP exposure induced HO-1 expression in a dose- and time-dependent manner *via* activating the translocation of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) from cytoplasm to nucleus. Furthermore, inhibiting HO-1 activation with its specific inhibitor, ZnPP, increased "killing" effect of NTP. Knocking down HO-1 or Nrf2 with the special siRNA also led to elevated ROS level and enhanced NTP-induced cell death. In addition, the c-JUN N-terminal kinase (JNK) signaling pathway was shown to be involved in NTP-induced HO-1 expression. Interestingly, a higher resistance to NTP exposure of A549 cell compared to H1299 and H322 cells was found to be linked to its higher basal level of HO-1 expression. These findings revealed that HO-1 could be considered as a potential target to improve the effect of NTP in cancer therapy.

1. Introduction

Non-thermal plasma (NTP) treatment, which selectively kill cancer cells with minimal adverse effects on normal cells, have emerged as a promising approach for cancer therapy in recent years [1–3]. The anti-cancer effect of NTP has been demonstrated in various cancer cells *in vitro* such as breast, lung, liver, melanoma, and colorectal cancers [4–9], and also in tumors *in vivo* [10,11]. NTP is in fact a "cocktail" which contains various components including electrons, ions, UV photons, charged particles, etc. [12] and will induce generation of intracellular reactive oxygen species (ROS) [13,14]. It is well established that ROS has a dual role in regulating multiple cellular activities, including triggering cell death and activation of antioxidant processes. Numerous studies revealed that NTP exposures increased ROS generation which caused DNA damages, arrested cell cycle, induced apoptosis and inhibited tumor invasion [15–19]. Furthermore, NTP-induced DNA damages and apoptosis mediated by ROS could be reduced through pre-treatment with the ROS scavenger N-acetyl-L-cysteine (NAC) [20]. However, whether the intracellular antioxidant system was also activated and involved in resisting NTP-induced cell death was not fully understood.

Heme oxygenase-1 (HO-1), a rate-limiting enzyme, catalyzes heme into biliverdin, carbon monoxide (CO) and free iron [21,22]. HO-1 has been found to express highly in various solid tumors, such as lung cancer and prostate cancer [23,24], or to be induced by oxidative stress to provide favorable conditions for tumor growth [25–27]. Furthermore, induction of HO-1 expression could provide an adaptive protection against apoptosis [27] and DNA damages [25]. Accumulated evidence suggested that inhibition of HO-1, with its specific inhibitors such as ZnPP or by knocking down through siRNA interference, increased the sensitivity to chemotherapy [28,29] and radiotherapy [30]. These findings hinted that HO-1 could be considered as a target for cancer therapy.

Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2), a

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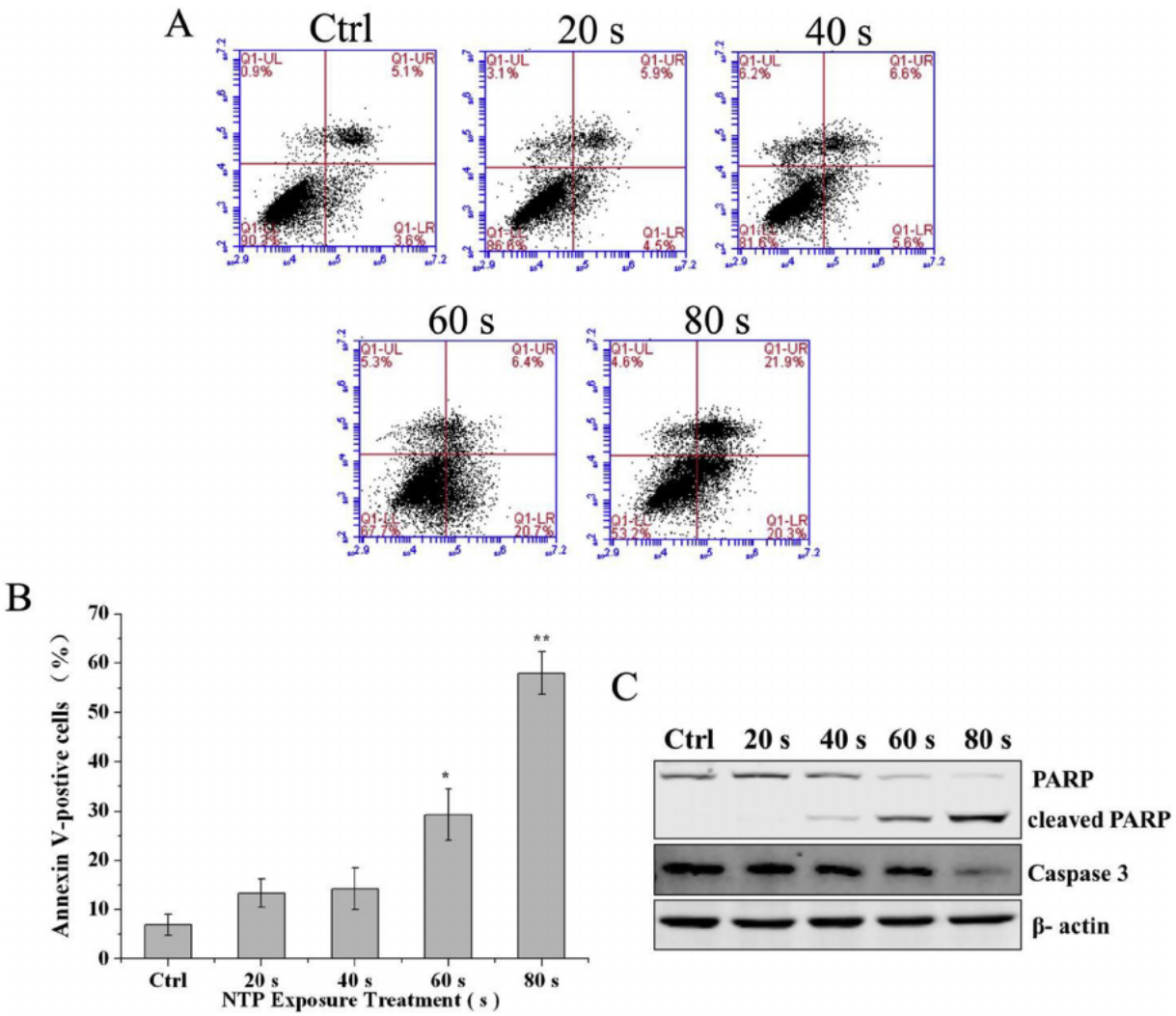

Fig. 1. Induction of apoptosis in A549 cells after NTP exposure. (A&B) Apoptosis was detected using Annexin V/PI double staining assay; (C) Expression levels of apoptosis-associated proteins, cleaved PARP and Caspase 3, were detected with Western Blot. $^{*}p < 0.05$, $^{**}p < 0.01$.

leucine zipper (bZIP) transcription factor, regulates the expression of antioxidant proteins and protects cells from oxidative damages. Normally, Nrf2 combines with Kelch-like ECH-associated protein 1 (Keap1) which retains Nrf2 in the cytoplasm [31]. In response to oxidative stress, Nrf2 is activated and translocated from cytoplasm into the nucleus, binds to the antioxidant response element (ARE) and then initiates the transcription of antioxidant genes, including HO-1, NADPH quinone oxidoreductase 1 (NQO-1), sulfiredoxin (Srx) etc. [32,33]. Activation of MAPK signaling pathways is demonstrated to play vital roles in activating the Nrf2 transcriptional factor and subsequently inducing HO-1 expression [34,35].

In this study, we aimed to investigate the effects and the molecular mechanism underlying the induction of HO-1 expression in the human lung cancer cell line (A549) by NTP, and to study the protective role of Nrf2/HO-1 against NTP-induced cell death. We hope these results can provide mechanistic information on NTP-induced cell death, which will be vital for potential clinical applications of NTP in cancer therapy.

2. Materials and methods

2.1. Cell culture and reagents

The human lung cancer cell lines A549, H1299 and H322 cells, obtained from the Type Culture Collection of the Chinese Academy of Sciences, were maintained in high glucose Dulbecco's modified Eagle medium (DMEM, Hyclone, Logan, USA), supplemented with 10% fetal bovine serum (FBS, Thermo Scientific Hyclone, Logan, UT, USA), 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco, Carlsbad, CA, USA). All cells were maintained in a humidified incubator under 5% CO_2 at 37 °C.

Counting Kit-8 (CCK-8) and NAC (ROS scavenger) were purchased from Beyotime Biotechnology (Beyotime Biotechnology, Shanghai, China). ERK1/2 inhibitor (SCH772984), p38 MAPK inhibitor (SB203580) and JNK inhibitor (SP600125) were purchased from Selleck Chemicals (Houston, TX, USA). The primary antibodies against Caspase3, PARP, HO-1, ERK1/2, *p*-ERK1/2, JNK, *p*-JNK, p38 MAPK, phosphorylated p38 MAPK, β-actin and Histone H3 were purchased from Cell Signaling Technology (Danvers, MA, USA), and primary antibody against Nrf2 was purchased from Abcam (Abcam, Cambridge, MA, USA).

2.2. NTP treatment

The NTP generator consists of a hollow plexiglass as a reactor chamber with four electrodes, one air inlet hole and one outlet hole, which was sufficiently described in our previous studies [19,36,37]. The high-voltage electrode is a 32 mm diameter copper cylinder covered by a layer of dielectric materials quartz glass (1 mm). The ground

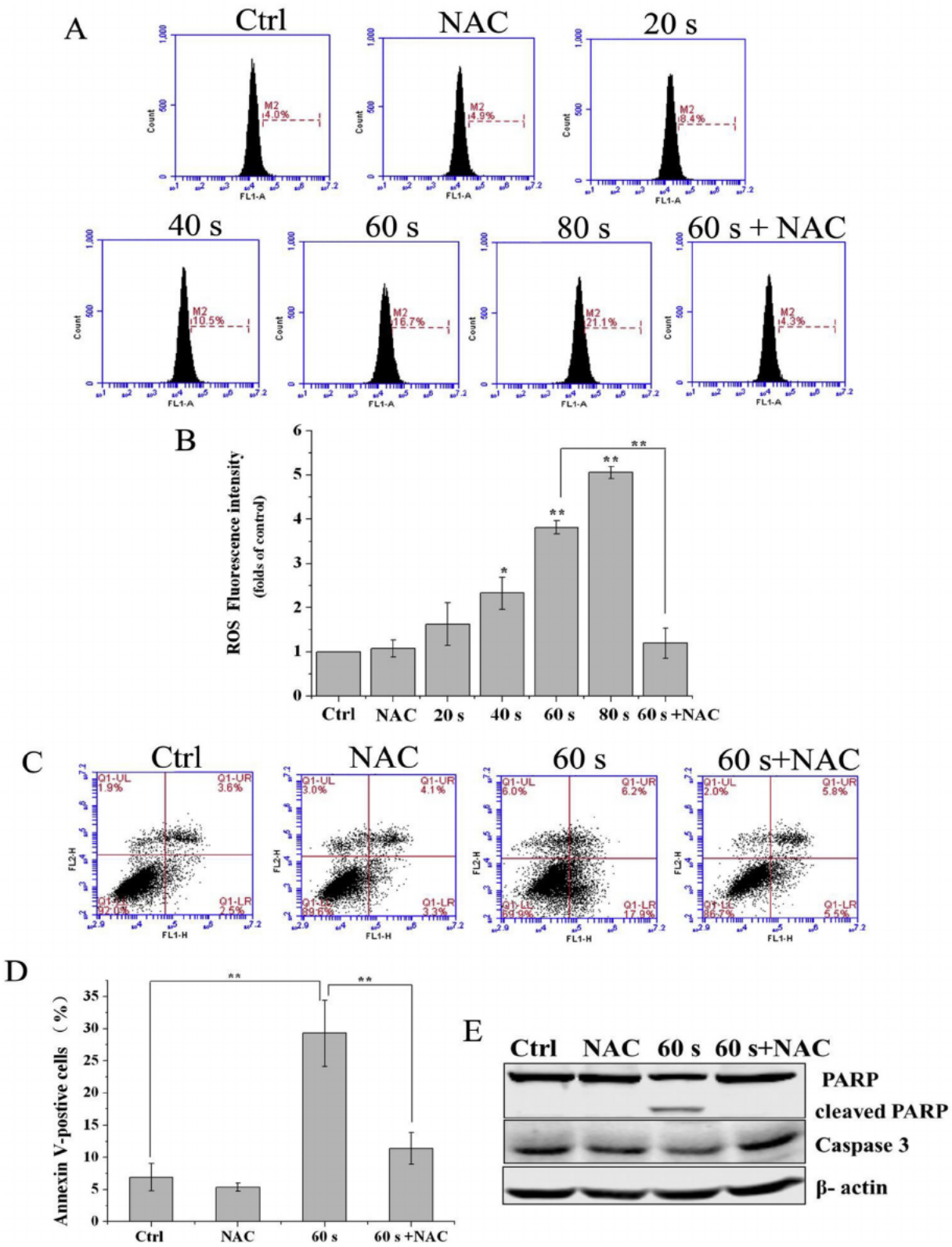

Fig. 2. ROS levels in A549 cells after NTP exposure. (A&B) ROS level was detected with 10 μM DCFDA staining using flow cytometry (2 h after NTP treatment); (C& D) Effects of NAC on apoptosis of A549 cells after NTP exposure. NAC (10 mM) treatment was performed at 2 h before NTP treatment; (E) Expression levels of apoptosis-associated proteins, PARP and Caspase 3. $*p < 0.05$, $**p < 0.01$.

electrode is a 37 mm diameter copper cylinder. NTP was generated by a voltage of 12 kV (peak to peak) with a frequency of 24 kHz. The discharge power density was measured to be about 0.9 W/cm^2. The discharge gap between the bottom of the quartz glass and medium surface was maintained at 5 mm. Helium gas (99.99%) was injected into the chamber at a rate of 80 L/h at 5 min before the experiment to expel air. For NTP exposures, cells were seeded into 35 mm diameter Petri dishes with 2 ml complete culture medium and exposed to NTP for 20, 40, 60

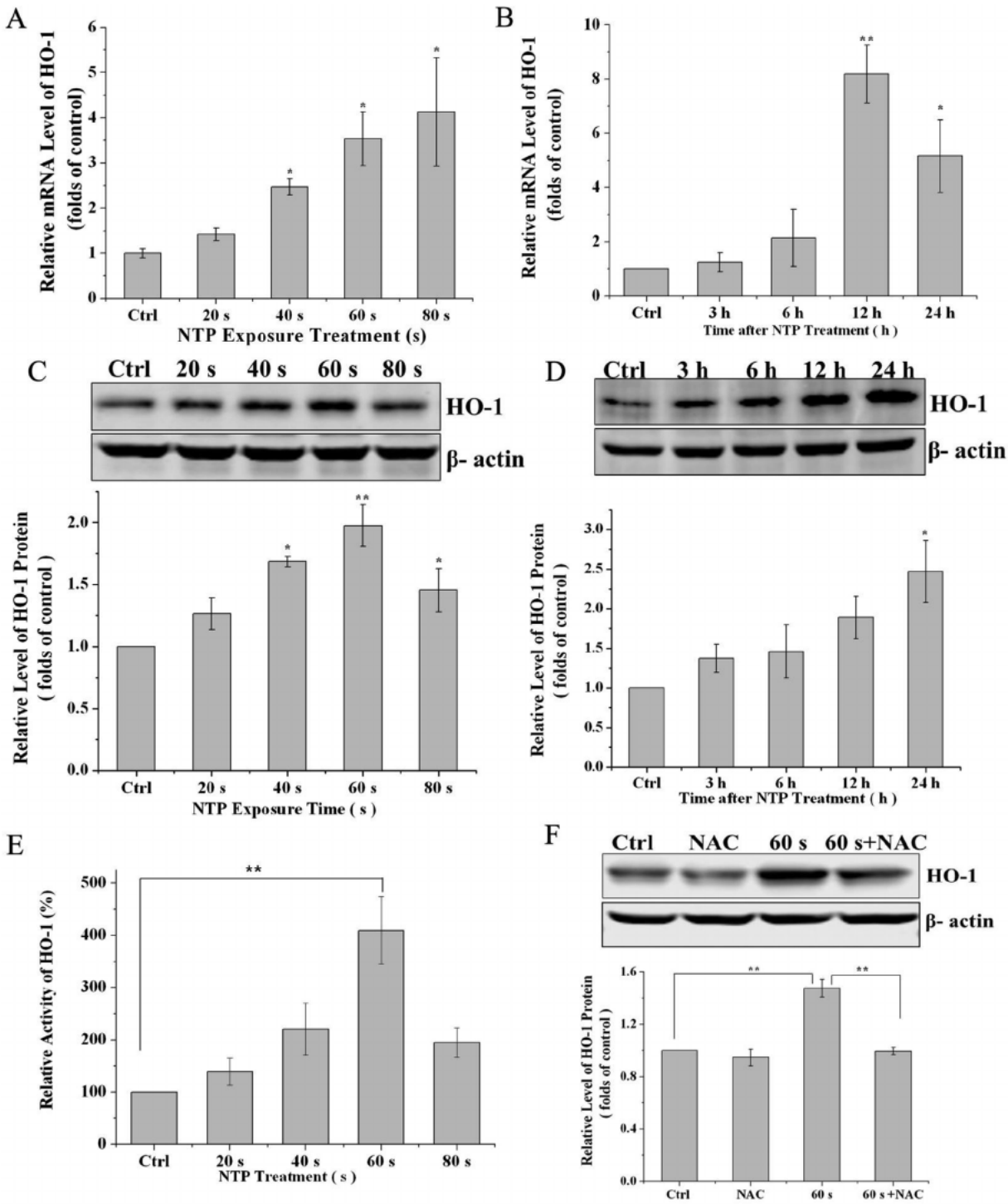

Fig. 3. Expression of HO-1 in A549 cells after NTP exposure. (A)Effect of exposure dose on HO-1 transcription; (B) Level of HO-1 transcript at different time points after NTP exposure (60 s); (C) Effect of exposure dose on HO-1 protein expression; (D) Expression level of HO-1 protein at the different time points after NTP exposure (60 s); (E) The changes of HO-1 activity after NTP treatment. (F) Effect of NAC pretreatment on HO-1 protein expression after NTP exposure. $*p < 0.05$, $**p < 0.01$.

or 80 s after overnight incubation. A Helium-only treatment was used as the control experiment.

2.3. Cell viability assay

After exposures to NTP for 20, 40, 60 or 80 s, the cells were returned to the incubator for further incubation for 24 h. The cells were incubated with the CCK-8 reagent for 1 h at 37 °C and the supernatant (100 μl) was then transferred into a 96-well plate. The absorbance was measured at 450 nm using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Rockford, IL, USA).

2.4. ROS detection with fluorescent probe

Intracellular levels of ROS were measured with the fluorescent probe CM-H2DCFDA (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. At 2 h post NTP exposures, the cells were incubated with CM-H2DCFDA (10 μM) for 30 min (37 °C) and then

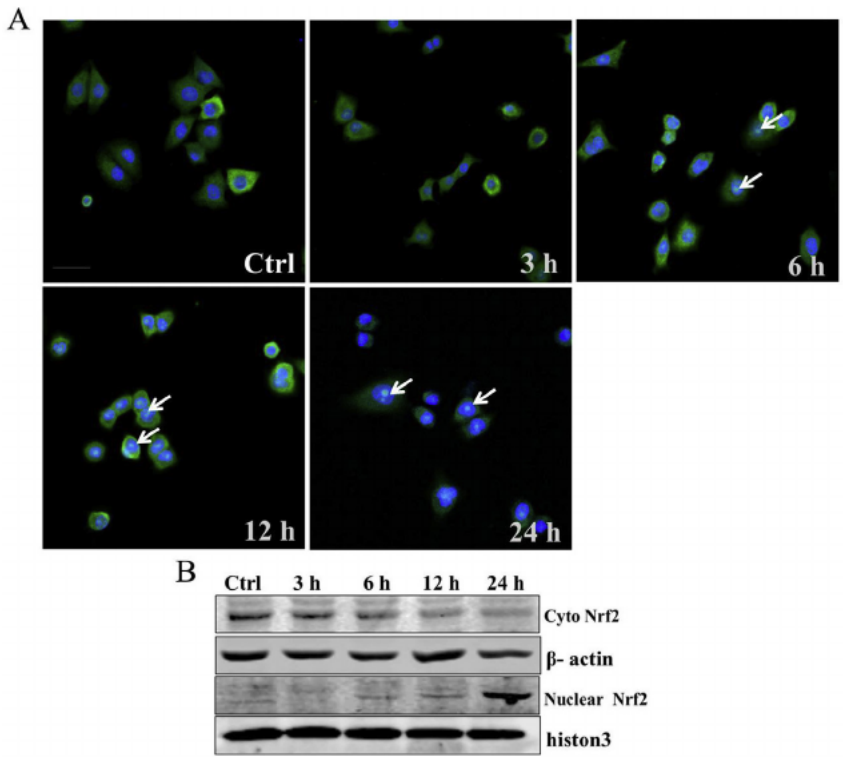

Fig. 4. NTP-induced expression of HO-1 is mediated by activation of Nrf2. (A) Cells were treated with NTP for 60 s at the indicated time, with the localization of Nrf2 determined by immunofluorescence and images captured with a confocal microscope; (B) Cellular nuclear and cytosolic lysates were separated and the level of Nrf2 in cytoplasm and nucleus were determined by Western blot.

quantified using flow cytometry (Accuri C6, BD Biosciences, Bedford, MA, USA).

2.5. Cell apoptosis detection

Cell apoptosis was detected with Annexin V- FITC/PI apoptosis detection kit (BD Biosciences, Bedford, MA, USA) according to the manufacturer's instruction. Briefly, the cells were incubated for 24 h after exposures to NTP or gas only (control). The cells were harvested, washed with PBS, and stained with Annexin V-FITC and propidium iodide (PI) for 15 min at room temperature in dark, and then analyzed using flow cytometry (Accuri C6, BD Biosciences, Bedford, MA, USA).

2.6. RNA isolation and RT-PCR analysis

Total RNA was extracted using TRIzol Reagent (Life technologies, Carlsbad, CA, US) according to the manufacturer's instructions. RT-PCR was performed with the One Step PrimeScript RT-PCR Kit (TaKaRa, Japan) and a Light Cycler 480IIinstrument (Roche, Indianapolis, IN). Gene expression levels were normalized by using a relative quantification approach (2-ΔΔCt method) with β-actin as control. The primers used in PCR amplification were shown as follows: human HO-1: 5′-GCCAGCAACAAAGTGCAAGAT -3′, 5′-GGTAAGGAAGCCAGCCAAGAG-3′, β-actin: 5′-CCACACCTTCTACAATGAGC-3′, 5′-GGTCTCAAACATGATCT GGG-3’. Each measurement was performed at least three times.

2.7. RNA interference

Specific siRNAs for HO-1 (sequence: 5′ UGCUCAACAUCCAGCU CUUtt 3′ and 5′ AAGAGCUGGAUGUUGAGCAtt 3′), Nrf2 (sequence: 5′ GCAUGCUACG UGAUGAAGAtt 3′ and 5′ UCUUCAUCACGUAGCA UGCtt 3′) and the control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection medium and transfection reagent were also purchased from Santa Cruz Biotechnology. The cells were transfected with double-stranded siRNAs for 24 h with the transfection reagent according to the manufacturer's protocols and recovered in fresh media for 24 h. The cells were then exposed to NTP and the proteins were collected at the indicated time points for further experiments.

2.8. Immunofluorescence assay

A549 cells (2×10^4 cells) were seeded onto slides and grown to 80% confluence before being exposed to NTP. The culture medium was removed, and the cells were washed twice with cold PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were then rinsed three times with cold PBS and permeabilized with PBST (PBS supplemented with 0.1% Triton X-100) for 30 min and then blocked with PBS^+ solution (PBS supplemented with 0.1% BSA) for 1 h at room temperature. After incubated with rabbit anti-Nrf2 antibady (1:200, Abcam, Cambridge, MA, USA) at 4 °C overnight and then washed three times with PBST, the cells were incubated with goat anti-rabbit IgG-FITC secondary antibody (1:500, Santa Cruz, Paso Robles, CA, USA) for 1 h at room temperature. After washed with PBST for another three times, the cells were counterstained with Hoechst 33342 (10 μg/ml, Invitrogen, Eugene, OR, USA) for 30 min at room temperature. The stained cells were mounted by a fluoroguard antifade reagent (Bio-Rad, Hercules, CA, USA) after a final wash with PBS, and fluorescent images were captured with a laser scanning confocal microscope (LSM510 META; Zeiss, Oberkochen, Germany).

2.9. HO-1 activity

HO-1 activity was evaluated by measuring bilirubin production as previously described [38]. Cells were harvested and homogenized with homogenation buffer (20 mM potassium phosphate buffer (pH 7.4), 250 mM sucrose, 2 mM EDTA, 10 μg/ml leupeptin, 2 mM PMSF). Homogenates were centrifuged 30 min at 4 °C, 10,000 g, then the supernatant were centrifuged 1h at 4 °C, 100,000g. The microsomal proteins were suspended with potassium phosphate buffer (pH 7.4). The reaction mixture containing microsomal protein, cytosolic fraction of rat liver (source of biliverdine reductase), 10 μM Hemin and 0.8 mM NADPH was incubated for 60 min at 37 °C in dark and was terminated by ice bath for 2 min. The production of bilirubin was calculated by

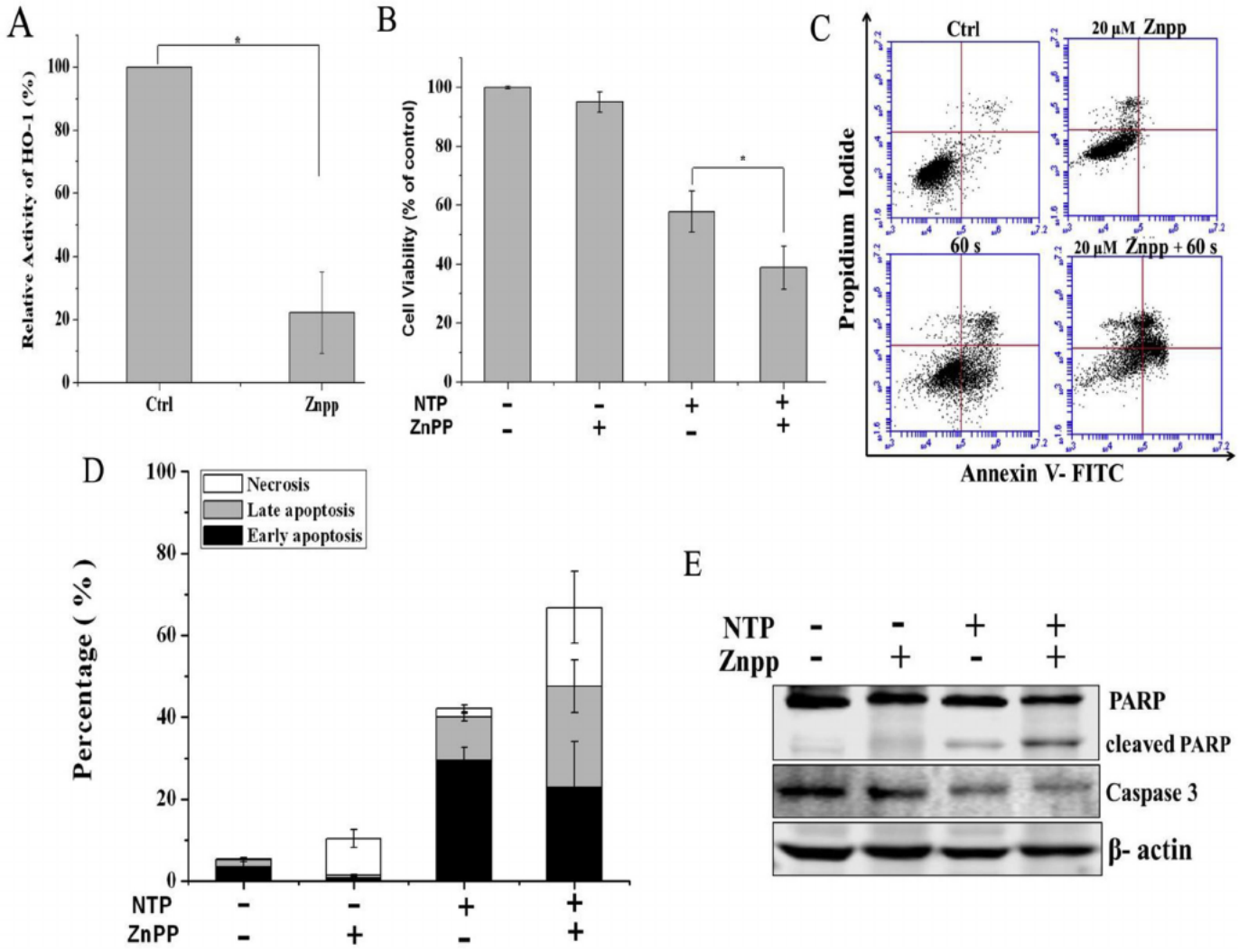

Fig. 5. Effects of ZnPP on cell death induced by NTP exposure in A549 cells. (A) The change of HO-1 activity after ZnPP treatment 18 h; (B) Effect of ZnPP on cell viability (assessed with CCK-8 assay) exposed to NTP (60 s); (C &D) Effect of ZnPP (20 μM) on apoptosis and necrosis induced by NTP exposure; (E) Effect of ZnPP (20 μM) on the expression of apoptosis associated proteins, cleaved PARP and Caspase 3. *$p < 0.05$.

measuring the difference absorbance between 465 and 530 nm by spectrophotometer, with a molar extinction coefficient of 40/mM/cm.

2.10. Western blot

Total protein was extracted with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and the protein concentration was determined with BCA Protein Assay Reagent Kit (Beyotime Biotechnology, Shanghai, China). The nuclear and cytosolic protein was extracted separately with nuclear and cytoplasmic protein extraction kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Equal amounts of protein extracts (50 μg) were fractionated by 10% SDS-PAGE, then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA, USA) and blocked with 5% non-fat dry milk for 1 h at room temperature. The membranes were incubated with the corresponding primary antibodies with appropriate dilution at 4 °C overnight, then washed three times with TBST (0.1% Tween-20 in Tris-HCl buffer) and developed with the fluorescent secondary antibodies (Alex Fluor® 790 goat anti-rabbit IgG, 1:10,000, Alex Fluor® 680 goat anti-mouse IgG, 1:10,000, LI-COR, Lincoln, NE, USA). The membranes were detected and analyzed with an Odyssey® CLx Infrared Imaging System (LI-COR, Lincoln, NE, USA).

2.11. Statistical analysis

Statistical analysis was performed on the data obtained from at least three independent experiments. The data were presented as mean ± SD. The significance was accessed using Student's *t*-test. A *p*-value less than 0.05 between two independent groups was considered statistically significant.

3. Results

3.1. ROS mediated A549 cell apoptosis after NTP exposure

Previous studies have reported that NTP treatment increased ROS production and induced apoptosis in cancer cells [8,14]. In this study, human lung cancer A549 cells (3×10^5 cells/dish) were seeded into 35 mm Petri dishes and then exposed to NTP, which was generated with an dielectric barrier discharge device described in our previous study [19,36,37] for 20, 40, 60 or 80 s after overnight incubation. Then, cell apoptosis were detected with flow cytometry (Annexin V-FITC/PI staining) and western blot (PARP and Caspase 3 expression). As shown in Fig. 1A and B, the ratio of apoptotic cells increased with NTP exposure time. When the exposure time increased to 60 or 80 s, the ratio of Annexin V positive cells significantly increased from 6.92% (control

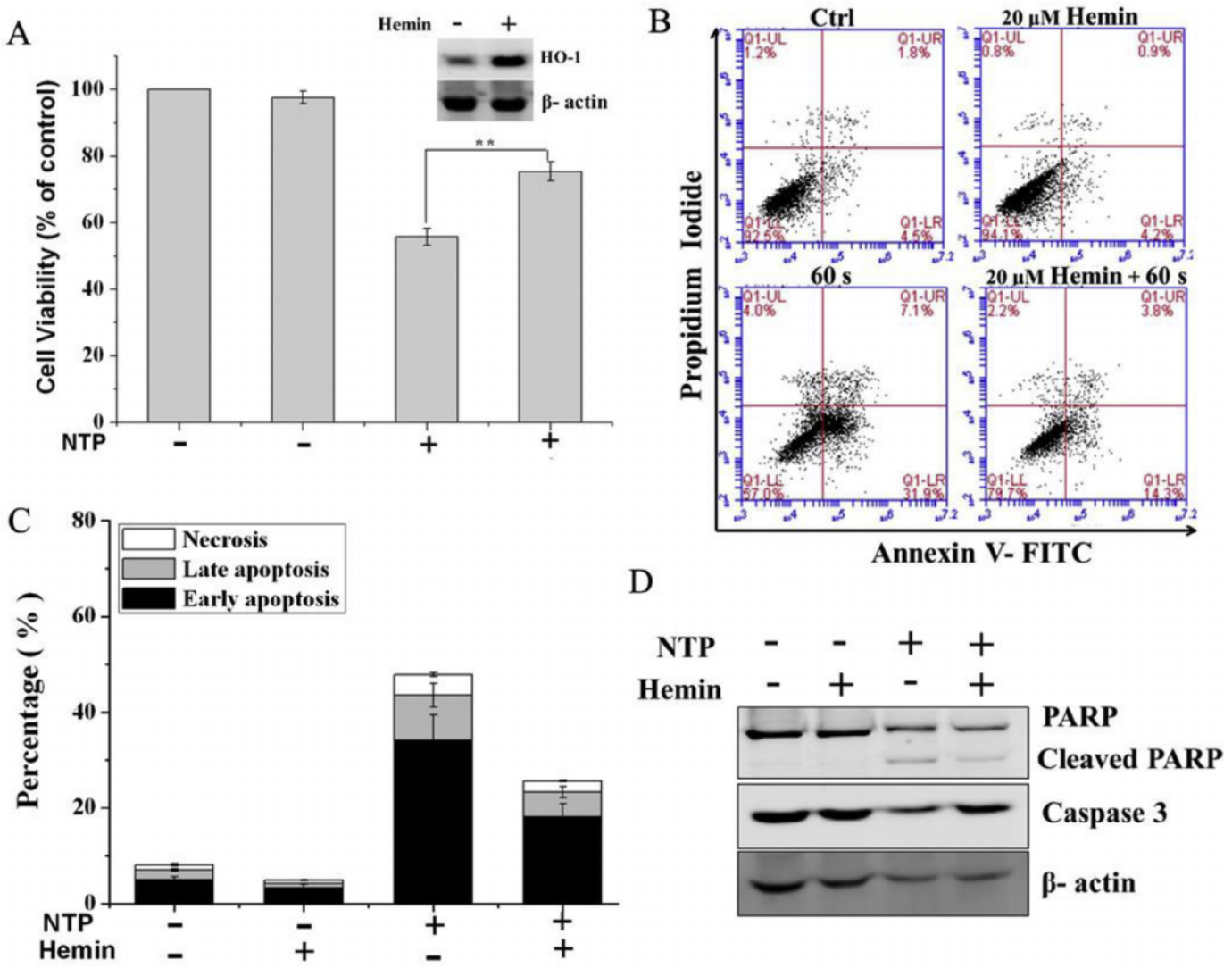

Fig. 6. Effects of Hemin on cell death induced by NTP exposure in A549 cells. (A) Effect of Hemin on cell viability (assessed with CCK-8 assay) exposed to NTP (60 s); (B &C) Effect of Hemin (20 μM) on apoptosis and necrosis induced by NTP exposure; (D) Effect of Hemin (20 μM) on the expression of apoptosis associated proteins, cleaved PARP and Caspase 3. *p < 0.05.

level) to 29.29% and 57.95%, respectively. Moreover, similar patterns were also observed in the activation of apoptosis-associated proteins, PARP and Caspase 3, in the A549 cells exposed to NTP (Fig. 1C).

To verify whether ROS-mediated apoptosis induced by NTP, the production of ROS was examined at 1–2 h after NTP treatment with the ROS fluorescent probe 2,7-dichlorofluorescein diacetate (H2DCFDA). Results in Fig. 2A and B showed that NTP treatment caused significant increase in ROS level in A549 cells, which could significantly inhibited by pre-treatment with NAC (10 mM), a ROS scavenger. The NAC treatment also significantly decreased the ratio of apoptotic cells induced by NTP (Fig. 2C and D), and decreased the level of cleaved PARP and Caspase 3 in the cells exposed to NTP (Fig. 2E). These results indicated that NTP exposures induced apoptosis, which was mediated by ROS, in A549 cells.

3.2. NTP exposure induced HO-1 expression in A549 cells

To investigate whether NTP treatment altered the expression of HO-1, the transcription level of HO-1 was measured with RT-PCR after NTP exposure in A549 cells. Results showed that NTP exposure increased the mRNA level of HO-1 in A549 cells in an exposure time dependent manner (Fig. 3A). After exposed to NTP for 60 s, the mRNA level of HO-1 increased with the time and reached the peak value at 12 h after exposure (Fig. 3B). The protein level of HO-1 was also observed to increase with the NTP exposure time (Fig. 3C) and the protein level of HO-1 kept increasing for 24 h after NTP exposure (60 s) (Fig. 3D). Moreover, HO-1 activity was significantly accelerated after NTP exposure (60 s) in A549 cells (Fig. 3E). With a pretreatment of NAC, induction of the HO-1 protein was decreased comparing with the case without NAC treatment (Fig. 3F). These results indicated that ROS generation played a critical role in HO-1 induction after NTP exposure.

It is well known that Nrf2 is an important transcriptional factor which regulate the expression of antioxidant phase II detoxifying enzymes such as HO-1, GCL, Trx to resistance oxidative stress [32]. To determine whether Nrf2 was involved in the activation of HO-1 induced by NTP, the nuclear and cytosolic levels of Nrf2 in control and NTP-treated cells was determined through immunofluorescence and western blot. The results in Fig. 4 A and B showed that the level of Nrf2 protein in the cytoplasm decreased with time after NTP exposure (60 s), but that in the nucleus showed an opposite trend. These observations indicated that Nrf2 activation, evidenced by a translocation of the protein from cytoplasm to nucleus, was initiated after the NTP exposure.

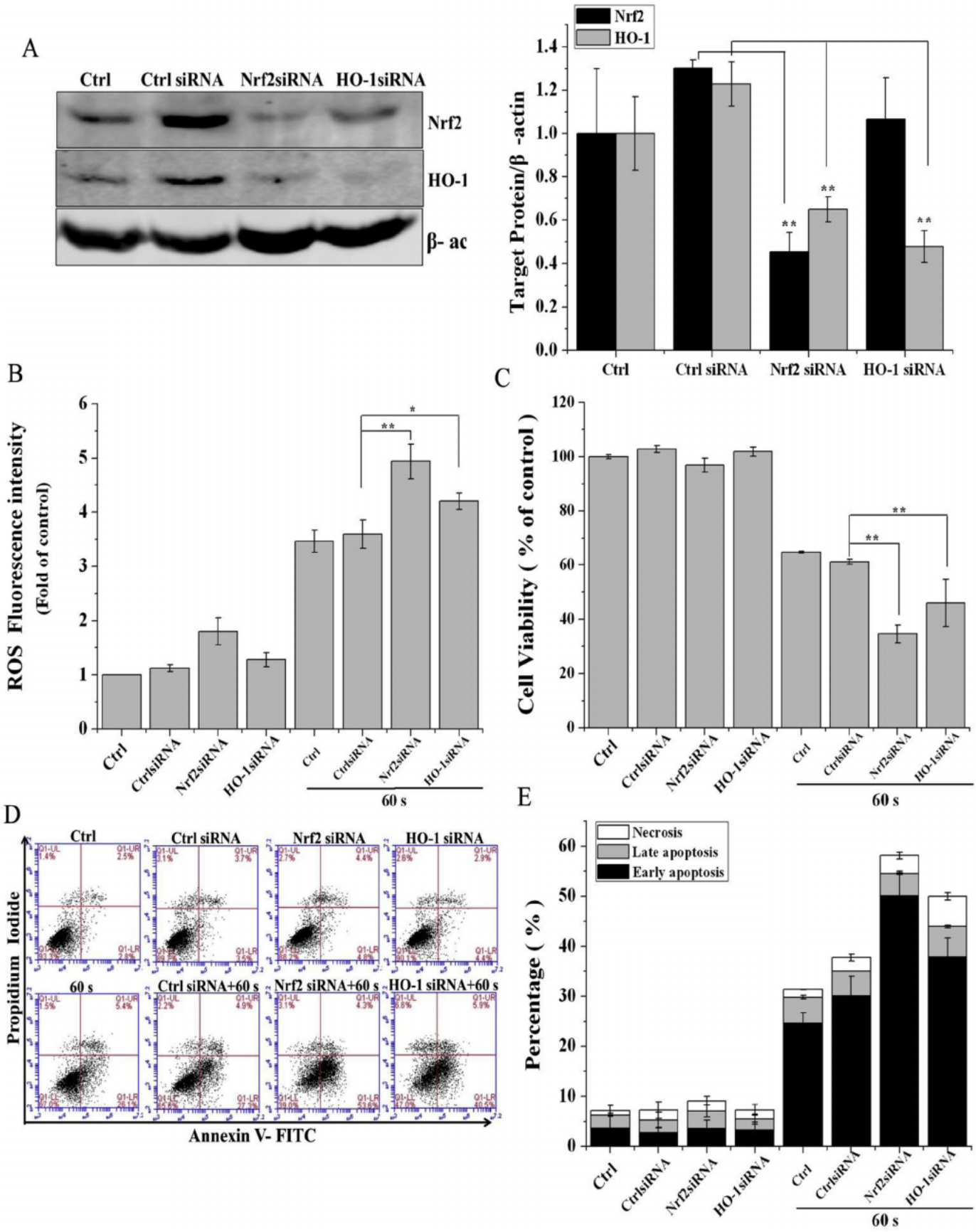

Fig. 7. Effects of knocking down Nrf 2 or HO-1 in A549 cells on cell death induced by NTP exposure. (A) Expressions of Nrf2 or HO-1 proteins after transfected with specific siRNAs or control siRNA; (B) ROS levels in control or siRNA-transfected A549 cells after NTP exposure; (C) Cell viability of control or siRNA-transfected A549 cells after NTP exposure; (D & E) Apoptosis and necrosis in control or siRNA-transfected A549 cells after NTP exposure. $^{*}p < 0.05$, $^{**}p < 0.01$.

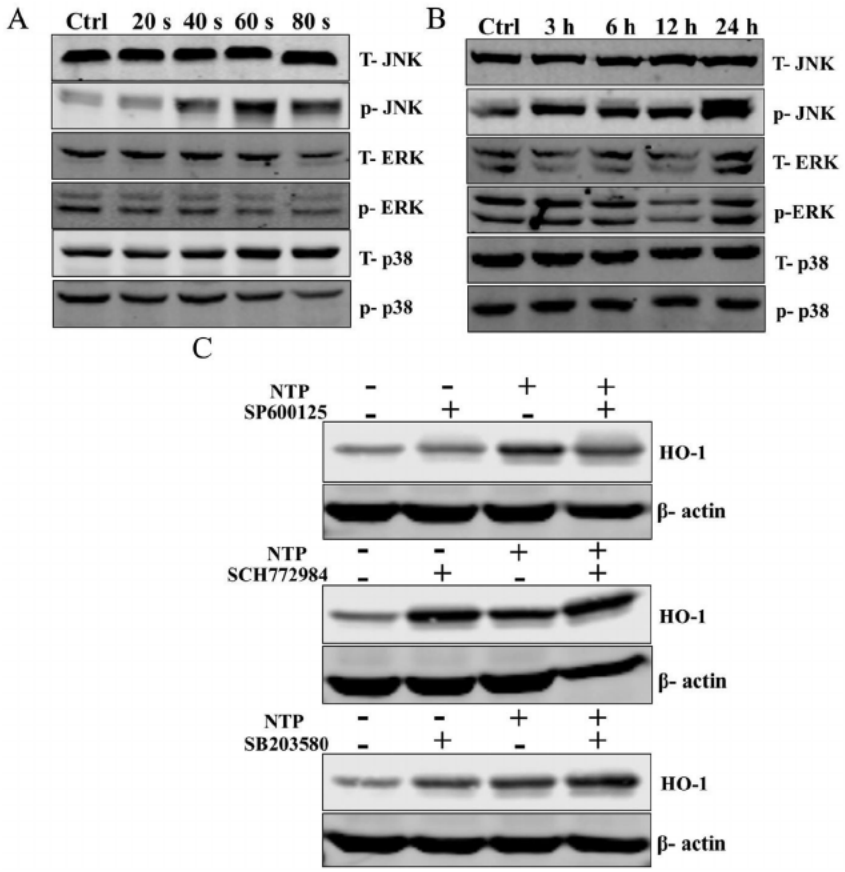

Fig. 8. Role of MAPKs pathway in the activation of Nrf2/HO-1 in A549 cells after NTP exposure. (A) Expression of critical proteins associated with the MAPK pathway at 24 after NTP exposure (0–80 s); (B) Expression of critical proteins associated with the MAPK pathway at indicated time points after NTP exposure (60 s); (C) Effects of specific inhibitors of MAPK signaling pathways on the expression of HO-1.

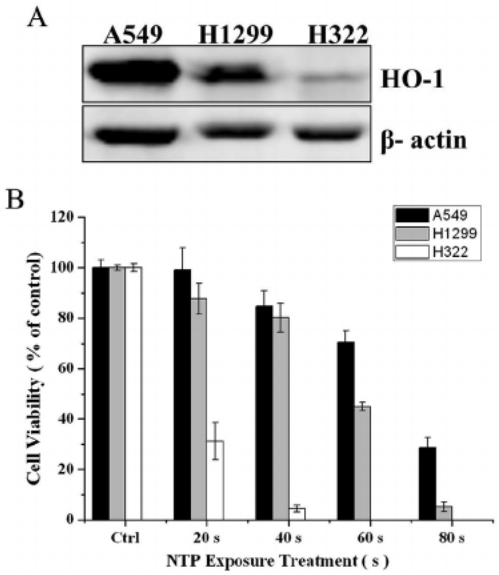

Fig. 9. Constitutive expression of HO-1 in three lung cancer cell lines. (A) Cells were harvested and total proteins were extracted to carry out western blot analysis for HO-1 protein; (B) NTP treatment inhibited cell viability in a dose-dependent manner. At 24 h after NTP exposures for 20 s, 40 s, 60 s and 80 s, the viability of cells was measured by CCK-8 assay. $*p < 0.05$, $**p < 0.01$.

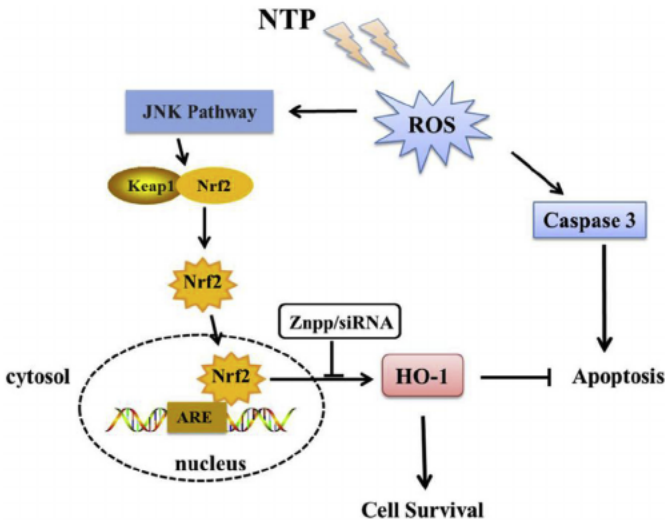

Fig. 10. Possible mechanisms of NTP-induced cell death and protective effects in A549 cells. NTP treatment can induce ROS-mediated apoptosis in A549 cells. On the other hand, ROS can also activate the antioxidant system to protect cells from oxidative damages. Accumulated intracellular ROS activates the JNK/Nrf2 pathway following upregulated expression of HO-1, while inhibition or knockdown of HO-1 enhances the cytotoxicity effect of NTP in A549 cells.

3.3. Inhibition of HO-1 enhanced the death of A549 cells exposed to NTP

To ascertain the roles of HO-1 in cell-death induction by NTP exposures in A549 cells, Zinc protoporphyrin IX, a specific competitive inhibitor of HO-1 was used. Cells were pretreated with Znpp (20 μM) for 18 h, followed by treatment with NTP (60 s). Cell viability was detected by CCK-8 assay. The results showed that HO-1 activity was inhibited in Znpp treated cells (Fig. 5A). Pretreatment with ZnPP (20 μM) led to significantly reduced cell viability compared with ZnPP (20 μM) treatment alone or NTP exposure (60 s) alone (Fig. 5A). The results on apoptosis also showed a similar trend. NTP and ZnPP co-treatment increased the ratio of apoptotic and necrotic death in A549 cells (Fig. 5C and D) and the apoptosis associated proteins (PARP

and Caspase 3) were also activated (Fig. 5E).

HO-1 inducer Hemin was used to determine the protective role of HO-1 induced by NTP. Hemin (20 μM) was added 18 h before NTP exposure. As shown in Fig. 6A, pretreatment with Hemin increased the expression of HO-1 and partly reversed NTP-induced cell death. The results also showed that pretreatment with Hemin effectively decreased the ratio of apoptotic cells induced by NTP (Fig. 6B and C) and the level of cleaved PARP and Caspase 3 (Fig. 6D). These results indicated that the normal function of HO-1 was important in helping A549 cells to defend against the stress of NTP.

3.4. Knocking down HO-1 or Nrf2 enhanced the death of A549 cells exposed to NTP

To investigate the functional role of HO-1 in A549 cells, the HO-1/Nrf2 special siRNA was utilized to knockdown the HO-1/Nrf2 expression. The negative control siRNA was used as negative control. After transfection with HO-1 siRNA for 24 h, HO-1 expression was confirmed by Western blot analysis. By transfection of specific siRNA, the gene expression of Nrf2 or HO-1 was significantly knocked down compared with the transfection with control siRNA (Fig. 7A). After NTP exposure, the ROS levels in A549 cells with knocked down Nrf2 or HO-1 distinctly increased when compared to the control (Fig. 7B). On the contrary, the viability of A549 cells with knocked down Nrf2 or HO-1 significantly decreased (Fig. 7C). The results on apoptosis showed that the ratios of necrotic and apoptotic cells were also higher than those of the control after NTP exposure (Fig. 7D and E).

3.5. JNK pathway may be involved in activation of Nrf2/HO-1 in A549 cells after NTP exposure

MAPK signaling pathways (p38 MAPK, ERK1/2 or JNK) were reported to regulate the activation of Nrf2/HO-1 [39]. To confirm which pathway was involved in the activation of Nrf2/HO-1 after NTP exposure, we determined the critical proteins in the MAPK pathway with western blot. The results in Fig. 8A and B showed that NTP exposures significantly increased the expression of phosphorylated JNK, but not phosphorylated ERK1/2 and p38 MAPK. With pretreatment with the JNK specific inhibitor (SP600125), ERK1/2 specific inhibitor (SCH772984) or p38 MAPK specific inhibitor (SB203580) at 2 h before NTP exposures, the expression of HO-1 protein was determined. The results in Fig. 8C showed that pretreatment with SP600125, a specific inhibitor of JNK, decreased the expression of HO-1, but SCH772984 or SB203580, specific inhibitors of ERK1/2 or p38 MAPK did not.

3.6. Basal level of HO-1 indicated the cell sensitivity to NTP exposure

Accumulated evidence showed that HO-1 overexpression played a key role in development of resistance to chemotherapy or radiotherapy [23,40]. To further explore whether overexpression of HO-1 in cancer cells be associated with more resistance to NTP-caused cell death, the basal expression of HO-1 was determined in three lung cancer cell lines (A549, H1299 and H322) and the corresponding cell viability was measured after NTP exposure (60 s). The results in Fig. 9A showed that the basal levels of HO-1 was A549 > H1299 > H322. The viability of these three cell lines exposed to NTP also showed the trend A549 > H1299 > H322 (Fig. 9B). These results indicated that the protein level of basal HO-1 had tight relationship with the sensitivity or resistance to NTP exposures, which could potentially be a useful indicator of prognosis for NTP cancer therapy.

4. Discussion

NTP treatment has been proved very effective against cancer cells in *in vitro* studies, and has thus attracted the attention of the research community as a promising novel cancer therapy technique. Numerous studies showed that NTP treatment enhanced intracellular ROS generation, caused DNA damages and induced apoptotic cancer cell death [15,20,41]. Several studies also showed that NTP induced cancer cell apoptosis through various signaling pathway: activating TNF-ASK1 pathway [8], targeting cell cycle [17], inhibiting cell invasion [42,43], involving DNA damages [16,44], triggering sub-G1 arrest *via* the ATM/p53 pathway [45] and so on. In the present study, we also demonstrated that NTP up-regulated intracellular ROS production, and induced apoptotic cell death in a dose-dependent manner while pretreatment with NAC (a ROS scavenger) could effectively repress cell death. However, the intracellular mechanism involved in NTP-induced antioxidant responses is still unclear.

The specific mechanisms of HO-1 induction might depend on the types of inducers and cells. Previous studies reported that hydrogen peroxide (H_2O_2) up-regulated HO-1 expression through activation of JNK and p38-MAPK pathways in H9c2 cells [34], whereas caffeic acid phenethyl ester (CAPE) induced HO-1 expression through activation of the ERK pathway [35]. The effects and mechanisms of HO-1 induction by NTP in A549 cells are still unknown.

HO-1 is one of the cytoprotective enzymes and its up-regulation protects against cell death induced by oxidative stress [46,47]. Previous studies also found that HO-1 overexpression was related to resistance to cancer therapy [23,48]. Up-regulation of HO-1 induced by Fisetin was also identified as a protection mechanism against cell death induced by H_2O_2 in human umbilical vein endothelial cells (HUVECs) [47]. Overexpression of HO-1 with transfection of a full-length human HO-1 cDNA exhibited more resistance to hyperbaric oxygen (HBO)-induced DNA damages in V79 cells [49]. Furthermore, suppression of HO-1 enhanced the sensitivity to radiotherapy [30] and chemotherapy [29,50], and reduced tumor growth [28]. Our results showed that NTP treatment induced HO-1 expression in dose- and time-dependent manners. Furthermore, our data revealed that knockdown of HO-1 with the special siRNA or the combination treatment with ZnPP significantly enhanced the cytotoxicity effect of NTP in A549 cells. Pretreatment with HO-1 inducer Hemin decreased NTP-induced cell death and the ratio of cell apoptosis. We also found that a higher basal level of HO-1 expression might contribute to resistance in A549 cells against cell death induced by NTP treatment. Therefore, HO-1 can serve as effective target to improve NTP therapy.

Nrf2 is an important transcription factor which regulates the expression of the HO-1 gene against oxidative damages [51]. Numerous studies reported that some chemicals induced the expression of HO-1 *via* ROS generation and induction of Nrf2 for protection against oxidative stress [52,53]. For example, Celastrol induced up-regulation of HO-1 expression in HaCaT cells *via* ROS generation upon activation of the ERK/p38-Nrf2-ARE pathway [53]. Anke et al. revealed that NTP treatment activated the Nrf2/Keap1 pathway and up-regulated the mRNA and protein expression of NQO-1 and HO-1 to protect HaCaT cells against oxidative stress, which facilitated wound healing [54]. Musarat et al. found that the up-regulation of sulfiredoxin (Srx) as a protective factor against NTP-induced oxidative stress was mediated by activation of Nrf2 [9]. Our results showed that the induction of HO-1 expression by NTP treatment was mediated by ROS generation, which activated Nrf2 translocation from cytoplasm to nucleus. Furthermore, knockdown of Nrf2 with the special siRNA led to ROS overproduction and increased the cytotoxicity effect of NTP in A549 cells.

MAPK pathways, including JNK, ERK and p38 MAPK, are involved in regulating many cellular processes, including cell proliferation, cell death and metastasis [55]. Numerous studies reported that MAPK pathways were involved in regulating the HO-1 expression [34,48,56]. Celastrol protected HaCaT cells against inflammation by up-regulating HO-1 expression *via* activation of ERK and p38 MAPK pathways [53]. The JNK pathway was also found involved in the up-regulation of HO-1 induced by H_2O_2 in H9c2 cells [34]. Our results demonstrated that NTP treatment enhanced the phosphorylation of JNK and pre-treatment with JNK inhibitors (SP600125) decreased the NTP-induced HO-1

expression. These results suggested that up-regulation of HO-1 by NTP could be mediated through activation of the JNK signaling pathway in A549 cells.

It was reported that NTP can altered antioxidant system, including glutathione peroxidases (GPX), glutathione reductase (GSR), glutathione S-transferases (GST), cytochrome P450 family members, which may contribute to reducing NTP-induced ROS level in cells [57,58]. Previous studies also proved that iron produced by heme metabolism can up regulation of ferritin expression which plays protective role, not HO-1 [59–61]. However, our results found that knocked down ferritin expression with special siRNA did not increased NTP-induced cell death (Fig. S1). This finding indicated that ferritin may not play the protective role in NTP-induced cell death. Our study also found that pretreatment with HO-1 inducer Hemin did not completely recovered NTP-induced cell death. This study found that HO-1 play an important protective role against NTP-induced cell death, however, there still some other antioxidant proteins involved in this process.

In conclusion, the present study demonstrated that NTP exposures caused two opposite effects in A549 cells, including induction of cell death and activation of antioxidant response. The possible mechanisms are depicted in Fig. 10. Induction of HO-1 expression plays a protective role against NTP-induced cell death *via* the JNK-Nrf2 pathway in A549 cells. On the other hand, inhibition of Nrf2/HO-1 leads to ROS overproduction and enhances the cytotoxicity effect of NTP. These findings facilitates a better understanding on the interaction between NTP and cancer cells, and provides useful information to guide clinical applications of NTP in future.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2018.09.015.

References

[1] M. Keidar, A. Shashurin, O. Volotskova, M.A. Stepp, P. Srinivasan, A. Sandler, B. Trink, Cold atmospheric plasma in cancer therapy, Phys. Plasmas 20 (2013) 057101.
[2] K. Song, G. Li, Y. Ma, A review on the selective apoptotic effect of nonthermal atmospheric-pressure plasma on cancer cells, Plasma Med. 4 (2014) 193–209.
[3] D. Yan, J.H. Sherman, M. Keidar, Cold atmospheric plasma, a novel promising anti-cancer treatment modality, Oncotarget 8 (2017) 15977–15995.
[4] M. Wang, B. Holmes, X. Cheng, W. Zhu, M. Keidar, L.G. Zhang, Cold atmospheric plasma for selectively ablating metastatic breast cancer cells, PLoS One 8 (2013) e73741.
[5] J. Huang, W. Chen, H. Li, X.Q. Wang, G.H. Lv, M.L. Khohsa, M. Guo, K.C. Feng, P.Y. Wang, S.Z. Yang, Deactivation of A549 cancer cells in vitro by a dielectric barrier discharge plasma needle, J. Appl. Phys. 109 (2011) 053305.
[6] X. Zhang, M. Li, R. Zhou, K. Feng, S. Yang, Ablation of liver cancer cells in vitro by a plasma needle, Appl. Phys. Lett. 93 (2008) 021502.
[7] G. Fridman, A. Shereshevsky, M.M. Jost, A.D. Brooks, A. Fridman, A. Gutsol, V. Vasilets, G. Friedman, Floating electrode dielectric barrier discharge plasma in air promoting apoptotic behavior in melanoma skin cancer cell lines, Plasma Chem. Plasma Process. 27 (2007) 163–176.
[8] M. Ishaq, S. Kumar, H. Varinli, Z.J. Han, A.E. Rider, M.D. Evans, A.B. Murphy, K. Ostrikov, Atmospheric gas plasma-induced ROS production activates TNF-ASK1 pathway for the induction of melanoma cancer cell apoptosis, Mol. Biol. Cell 25 (2014) 1523–1531.
[9] M. Ishaq, M.D. Evans, K.K. Ostrikov, Atmospheric pressure gas plasma-induced colorectal cancer cell death is mediated by Nox2-ASK1 apoptosis pathways and oxidative stress is mitigated by Srx-Nrf2 anti-oxidant system, Biochim. Biophys. Acta 1843 (2014) 2827–2837.
[10] M. Keidar, R. Walk, A. Shashurin, P. Srinivasan, A. Sandler, S. Dasgupta, R. Ravi, R.G. Preston, B. Trink, Cold plasma selectivity and the possibility of a paradigm shift in cancer therapy, Br. J. Canc. 105 (2011) 1295–1301.
[11] S.Y. Kim, H.J. Kim, S.U. Kang, Y.E. Kim, J.K. Park, Y.S. Shin, Y.S. Kim, K. Lee, C.H. Kim, Non-thermal plasma induces AKT degradation through turn-on the MUL1 E3 ligase in head and neck cancer, Oncotarget 6 (2015) 33382–33396.
[12] C. Tendero, C. Tixier, P. Tristant, J. Desmaison, P. Leprince, Atmospheric pressure plasmas: a review, Spectrochim. Acta B. 61 (2006) 2–30.
[13] X. Lu, G.V. Naidis, M. Laroussi, S. Reuter, D.B. Graves, K. Ostrikov, Reactive species in non-equilibrium atmospheric-pressure plasmas: generation, transport, and biological effects, Phys. Rep. 630 (2016) 1–84.
[14] X. Yan, Z. Xiong, F. Zou, S. Zhao, X. Lu, G. Yang, G. He, K. Ostrikov, Plasma-induced death of HepG2 cancer cells: intracellular effects of reactive species, Plasma Process. Polym. 9 (2012) 59–66.
[15] M. Vandamme, E. Robert, S. Lerondel, V. Sarron, D. Ries, S. Dozias, J. Sobilo, D. Gosset, C. Kieda, B. Legrain, J.M. Pouvesle, A. Le Pape, ROS implication in a new antitumor strategy based on non-thermal plasma, Int. J. Canc. 130 (2012) 2185–2194.
[16] X. Han, M. Klas, Y. Liu, M. Sharon Stack, S. Ptasinska, DNA damage in oral cancer cells induced by nitrogen atmospheric pressure plasma jets, Appl. Phys. Lett. 102 (2013) 233703.
[17] O. Volotskova, T.S. Hawley, M.A. Stepp, M. Keidar, Targeting the cancer cell cycle by cold atmospheric plasma, Sci. Rep. 2 (2012) 636.
[18] K. Panngom, K.Y. Baik, M.K. Nam, J.H. Han, H. Rhim, E.H. Choi, Preferential killing of human lung cancer cell lines with mitochondrial dysfunction by nonthermal dielectric barrier discharge plasma, Cell Death Dis. 4 (2013) e642.
[19] W. Li, K.N. Yu, L. Bao, J. Shen, C. Cheng, W. Han, Non-thermal plasma inhibits human cervical cancer HeLa cells invasiveness by suppressing the MAPK pathway and decreasing matrix metalloproteinase-9 expression, Sci. Rep. 6 (2016) 19720.
[20] R. Sensenig, S. Kalghatgi, E. Cerchar, G. Fridman, A. Shereshevsky, B. Torabi, K.P. Arjunan, E. Podolsky, A. Fridman, G. Friedman, J. Azizkhan-Clifford, A.D. Brooks, Non-thermal plasma induces apoptosis in melanoma cells via production of intracellular reactive oxygen species, Ann. Biomed. Eng. 39 (2011) 674–687.
[21] J. Ye, S.G. Laychock, A protective role for heme oxygenase expression in pancreatic islets exposed to Interleukin-1β, Endocrinology 139 (1998) 4155–4163.
[22] T. Yoshida, N. Maulik, Y.S. Ho, J. Alam, D.K. Das, Hmox-1 constitutes an adaptive response to effect antioxidant cardioprotection: a study with transgenic mice heterozygous for targeted disruption of the heme Oxygenase-1 gene, Circulation 103 (2001) 1695–1701.
[23] M.H. Kweon, V.M. Adhami, J.S. Lee, H. Mukhtar, Constitutive overexpression of Nrf2-dependent heme oxygenase-1 in A549 cells contributes to resistance to apoptosis induced by epigallocatechin 3-gallate, J. Biol. Chem. 281 (2006) 33761–33772.
[24] M.D. Maines, P.A. Abrahamsson, Expression of heme oxygenase-1 (HSP32) in human prostate: normal, hyperplastic, and tumor tissue distribution, Urology 47 (1996) 727.
[25] G. Speit, C. Dennog, Eichhorn, A. Rothfuβ, B. Kaina, Induction of hemo oxygenase-1 and adaptive protection againse the idnuction of DNA damage after hyperbaric oxygen treatment, Carcinogenesis 21 (2000) 1795–1799.
[26] K. Doi, T. Akaike, S. Fujii, S. Tanaka, N. Ikebe, T. Beppu, S. Shibahara, M. Ogawa, H. Maed, Induction of haem oxygenase-1 by nitric oxide andischaemia in experimental solid tumours and implications for tumour growth, Br. J. Canc. 80 (1999) 1945–1954.
[27] D. Lang, S. Reuter, T. Buzescu, C. August, S. Heidenreich, Heme-induced heme oxygenase-1 (HO-1) in human monocytes inhibits apoptosis despite caspase-3 up-regulation, Int. Immunol. 17 (2005) 155–165.
[28] K. Hirai, T. Sasahira, H. Ohmori, K, Fujii H. Kuniyasu, Inhibition of heme oxygenase-1 by zinc protoporphyrin IX reduces tumor growth of LL/2 lung cancer in C57BL mice, Int. J. Canc. 120 (2007) 500–505.
[29] H.R. Kim, S. Kim, E.J. Kim, J.H. Park, S.H. Yang, E.T. Jeong, C. Park, M.J. Youn, H.S. So, R. Park, Suppression of Nrf2-driven heme oxygenase-1 enhances the chemosensitivity of lung cancer A549 cells toward cisplatin, Lung Canc. 60 (2008) 47–56.
[30] W. Zhang, T. Qiao, L. Zha, Inhibition of heme oxygenase-1 enhances the radiosensitivity in human nonsmall cell lung cancer a549 cells, Canc. Biother. Rad. 26 (2011) 639–645.
[31] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J.D. Engel, M. Yamamoto, Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, Gene Dev. 13 (1999) 76–86.
[32] T.C. Huang, Y.L. Chung, M.L. Wu, S.M. Chuang, Cinnamaldehyde enhances Nrf2 nuclear translocation to upregulate phase II detoxifying enzyme expression in HepG2 cells, J. Agric. Food Chem. 59 (2011) 5164–5171.
[33] K. Itoh, T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamotoa, Y. Nabeshimab, An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements, Biochem. Bioph. Res. Co. 236 (1997) 313.
[34] K.S. Aggeli I, C. Gaitanaki, I. Beis, Involvement of JNKs and p38-MAPK/MSK1 pathways in H2O2-induced upregulation of heme oxygenase-1 mRNA in H9c2 cells, Cell. Signal. 18 (2006) 1801–1812.
[35] J.K. Kim, H.D. Jang, Nrf2-mediated HO-1 induction coupled with the ERK signaling

pathway contributes to indirect antioxidant capacity of caffeic acid phenethyl ester in HepG2 cells, Int. J. Mol. Sci. 15 (2014) 12149–12165.
[36] J. Hou, J. Ma, K.N. Yu, W. Li, C. Cheng, L. Bao, W. Han, Non-thermal plasma treatment altered gene expression profiling in non-small-cell lung cancer A549 cells, BMC Genom. 16 (2015) 435.
[37] J. Ma, H. Zhang, C. Cheng, J. Shen, L. Bao, W. Han, Contribution of hydrogen peroxide to non-thermal atmospheric pressure plasma induced A549 lung cancer cell damage, Plasma Process. Polym. 9999 (2016) 1–12.
[38] K. Hirai, T. Sasahira, H. Ohmori, et al., Inhibition of heme oxygenase-1 by zinc protoporphyrin IX reduces tumor growth of LL/2 lung cancer in C57BL mice, Int. J. Canc. 120 (2007) 500.
[39] M. Kobayashi, M. Yamamoto, Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation, Antioxidants Redox Signal. 7 (2005) 385–394.
[40] P.O. Berberat, Z. Dambrauskas, A. Gulbinas, T. Giese, N. Giese, B. Künzli, F. Autschbach, S. Meuer, M.W. Büchler, H. Friess, Inhibition of heme Oxygenase-1 increases responsiveness of pancreatic cancer cells to anticancer treatment, Clin. Canc. Res. 11 (2005) 3790–3798.
[41] S.U. Kang, J.H. Cho, J.W. Chang, Y.S. Shin, K.I. Kim, J.K. Park, S.S. Yang, J.S. Lee, E. Moon, K. Lee, C.H. Kim, Nonthermal plasma induces head and neck cancer cell death: the potential involvement of mitogen-activated protein kinase-dependent mitochondrial reactive oxygen species, Cell Death Dis. 5 (2014) e1056.
[42] J.W. Chang, S.U. Kang, Y.S. Shin, K.I. Kim, S.J. Seo, S.S. Yang, J.S. Lee, E. Moon, K. Lee, C.H. Kim, Non-thermal atmospheric pressure plasma inhibits thyroid papillary cancer cell invasion via cytoskeletal modulation, altered MMP-2/-9/uPA activity, PLoS One 9 (2014) e92198.
[43] C.H. Kim, S. Kwon, J.H. Bahn, K. Lee, S.I. Jun, P.D. Rack, S.J. Baek, Effects of atmospheric nonthermal plasma on invasion of colorectal cancer cells, Appl. Phys. Lett. 96 (2010) 243701.
[44] X. Yan, F. Zou, X.P. Lu, G. He, M.J. Shi, Q. Xiong, X. Gao, Z. Xiong, Y. Li, F.Y. Ma, M. Yu, C.D. Wang, Y. Wang, G. Yang, Effect of the atmospheric pressure nonequilibrium plasmas on the conformational changes of plasmid DNA, Appl. Phys. Lett. 95 (2009) 083702.
[45] J.W. Chang, S.U. Kang, Y.S. Shin, K.I. Kim, S.J. Seo, S.S. Yang, J.S. Lee, E. Moon, S.J. Baek, K. Lee, C.H. Kim, Non-thermal atmospheric pressure plasma induces apoptosis in oral cavity squamous cell carcinoma: involvement of DNA-damage-triggering sub-G(1) arrest via the ATM/p53 pathway, Arch. Biochem. Biophys. 545 (2014) 133–140.
[46] S.E. Lee, S.I. Jeong, G.D. Kim, H. Yang, C.S. Park, Y.H. Jin, Y.S. Park, Upregulation of heme oxygenase-1 as an adaptive mechanism for protection against crotonaldehyde in human umbilical vein endothelial cells, Toxicol. Lett. 201 (2011) 240–248.
[47] S.E. Lee, S.I. Jeong, H. Yang, C.S. Park, Y.H. Jin, Y.S. Park, Fisetin induces Nrf2-mediated HO-1 expression through PKC-δ and p38 in human umbilical vein endothelial cells, J. Cell. Biochem. 112 (2011) 2352–2360.
[48] M.S. Degese, J.E. Mendizabal, N.A. Gandini, J.S. Gutkind, A. Molinolo, S.M. Hewitt, A.C. Curion, O.A. Coso, M.M. Facchinetti, Expression of heme oxygenase-1 in non-small cell lung cancer (NSCLC) and its correlation with clinical data, Lung Canc. 77 (2012) 168–175.
[49] A. Rothfuss, G. Speit, Overexpression of heme oxygenase-1 (HO-1) in V79 cells results in increased resistance to hyperbaric oxygen (HBO)-induced DNA damage, Environ. Mol. Mutagen. 40 (2002) 258–265.
[50] X.F. Zhu, W. Li, J.Y. Ma, N. Shao, Y.J. Zhang, R.M. Liu, W.B. Wu, Y. Lin, S.M. Wang, Knockdown of heme oxygenase-1 promotes apoptosis and autophagy and enhances the cytotoxicity of doxorubicin in breast cancer cells, Oncol. Lett. 10 (2015) 2974–2980.
[51] J.J. Sun, S. Piao, Y.N. Cha, C. Kim, Taurine chloramine activates Nrf2, increases HO-1 expression and protects cells from death caused by hydrogen peroxide, J. Clin. Biochem. Nutr. 45 (2009) 37–43.
[52] Y.J. Lee, H.Y. Jeong, Y.B. Kim, Y.J. Lee, S.Y. Won, J.H. Shim, M.K. Cho, H.S. Nam, S.H. Lee, Reactive oxygen species and PI3K/Akt signaling play key roles in the induction of Nrf2-driven heme oxygenase-1 expression in sulforaphane-treated human mesothelioma MSTO-211H cells, Food Chem. Toxicol. 50 (2012) 116–123.
[53] W.Y. Seo, A.R. Goh, S.M. Ju, H.Y. Song, D.J. Kwon, J.G. Jun, B.C. Kim, S.Y. Choi, J. Park, Celastrol induces expression of heme oxygenase-1 through ROS/Nrf2/ARE signaling in the HaCaT cells, Biochem. Bioph. Res. Co. 407 (2011) 535–540.
[54] A. Schmidt, S. Dietrich, A. Steuer, K.D. Weltmann, W.T. von, K. Masur, K. Wende, Non-thermal plasma activates human keratinocytes by stimulation of antioxidant and phase II pathways, J. Biol. Chem. 290 (2015) 6731–6750.
[55] J.R. Woodgett, J.M. Kyriakis, J. Avruch, L.I. Zon, B. Zanke, D.J. Templeton, Reconstitution of novel signalling cascades responding to cellular stresses, Philos. T. R. Soc. B. 351 (1996) 135–142.
[56] E.J. Park, Y.M. Kim, S.W. Park, H.J. Kim, J.H. Lee, D.U. Lee, K.C. Chang, Induction of HO-1 through p38 MAPK/Nrf2 signaling pathway by ethanol extract of Inula helenium L. reduces inflammation in LPS-activated RAW 264.7 cells and CLP-induced septic mice, Food Chem. Toxicol. 55 (2013) 386–395.
[57] N.K. Kaushik, N. Kaushik, D. Park, et al., Altered antioxidant system stimulates dielectric barrier discharge plasma-induced cell death for solid tumor cell treatment, PLoS One 9 (2014) e103349.
[58] A. Schmidt, K. Wende, S. Bekeschus, et al., Non-thermal plasma treatment is associated with changes in transcriptome of human epithelial skin cells, Free Radic. Res. 47 (2013) 577–592.
[59] G. Balla, H.S. Jacob, J. Balla, et al., Ferritin: a cytoprotective antioxidant strategem of endothelium, J. Biol. Chem. (1992) 18148–18153.
[60] J. Cermak, J. Balla, H.S. Jacob, et al., Tumor cell heme uptake induces ferritin synthesis resulting in altered oxidant sensitivity: possible role in chemotherapy efficacy, Canc. Res. 53 (1993) 5308–5313.
[61] T. Wu, Y. Li, B. Liu, et al., Expression of ferritin Light chain (FTL) is elevated in glioblastoma, and FTL silencing inhibits glioblastoma cell proliferation via the GADD45/JNK pathway, PLoS One (2016) e0149361.