

# Therapeutic efficacy of hydrogen-rich saline alone and in combination with PI3K inhibitor in non-small cell lung cancer

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**Abstract.** The aim of the present study was to investigate the effects of combination therapy of LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K), with hydrogen-rich saline on the proliferation and apoptosis of the non-small cell lung cancer (NSCLC) A549 cell line and the mechanisms underpinning this. Excessive production of reactive oxygen species (ROS) may induce DNA mutations, DNA damage, genomic instability and cell proliferation, and ROS are involved in several types of cancer, particularly lung cancer. In a previous study, hydrogen was recognized as an antioxidant in preventive and therapeutic applications. The PI3K/protein kinase B (Akt) pathway is an important signaling pathway that may activate downstream of a series of extracellular signals and impact on cellular processes including cell proliferation, apoptosis and survival. To date, the PI3K/Akt signaling pathway has been indicated as a feasible target for novel antineoplastic drugs. Different strategies combining the two treatment modalities have been used in cancer therapy in order to achieve an improved therapeutic response and longer control of tumor modalities control. The present study investigated the effect of hydrogen-rich saline alone and in combination with the PI3K inhibitor, LY294002, on the proliferation, oxidative stress and apoptosis of NSCLC A549 cells. This combination therapy may be more effective than separate drug treatment; it decreased the malondialdehyde level and increased the superoxide dismutase activity. The combination therapy also enhanced the efficacy of anti-proliferation and apoptosis. Similarly, the results of the present study demonstrated that administration of the two agents in combination may inhibit phospho-Akt activity, and reduce expression of heme oxygenase-1 and nuclear factor- $\kappa$ B p65. The results

further suggested that the combination therapy may reduce cell proliferation and promote cell apoptosis by downregulating Akt phosphorylation and inhibiting the PI3K pathway in NSCLC cell lines. Therefore, the present study provided evidence that combined therapy may be a novel therapeutic option for patients with NSCLC.

## Introduction

Lung cancer is the leading cause of cancer death worldwide (1), including China (2). Non-small cell lung cancer (NSCLC) accounts for >80% of lung cancer cases. Despite advances in diagnosis and treatment, outcomes remain poor, with the 5-year survival rate generally being less than 15% (3). In order to improve the survival rate, numerous studies have aimed to identify novel antineoplastic therapies that have a wide range of biological activities, fewer side effects and low toxicity (2-4).

Oxidative stress is defined as an imbalance between the antioxidant defenses and the production of reactive oxygen species (ROS). It occurs when excessive production of ROS overwhelms their elimination by protective antioxidants or when there is a prominent decrease or lack of antioxidant defense activity (5). ROS are involved in a number of types of cancer (6). Additionally, oxidative stress may affect cancer initiation and progression through increasing DNA mutations or DNA damage, genomic instability and cell proliferation (6,7).

The lungs are directly exposed to higher concentrations of oxygen than other tissues and organs (8). Certain studies have demonstrated that the levels of oxidative stress increase in advanced stages of lung cancer, while the levels of antioxidant molecules decrease (9-11). In particular, patients with squamous cell carcinoma exhibit much higher oxidative stress and ROS, which may be linked to the fact that squamous cell carcinoma is associated with tobacco use (9,12). Enhanced ROS/RNS in the lung may increase the risk to lung cancer through recurring DNA damage, inhibition of apoptosis, and activation of proto-oncogenes by initiating signal transduction pathways (13). The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway is one of most important survival signaling pathways. It has been considered as the predominant growth-factor-activated pathway that may be activated

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downstream of a variety of extracellular signals and that impacts various aspects of cellular processes, including cell proliferation, apoptosis and survival (14). Therefore, numerous types of cancer have been linked to PI3K activity, including lung cancer (15), breast cancer (16), leukemia (17) and melanoma (18), among others (19,20). Therefore, this pathway presents a challenge and an opportunity for cancer therapy. A recent study demonstrated that the PI3K/Akt pathway may be a novel target in antineoplastic therapy (19). However, in a number of cases, activation of the PI3K/Akt pathway alone is not responsible for oncogenic transformation and therefore, the antineoplastic effect of PI3K/mechanistic target of rapamycin (mTOR) inhibitor is not satisfied. In addition, cancer cells resistant to PI3K inhibition remain a problem.

Hydrogen is an efficient antioxidant and has been identified as a novel therapeutic antioxidant recently, as it was revealed to selectively reduce cytotoxic ROS in tissues (21-23). In *in vivo* and *in vitro* studies, H<sub>2</sub> has exhibited protective antioxidant, anti-apoptotic and anti-inflammatory properties. Hydrogen is safe and effective for distribution into the cytoplasm without the need for specific receptors to overcome hydrophilicity (24,25). In rat models, inhaled hydrogen gas is able to reduce myocardial ischemia reperfusion injury and the infarct size of focal cerebral (26). Due to the fact that hydrogen gas is flammable and inconvenient for clinical use, hydrogen-rich saline with a therapeutic concentration of hydrogen is an alternative model of molecular hydrogen. The protective effect of hydrogen-rich saline is largely due to its ability to reduce the ROS-associated pathologies.

Clinically, combination therapy is most widely used in the treatment of cancer, including lung cancer. The main aim is to achieve synergistic therapeutic effect, to reduce dose and cytotoxicity, and to delay or minimize the induction of drug resistance (27,28).

In the present study, we hypothesized that the combination of hydrogen-rich saline and a highly potent PI3K inhibitor, LY294002, would decrease the proliferation of cancer cells more than single-agent therapy through anti-oxidation and regulation of apoptosis. The present study aimed to demonstrate the effect of the release of inflammatory and apoptosis cytokines. Furthermore, it focused on the influence of combination therapy on the PI3K/Akt signaling pathway and the mechanism underpinning this, which may be applied to clinical tumor therapy.

## Materials and methods

**Reagents.** Assay kits for superoxide dismutase (SOD) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). A propidium iodide (PI) double staining kit and Annexin V-fluorescein isothiocyanate (FITC) were purchased from Nanjing KeyGene Biotech Co., Ltd. (Nanjing, China). PI was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). LY-294002 was purchased from Sigma-Aldrich (Merck KGaA), Stock solution (50 mM) of LY-294002 was dissolved in DMSO and further diluted to a final concentration of 20  $\mu$ M. Antibodies were obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). Other reagents were obtained locally.

**Cell lines and cell culture.** The human NSCLC A549 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The A549 cell line was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air.

**Hydrogen saline.** Molecular hydrogen (H<sub>2</sub>) was dissolved in saline under high pressure (0.6 MPa) to a supersaturated level for 2 h. Hydrogen-rich saline was freshly prepared each week and was sterilized by  $\gamma$  radiation. It was ensured that hydrogen-rich saline was maintained at a concentration of more than 0.6 mmol/l. The hydrogen concentration was detected by gas chromatography using the method previously described by Ohsawa *et al* (22).

**Cell proliferation inhibition assay.** The inhibition rate of cell proliferation was tested using an MTT assay. A549 cells were grown in 96-well plates at a density of 5x10<sup>3</sup> cells per well for 24 h, prior to being treated with different concentrations of LY294002 (primary concentration, 20  $\mu$ M, the concentration of each well is 1/3 of the previous well), hydrogen-rich saline (with a hydrogen concentration of 0.6 mmol/l) or a combination of hydrogen-rich saline and LY294002. After 48 h, 20  $\mu$ l MTT solution [2 mg/ml in phosphate-buffered saline (PBS)] was added into each well and the cells were incubated for an additional 4 h at 37°C. The medium was completely removed and then 150  $\mu$ l DMSO was added to solubilize the MTT formazan crystals. Finally, the optical density was read at 570 nm (OD<sub>570</sub>) using a Microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, MA, USA). Results were presented as the mean of 3 independent experiments each conducted in duplicate. The half-maximal inhibitory concentration (IC<sub>50</sub>) values were calculated from dose-response curves utilizing GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

**SOD and MDA measurement.** A549 cells were grown in 6-well plates at a density of 1x10<sup>6</sup> cells per well for 24 h, prior to being treated with hydrogen-rich saline (0.6 mmol/l), LY294002 (20  $\mu$ M) or a combination of hydrogen-rich saline and LY294002 for 24 h. The cells were subsequently harvested, disrupted ultrasonically on ice and centrifuged at 2,500 x g for 10 min at 4°C. The supernatants were collected and stored at -20°C for subsequent analysis. The concentrations of MDA and SOD were evaluated using an MDA and SOD detection kit according to the manufacturer's protocols.

**Annexin V/PI flow cytometry analysis.** Cell apoptosis was measured using an Annexin V-FITC Apoptosis kit, according to the manufacturer's protocols. A549 cells were grown in 6-well plates at a density of 1x10<sup>6</sup> cells per well for 24 h, prior to being treated with hydrogen-rich saline (0.6 mmol/l), LY294002 (20  $\mu$ M) or a combination of hydrogen-rich saline and LY294002 for 24 h. Following staining, flow cytometry was performed using a FACScan flow cytometer. Cells stained as Annexin V-positive and PI-negative were considered apoptotic, while cells that double stained as Annexin V-positive and PI-positive were considered late apoptotic or necrotic. Data were analyzed using the Cell Quest Software

Program (FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA).

**Western blot analysis.** The cells were washed twice with ice-cold PBS following treatment for 24 h. Total protein was extracted using cell lysis buffer (1 M Tris.HCl pH 7.5, 8M Urea, 150 mM  $\beta$ -ME and 1% protease inhibitor cocktail). Protein concentration was determined by bicinchoninic acid (BCA) protein assay. Equal amounts of protein (50  $\mu$ g) from each sample were separated by SDS-PAGE. Proteins were subsequently transferred onto polyvinylidene difluoride membranes. Next, the membranes were blocked with 5% skimmed milk for half an hour in room temperature, prior to being incubated with primary antibodies against HO-1, p65 and p-Akt separately overnight at 4°C, followed by being washed three times with PBS. The membranes were subsequently incubated with secondary antibodies for 2 h in room temperature. Following washing, the membranes were visualized with an ECL kit and quantified using ImageJ Software (National Institutes of Health, Bethesda, MD, USA). Experiments were repeated at least three times.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from cells using an mirVana miRNA Isolation kit (Qiagen GmbH, Hilden, Germany). Equal amounts of RNA samples were reverse transcribed into cDNA using a high-capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RT-qPCR was performed using first-strand cDNA with TaqMan probes and TaqMan Universal PCR Master mix (Roche Diagnostics, Basel, Switzerland) with the following conditions: Denaturation at 95°C for 10 sec, annealing at 58°C for 20 sec and elongation at 72°C for 20 sec, and GAPDH served as an internal control. PCR was performed using the following primers: HO-1 forward, 5'-GATAGAGCGCAACAAGCAGAA-3' and reverse, 5'-CAGTGAGGCCCATACCAGAAG-3'; P65 forward, 5'-GGGAAGGAACGCTGTCAGAG-3' and reverse, 5'-TAGCCTCAGGGTACTCCATCA-3'; and GAPDH forward, 5'-TGTTGCCATCAATGACCCCTT-3' and reverse, 5'-CTCCACGACGTA CTCAGCG-3'. Experiments were repeated at least three times. The relative mRNA expression was measured using the  $\Delta\Delta C_q$  method (29).

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.). Data are presented as the mean  $\pm$  standard error of the mean. The statistical significance of differences between groups *in vitro* experiments was determined by the Student t-test. The statistical significance of differences between combinations with theoretical combination *in vitro* experiments was determined by one-way analysis of variance followed by a Turkey's post-hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Inhibition of cell proliferation by hydrogen-rich saline and the PI3K inhibitor, LY294002, in A549 cells.** To investigate the proliferation inhibition effects of hydrogen-rich saline

alone and in combination with LY-294002 in A549 cells, the cells were treated with hydrogen-rich saline, LY-294002 or a combination of the two for 48 h, and the inhibition of cell proliferation rate was measured using an MTT assay. We observed that growth of A549 cells was suppressed in treatment group (Fig. 1A). Cell proliferation was suppressed following treatment with either hydrogen-rich saline or LY-294002 alone (Fig. 1B). However, compared with treatment with hydrogen-rich saline or LY-294002 alone (Fig. 1C), the combination of the two resulted in increased inhibition.

**Effect of hydrogen-rich saline and the PI3K inhibitor, LY294002, on the concentrations of ROS and MDA in A549 cells.** To further investigate the effect of a combination of hydrogen-rich saline and the PI3K inhibitor, LY294002, on intracellular oxidant generation in A549 cells, the concentrations of ROS and MDA were examined. The results indicated that the combination of hydrogen-rich saline and LY294002 increased the concentrations of SOD (Fig. 2A), and markedly decreased the levels of MDA, compared with the control group and the hydrogen-rich saline monotherapy group (Fig. 2B). These results demonstrated that the combination of the two treatments suppressed intracellular oxidant generation in A549 cells.

**Induction of apoptosis by hydrogen-rich saline and the PI3K inhibitor, LY294002, in A549 cells.** A549 cells were treated with hydrogen saline, the PI3K inhibitor, LY294002, or the combination of hydrogen saline with LY294002 for 24 h, prior to being analyzed by flow cytometry. In comparison with the control group, the numbers of early and late apoptotic cells were markedly increased (Fig. 3A and B). The proportion of early and late apoptotic cells in the combination treatment group was markedly increased, compared with the control group. The proportion of apoptotic cells was increased in the three groups, but the combination of the two treatments induced a more marked increase than treatment with hydrogen-rich saline or LY-294002 alone.

**Inhibition of HO-1 and NF- $\kappa$ B p65 protein and mRNA expression in A549 cells by combination of hydrogen-rich saline and the PI3K inhibitor, LY294002.** To investigate the mechanism of anti-oxidation induced by hydrogen-rich saline alone, LY294002 alone and the combination of hydrogen-rich saline and LY294002, the present study tested the effect of three treatment groups on HO-1 and NF- $\kappa$ B p65 protein and mRNA expression levels. Western blot analysis revealed that hydrogen-rich saline monotherapy led to a decrease in HO-1 and p65 levels as compared with the control cells (Fig. 4A and B). RT-qPCR analysis revealed that hydrogen-rich saline monotherapy led to a decrease in HO-1 and p65 mRNA expression levels, compared with the control cells (Fig. 5A and B). The protein and mRNA expression levels were higher following combined treatment with hydrogen-rich saline and LY294002, compared with treatment with either of the agents alone.

**Inhibition of the PI3K/Akt pathway by hydrogen-rich saline alone and in combination with LY294002.** In order to better understand the molecular basis of the anti-proliferation and apoptosis induced by hydrogen-rich saline alone and in

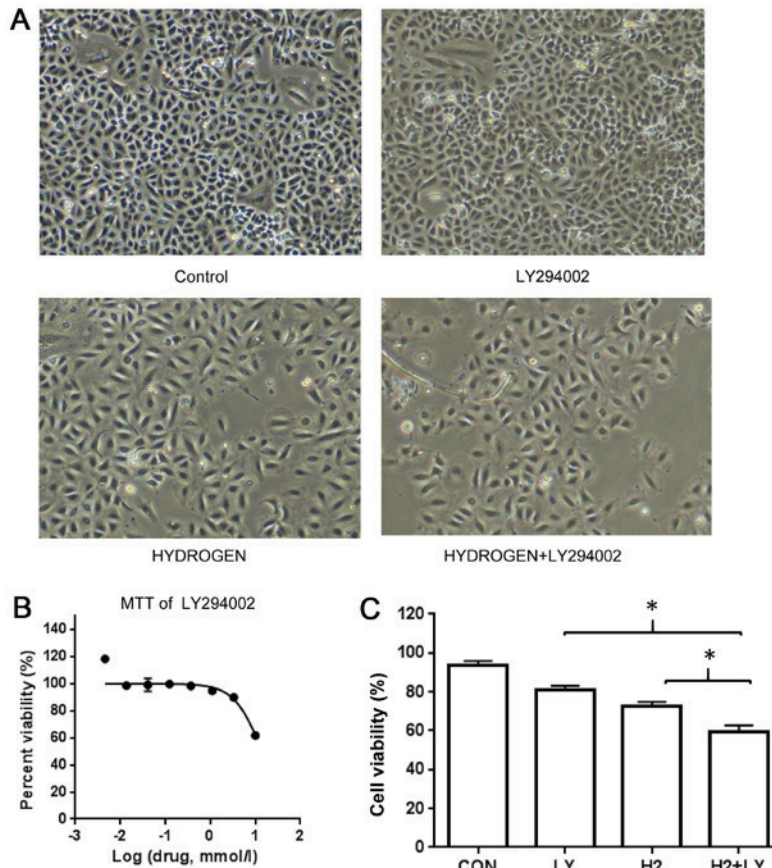


Figure 1. Effects of hydrogen-rich saline and in combination with the PI3K inhibitor LY294002 on cell proliferation in A549 cells. (A) NSCLC cell line (A549) treated with hydrogen-rich saline, LY294002 and combination was incubated for 24 h (magnification, x100). (B) Percentage viability of the cells was determined after cells were incubated with MTT, and (C) with the different groups. Optical density measurements were made at 570 nm. Data are presented as the mean ± standard deviation for 3 independent experiments. \*P<0.05, as indicated. PI3K, phosphatidylinositol 3-kinase; NSCLC, non-small cell lung cancer; CON, control; LY, LY294002; H2, molecular hydrogen.

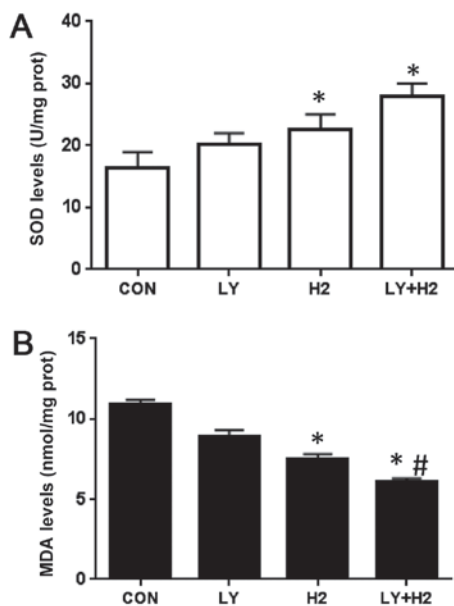


Figure 2. Effects of hydrogen-rich saline and in combination with PI3K inhibitor LY294002 on intracellular oxidant generation and antioxidant enzyme activity in A549 cells. (A) SOD and (B) MDA levels were measured using a microplate spectrophotometer. Data are presented as the mean ± standard deviation for 3 independent experiments. \*P<0.05 vs. CON; #P<0.05 vs. H2. PI3K, phosphatidylinositol 3-kinase; CON, control; LY, LY294002; H2, molecular hydrogen; SOD, superoxide dismutase; MDA, malondialdehyde.

combination with LY294002, the present study examined the expression of p-Akt, an effector of the PI3K/Akt/mTOR signaling pathway, following treatment with hydrogen-rich saline alone and in combination with LY294002 for 24 h. The protein expression of p-Akt was decreased in response to hydrogen-rich saline alone, LY294002 alone and a combination of the two, while the p-Akt protein expression level remained lower in the combined treatment group than following drug treatment alone (Fig. 6A and B). These results indicated that the combination therapy has a synergistic effect on anti-proliferation and the induction of apoptosis possibly through inhibiting the PI3K/Akt pathway.

### Discussion

To the best of our knowledge, oxidative stress play a key role in tumorigenesis (6,30-32). Oxidative stress may affect the initiation and progression of cancer by leading to DNA mutations, DNA damage, inducing genomic instability and cell proliferation (33-36).

In particular, oxidative damage serves a pivotal role in pulmonary disease as the lungs are exposed daily to oxidants, which are generated either endogenously or exogenously (e.g., air pollutants and cigarette smoke) (10); tobacco smoke and particulate matter in air pollution may generate ROS and therefore increase the incidence of lung cancer and

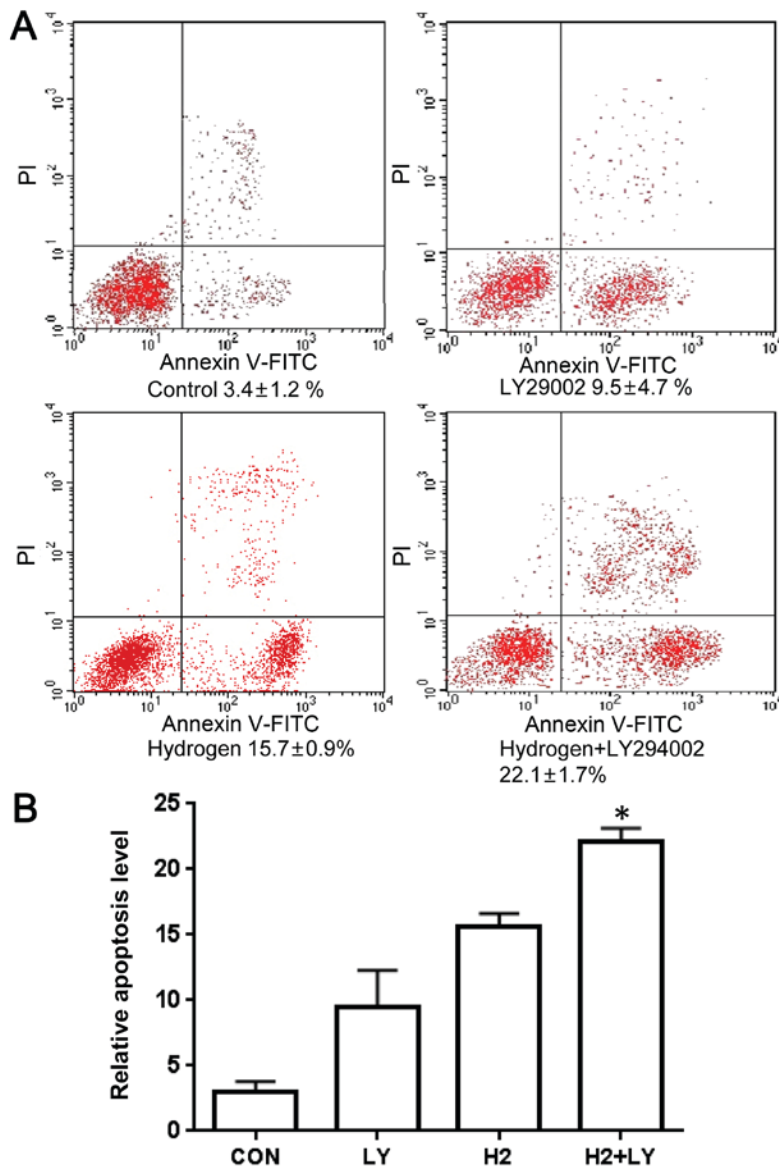


Figure 3. Effects of hydrogen-rich saline and LY294002 as single-agents and in combination on the survival of A549 cell line. A549 non-small-cell lung cancer cells were treated with hydrogen-rich saline (0.6 mmol/l) and LY294002 (1  $\mu$ mol/l), each alone or in combination, for 24 h. (A) Single drug or combination-induced apoptosis as analyzed by Annexin V-FITC and PI staining and flow cytometry. (B) The percentage of apoptotic cells was determined by Annexin V and PI staining. Data are presented as the mean  $\pm$  standard deviation for 3 independent experiments. \* $P$ <0.05 vs. single drug. CON, control; LY, LY294002; H2, molecular hydrogen; FITC, fluorescein isothiocyanate; PI, propidium iodide.

respiratory diseases (37). Furthermore, synergistic effects in the generation of ROS contribute toward lung cancer through inducing oxidative stress and inflammation with a high DNA damage potential (10). Since oxidative stress is a crucial event in cancer cells, this may also provide an opportunity to kill malignant cells.

Hydrogen has been generally recognized as a therapeutic anti-oxidative and anti-apoptotic tool (22,38). Numerous studies have demonstrated that hydrogen may selectively react with exclusively detrimental ROS, while hydrogen does not disturb physiological metabolic oxidation-reduction reactions or disrupt the ROS involved in cell signaling (22,31,39). Hydrogen-rich saline, which is easily administered and is safe for clinical application, is a viable approach with the same properties of anti-oxidation, anti-inflammation and anti-apoptosis, and has been used in several recent animal

disease studies (40-42). However, a study has demonstrated that hydrogen therapy may inhibit the proliferation of tongue carcinoma cells (43) and reduce the size of skin tumors. Further studies will elucidate the molecular mechanism of hydrogen-mediated inhibition of cancer cell proliferation.

The present study initially applied hydrogen-rich saline alone to lung cancer cells. Subsequently, the effect of hydrogen-rich saline on apoptosis and inflammatory cytokines, and the pathway involved in this process, was investigated. The results demonstrated the following: i) Hydrogen-rich saline treatment alone inhibited A549 cell proliferation; ii) hydrogen-rich saline treatment alone decreased MDA expression and increased SOD activity; iii) hydrogen-rich saline treatment alone induced A549 cell apoptosis; iv) in *in vitro* experiment, treatment with hydrogen significantly suppressed the effect on protein and mRNA expression of

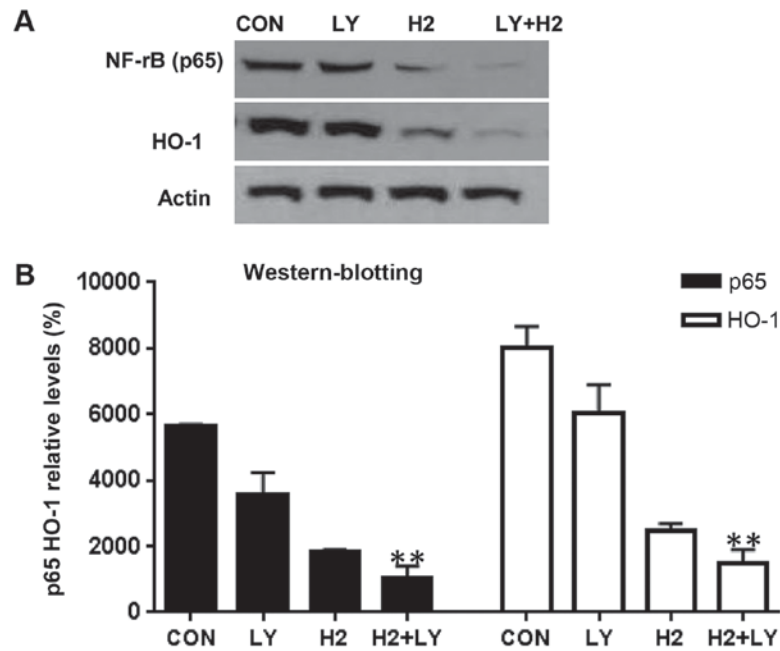


Figure 4. Western blotting analysis of A549 cells treated as indicated for 24 h. (A) Western blot results of the protein expression levels of HO-1 and NF- $\kappa$ B p65 in A549 cells treated with single agent and combination.  $\beta$ -actin was used as a loading control. Representative images from three independent experiments are shown. (B) Treatment with hydrogen-rich saline in combination with LY294002 led to a significant decrease in HO-1 and NF- $\kappa$ B p65. Data are presented as the mean  $\pm$  standard deviation for 3 independent experiments. \*\* $P$ <0.01 vs. control. CON, control; LY, LY294002; H2, molecular hydrogen; HO-1, heme oxygenase; NF-, nuclear factor.

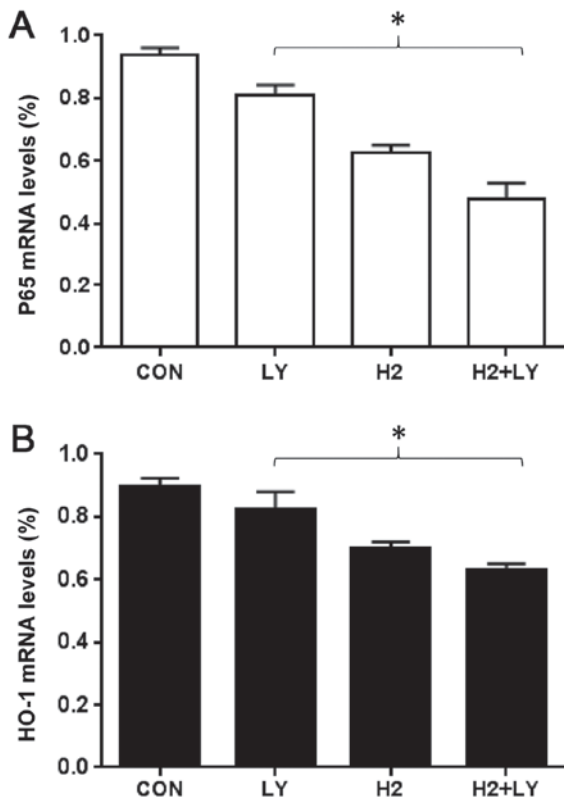


Figure 5. (A and B) Reverse transcription-quantitative polymerase chain reaction analysis of A549 cells treated as indicated. The mRNA expression of NF- $\kappa$ B p65 and HO-1 following treatment with hydrogen-rich saline, LY294002 alone and combination hydrogen-rich saline with LY294002 on A549 cells. Note that treatment with hydrogen-rich saline in combination with LY294002 led to a significant decrease in the mRNA expression of HO-1 and p65. Data are presented as the mean  $\pm$  standard deviation for 3 independent experiments. \* $P$ <0.05, as indicated. CON, control; LY, LY294002; H2, molecular hydrogen; HO-1, heme oxygenase; NF-, nuclear factor.

HO-1 and NF- $\kappa$ B p65 in A549 cell and v) hydrogen-rich saline suppressed the expression of p-Akt and the expression levels of HO-1 and p65.

The results of the present study demonstrated that hydrogen-rich saline alone may downregulate the expression of NF- $\kappa$ B p65, which is a redox-regulated transcription factor. The activation of NF- $\kappa$ B serves an important role in regulating the expression of a number of early stress response genes, which are associated with oxidative stress-induced cell death in a wide range of tumor types (44). Therefore, it is suggested that hydrogen-rich saline may attenuate oxidative stress by inhibiting the activation of NF- $\kappa$ B. The present study demonstrated that hydrogen-rich saline alone downregulated the expression of HO-1, which is a stress response protein (45). As HO-1 expression and activity are markedly increased by stressful conditions, the major role of HO-1 is involved in antioxidant and anti-inflammatory responses (46,47). However, the induction of HO-1, which may provide cells with a growth advantage, is not always adequate to protect the cells. HO-1 may be overexpressed in tumor cells (45,48,49). In the present study, HO-1 was downregulated *in vitro*, oxidative stress was markedly decreased by hydrogen-rich saline and the expression of p-Akt was suppressed. These results indicated that it is possible that the anti-proliferation and apoptosis inducing functions of hydrogen-rich saline are primarily achieved by downregulating transcriptional factors (NF- $\kappa$ B and p-Akt) via the PI3K/Akt pathway.

The PI3K/Akt pathway is involved crucially in the development and progression of several types of cancer by aiding in promoting cell proliferation and allowing cells to evade apoptosis. PI3K/Akt pathway activation is associated with poor outcomes in certain types of cancer (50). In addition, numerous novel 'targeted agents' have been specifically exploited to act

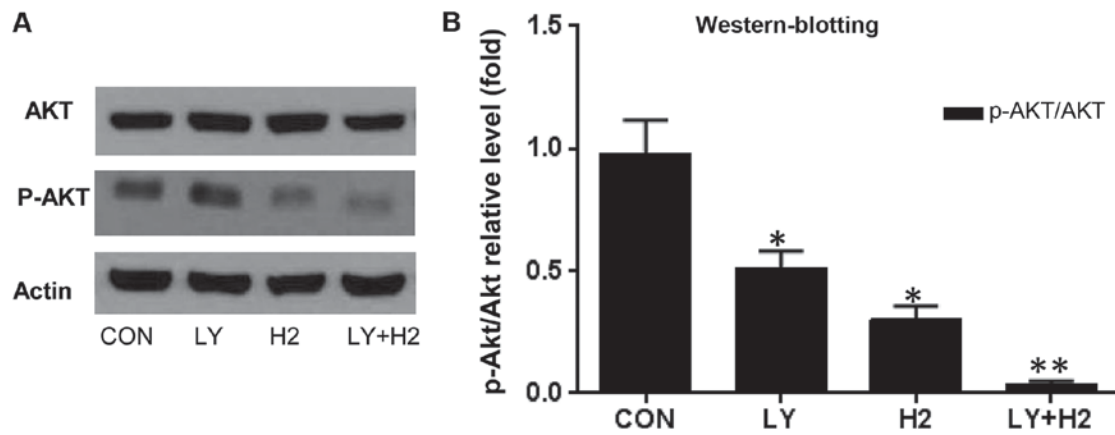


Figure 6. (A and B) Effects of hydrogen-rich saline and LY294002 as single-agents and in combination on PI3K/p-AKT pathway of NSCLC cells. (A) Western blot analysis of proteins (pAKT and AKT) was indicated in A549 cells treated with hydrogen-rich saline and LY294002 as single agents or in combination for 24 h. Actin was used as a loading control. (B) Treatment with hydrogen-rich saline in combination with LY294002 led to a significant decrease of protein expression of p-Akt/Akt. Data are presented as the mean  $\pm$  standard deviation for 3 independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  vs. CON. PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; NSCLC, non-small cell lung cancer; CON, control; LY, LY294002; H2, molecular hydrogen; p-, phosphorylated.

on PI3K/Akt and downstream component-related targets due to the ubiquitous nature of the activation of this pathway in cancer. Furthermore, multiple PI3K/Akt pathway inhibitors are now under active clinical development (20).

In NSCLC cells, these small molecule inhibitors have been demonstrated to shift the apoptotic threshold in cancer cells following treatment with other molecule inhibitors or standard chemotherapy (51).

The present study focused on the efficacy of a PI3K inhibitor (LY294002) in combination with anti-oxidative treatment with hydrogen-rich saline. Regarding the administration of hydrogen-rich saline or LY294002 separately as single agents, the results of the present study demonstrated that the combination treatment of A549 cells may be more effective than single treatment. Combination treatment enhanced the efficacy of anti-proliferation and apoptosis induction. Similarly, these data demonstrated that usage of the two agents in combination may decrease MDA expression levels, inhibit p-Akt activity, and reduce expression of HO-1 and NF- $\kappa$ B. The results of the present study supported the hypothesis that the combination therapy has the effect of anti-proliferation and apoptosis induction through inhibiting the activation of Akt phosphorylation and reducing the downstream expression of HO-1 and NF- $\kappa$ B. Therefore, combining PI3K inhibitor (LY294002) and hydrogen-rich saline may negatively regulate A549 cell proliferation through inhibition of the PI3K/Akt pathway. The present study has suggested that this combination is able to serve as dual PI3K pathway inhibitors, which may be attractive targets for the treatment of lung cancer, with a low risk of side effects. However, due to the fact that the present study using combination are unable to assay clear mechanisms of PI3K in the cells, clarification of the intracellular signaling network of cells should be verified in future experiments.

In conclusion, the present study demonstrated that hydrogen-rich saline in combination with LY294002 treatment in A549 cancer cells leads to constitutive inhibition of the PI3K/Akt signaling pathway and acts as a negative regulator for cell proliferation and expression of HO-1. Furthermore, the drug combination approach used in the present study may

be useful for the study of novel cancer therapeutics and may directly aid future drug development and patient stratification in clinical trials.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

YJ and SLG planned the experiments. GL, SC and CL performed the experiments. LZ and YL analyzed the data. YJ prepared the figures. YJ and LZ drafted the manuscript. YJ and LZ proof read and approved the final version of the manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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