Molecular Hydrogen Attenuates High Hydrostatic Pressure-Induced Neuronal Cell Damage by Reversing Dysfunction of Mitochondrial Electron Transfer Chain

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Research Article

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Abstract

Cellular hydrostatic pressure beyond its normal range can induce the accumulation of reactive oxidative species (ROS) generated by mitochondria and lead to pathological conditions such as glaucomatous optic neuropathy. However, little is known about how the mitochondrial electron transfer chain (ETC) is affected by elevated pressure. Moreover, the protective effects of hydrogen on various pathological conditions have been observed by reductions in ROS, yet the role of hydrogen in high hydrostatic pressure (HHP)-induced cell damage remains obscure. The goal of this study was to investigate the effect of HHP on ETC activity and whether hydrogen exerts protective effects against HHP-induced damage in cultured neuronal cells. Cultured SH-SY5Y human neuroblastoma cells were exposed to an elevated ambient hydrostatic pressure of 50 mmHg for a period of 2 to 6 h. HHP impaired the activities of ETC complexes, and these effects were reversed by hydrogen. Significant increases in apoptotic rates and intracellular ROS levels were observed in HHP-treated SH-SY5Y cells. Hydrogen significantly inhibited the apoptotic rates and reduced the levels of ROS. These findings suggest that HHP induces cell damage by causing ETC dysfunction to increase oxidative stress and that hydrogen may act as a protective agent to alleviate HHP-induced neuronal injury.

1. Introduction

Hydrostatic pressure is prevalent in all biological environments and determines the normal function of cells. Disorders in cell environmental hydrostatic pressure are closely associated with diverse physiological and pathological processes (Chen et al. 2020; Ehrlich et al. 2010; Golebiewska and Scarlata 2015; Hasel et al. 2005; Li et al. 2020; Maki et al. 2021; Wei et al. 2018; Yang et al. 2018). Elevated intraocular pressure (IOP) is generally considered the most essential risk factor in the pathogenesis of glaucoma, which leads to glaucomatous optic neuropathy (Kwon et al. 2009). Studies using high hydrostatic pressure (HHP) have shown that excessive production of reactive oxidative species (ROS) and oxidative stress are early events in HHP-exposed retinal ganglion cells *in vitro* (Liu et al. 2007). ROS are byproducts of electron transfer chain (ETC) and are involved in cellular signaling and excessive levels of ROS attack nucleic acids, lipids and proteins, resulting in severe cellular damage (Rani et al. 2016). Mitochondria are the major site of oxygen consumption and ROS production, and are also targets of ROS attack. Impaired mitochondrial functions, particularly ETC function impairments, may increase electron escape from the ETC and result in disturbed redox homeostasis, which may further cause oxidative damage and eventually induce cell death (Liu et al. 2012). Despite the central role of mitochondria in optic nerve damage during glaucoma pathogenesis and the prominence of HHP as an *in vitro* model of IOP, to our knowledge, little is known about the function of mitochondrial ETC under HHP.

Hydrogen (H_2) has gained considerable attention as an antioxidant because of its selective antioxidant properties and ability to penetrate cytomembranes and diffuse into organelles (James et al. 2005). Previous reports have demonstrated that H_2 can selectively quench intracellular free radicals without interfering with metabolic redox reactions (Ohsawa et al. 2007). Since its discovery, the effects of H_2 have been investigated in a wide range of oxidative stress and inflammation-related diseases (Cardinal et al.

2010; Shi et al. 2015; Sun et al. 2011; Wang et al. 2011a; Wang et al. 2011b; Zhang et al. 2018; Zheng et al. 2021). However, no experimental studies have explored the protective effects of H_2 against HHP-induced ROS generation and cellular oxidative damage in neuronal cells.

Herein, we used the human neuroblastoma cell line SH-SY5Y and evaluated the effects of H_2 on the cell damage induced by short-term HHP. We exposed SH-SY5Y cells to 2 h, 4 h and 6 h of 50 mmHg HHP to mimic the conditions observed in acute glaucoma patients with high IOP (Kwon et al. 2009). Protective effects of H_2 included reversing the HHP-induced compromise in mitochondrial ETC activity and mitochondrial membrane potential (MMP), reducing intracellular ROS and inhibiting the apoptotic rate. Our results suggest that mitochondrial ETC impairment plays a key role in HHP-induced cell damage and that H_2 has neuroprotective effects by protecting mitochondrial dysfunction.

2. Materials And Methods

2.1 Cell culture

Cultures of SH-SY5Y human neuroblastoma cells (CRL-2266, ATCC, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Corning, USA) supplemented with 10% (v/v) fetal bovine serum (Biological Industries, Israel) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2 (v/v).

2.2 Preparation of H_2-rich cell culture medium

H_2-rich cell culture medium was prepared as previously described (Yu et al. 2011). Briefly, H_2-rich cell culture medium was freshly prepared by dissolving pure H_2 gas (> 99.99%) into serum-free DMEM under 0.1 MPa of pressure to reach a supersaturated level. The H_2-rich DMEM was then filtered through a 0.22-μm syringe membrane filter (Sartorius, Germany).

2.3 HHP stimulation of cultured cells

Elevated stable hydrostatic pressure was achieved by culturing SH-SY5Y cells in a customized pressure chamber. In brief, the stainless steel pressure chamber was constructed with a gas inlet, a gas outlet and flow valves to seal the chamber. The chamber was connected to a high-precision digital output pressure sensor (PAA-33X/80794, Keller, Switzerland) that continuously monitors the pressure levels. The atmospheric pressure was calibrated to 0 mmHg. The chamber could be pressurized and maintained at a constant air pressure ranging from 0–5 MPa with a 5% CO_2 and 95% air gas mix. A pressure of 50 mmHg was used to mimic the condition of glaucomatous high IOP (Kwon et al. 2009). Compression to 50 mmHg was attained within 60 sec. The chamber was then placed in a customized water bath at 37°C. The cells in the normal hydrostatic pressure group were maintained in a normal cell incubator under normobaric conditions.

2.4 H_2 treatment of cultured cells

H_2 treatment of cultured cells was performed by replacing the normal cell culture medium with freshly prepared H_2-rich medium. To keep the concentration of H_2 in the medium at a high level, the medium was changed every 30 min during treatment.

2.5 Isolation of mitochondria

Mitochondria were isolated from SH-SY5Y cells by differential centrifugation methods (Shen et al. 2008). Briefly, cultured SH-SY5Y cells were collected and suspended in ice-cold hypotonic isolation buffer. After 5–8 min of incubation, the samples were homogenized with a Dounce homogenizer and fractionated by centrifugation at 1,300 g and 17,000 g.

2.6 Measurement of mitochondrial ETC activity

Activities of complex I (NADH-ubiquinone reductase), complex II (succinate-CoQ oxidoreductase), complex III (CoQ-cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase) were determined spectrophotometrically in accordance with the previously reported methods (Cao et al. 2014).

2.7 Fluorimetric detection of MMP, hydroxyl free radical (·OH), total ROS and apoptosis

SH-SY5Y cells were collected and loaded with fluorescence probe JC-1 (Invitrogen, USA), 2-[6-(4'-hydroxy) phenoxy-3*H*-xanthen-3-on-9-yl] benzoate (hydroxyphenyl fluorescein, HPF, AAT Bioquest, USA), ROS Brite 670 (AAT Bioquest, Inc., USA) and Annexin V and propidium iodide (PI) (BD Biosciences, USA), respectively. Cells were then incubated for 30 min at 37°C in the dark and analyzed immediately by flow cytometry (Novocyte, Agilent, USA). The specific fluorescence signals corresponding to the FITC, PI and APC channels were collected. A total of 1.2×10^4 events per sample were acquired.

2.8 Statistical analysis

All values are presented as the means with their standard errors. Statistical analysis was performed using commercial software GraphPad Prism 5 (GraphPad Software, USA). The results were analyzed by two-way analysis of variance (ANOVA) followed by a Bonferroni posttest for multiple comparisons. The two factors were HHP time and H_2 treatment. A p-value of less than 0.05 was considered to be statistically significant. The relative changes in enzymatic activity levels are presented as percentages of the control samples, which were assumed to be 100%.

3. Results

3.1 H_2 reversed HHP-induced impairment of mitochondrial ETC activity

There was no significant change in the activity of complex I in HHP-exposed cells (Fig. 1A). H_2 exhibited no significant effect on complex I activity (Fig. 1A). Exposure to 50 mmHg HHP resulted in significant

reductions in the levels of complex II activity (Fig. 1B). H_2-rich medium treatment significantly increased complex II activity after 4 h and 6 h of HHP and H_2 treatment (Fig. 1B). Stimulation of 50 mmHg HHP resulted in significantly diminished activity of complex III (Fig. 1C). H_2-rich medium treatment significantly increased complex III activity after 6 h of HHP and H_2 treatment (Fig. 1C). Exposure to 50 mmHg HHP resulted in significant reductions in the levels of complex IV activity (Fig. 1D). Complex IV did not show significant differences between cells with and without H_2-rich medium treatment (Fig. 1D).

3.2 H_2 reversed HHP-induced reductions of MMP

Exposure to 50 mmHg HHP resulted in significant reductions in the levels of MMP (Fig. 2). H_2-rich medium treatment significantly increased complex II activity after 6 h of HHP and H_2 treatment (Fig. 2).

3.3 H_2 reduced HHP-induced excessive ROS production

HHP is known to increase ROS production by the mitochondrial ETC system and lead to oxidative stress; however, the generation of specific species of free radicals, particularly ·OH, has not been directly investigated. H_2 has been shown to neutralize ·OH in living cells (Ohsawa et al. 2007). Herein, we assessed intracellular generation of··OH by using the fluorescent probe HPF. A significant increase in the spontaneous production of ·OH was observed in cells after 6 h of 50 mmHg HHP exposure (Fig. 3A). However, we did not observe decreased intracellular levels of ·OH after treatment with H_2-rich medium (Fig. 3A). We further evaluated the levels of total intracellular ROS production. Significant increases were observed in HHP-exposed cells, while H_2 significantly reduced total intracellular ROS levels (Fig. 3B).

3.4 H_2 inhibited HHP-induced elevated apoptotic rates

Flow cytometry analysis with Annexin V/PI staining showed that the apoptotic rate (the sum of early and late apoptotic cells) was greatly increased in SH-SY5Y cells exposed to 2 h, 4 h and 6 h of 50 mmHg HHP (Fig. 4). In contrast, treatment using H_2-rich medium markedly suppressed apoptotic rates in cells exposed to 6 h of HHP (Fig. 4).

4. Discussion

Despite the recognized central role of mitochondrial ETC in regulating redox homeostasis and mitochondrial functions, to our knowledge, alterations in mitochondrial ETC activity under HHP have not yet been reported. Moreover, although studies have revealed protective effects of a few antioxidants and mitochondrial nutrients in counteracting HHP-induced neuronal cell injury, the effects of H_2 are still not investigated (Liu et al. 2012; Liu et al. 2017). In this study, we explored the effects of HHP on mitochondrial ETC activity in cultured SH-SY5Y human neuroblastoma cells and the effects of H_2. Our results revealed that mitochondrial ETC impairment is involved in the HHP-induced cell damage and that H_2 has a cytoprotective effect against HHP-induced cell injury *in vitro*.

An essential function of mitochondria is energy production through ETC, which is carried out by complexes I-IV, the electron transporters ubiquinone and cytochrome *c* (Zhao et al. 2019). Complex I is the main site of electron leakage to oxygen and the source of superoxides, which are cytotoxic mediators of cellular oxidative damage (Zhao et al. 2019). The imbalanced decrease of ETC activity, *i.e.*, significant decrease in all ETC complexes except for complex I, may partially account for the increase in intracellular ROS production because of the decreased efficiency of electron transfer and the resulting electron leak from complex I. Complex II plays an important role in connecting the ETC with the tricarboxylic acid cycle (Zhao et al. 2019). The decrease in complex II activity provides evidence that HHP impairs the transfer of electrons from complex II to complex III. The unchanged complex I activity and decreased complex II activity also indicate that the HHP-stimulated SH-SY5Y cells rely more on complex I/III/IV electron transport pathway. The decrease in complex IV activity also indicated compromised mitochondrial ETC functionality. Mitochondrial ATP production depends on the proton electrochemical gradient across the inner mitochondrial membrane established by electrons passing through the ETC (Zhao et al. 2019). The decrease in MMP suggests that poor mitochondrial ETC functionality results in lower energy production, which is required for cells to defend against HHP-induced stress. The cells might thus be more susceptible to cell injury in this context.

Previous studies have demonstrated that H_2 can neutralize several cytotoxic free radicals such as ·OH, ·ONOO and O- 2 (Ohsawa et al. 2007; Yu et al. 2011). In this study, significant increase in total intracellular ROS production and significant elimination of ROS by H_2 were observed in HHP-stimulated SH-SY5Y cells. The free radical ·OH, among other ROS, is essential in causing oxidative damage, as ·OH is one of the strongest oxidants in nature and quickly causes cellular injury (Yu et al. 2011). However, the ·OH increased slightly after 6 h of HHP exposure in SH-SY5Y cells, but was not significantly affected by H_2. Considering that ·OH is generated through the dismutation of O- 2 to H_2O_2 followed by its partially reduction (Gaur et al. 2021), it can be inferred that H_2 might effectively eliminated O- 2 at the early stage of generation and inhibited its further conversion into ·OH and other free radical species.

Mitochondria also serve as regulators of apoptosis (Bock and Tait 2020). It has been reported that HHP can induce apoptosis in diverse cell types (Agar et al. 2000; Ju et al. 2009; Klett et al. 2004; Tök et al. 2014). Our study showed that HHP-induced apoptosis can be attenuated by H_2, demonstrating that H_2 has an antiapoptotic effect on pressure-induced cell death. Recent studies have revealed several mitochondria-mediated pathways that inhibit apoptosis in different cell and animal models (Chen et al. 2017; Guan et al. 2019; Li and Ai 2017; Wu et al. 2018). Further investigations are needed to better understand the mechanisms of HHP-induced apoptosis and the antiapoptotic effects of H_2 in detail.

The cell model used in this study did not originate from the retinal ganglion. Unfortunately, there is currently no proper *in vitro* or *in vivo* glaucoma model (Liu et al. 2017). Although the RGC-5 cell line has been used in most of the previous studies to examine HHP-induced cell damage (Ju et al. 2007; Ju et al. 2009; Liu et al. 2012; Liu et al. 2007; Shang et al. 2014), there have been significant concerns about the origin of RGC-5 cells (Krishnamoorthy et al. 2013; Van Bergen et al. 2009). Further work in primary retinal

ganglion cells *in vitro* and *in vivo* is necessary to better clarify the detailed mechanisms of HHP-induced cell damage and H_2-mediated protection.

5. Conclusions

In conclusion, the findings reported herein suggest that short-term HHP exposure compromises mitochondrial ETC function and elevates ROS production and apoptosis in the SH-SY5Y cell model. We further found that H_2 attenuates short-term HHP-induced cell damage by restoring mitochondrial ETC function, scavenging intracellular ROS and preventing apoptosis. Thus, H_2 may have therapeutic potential as a protective agent to mitigate HHP-induced neuronal damage.

Declarations

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Conflicts of interest/Competing interests

All authors declare that they have no conflict of interest.

Availability of data and material

All data are available upon request.

Code availability

Not applicable.

Authors' contributions

Jiangang Long, and Jiankang Liu conceived the idea, Zhuoyang Lu designed the experiments, Tiantian Zhang and Yachong Hu prepared mitochondria samples, Hui Liu and Li Cui designed the pressure chamber, Zhuoyang Lu performed biochemical assays, analyzed the data and wrote the paper, and Jiangang Long and Jiankang Liu supervised the project and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

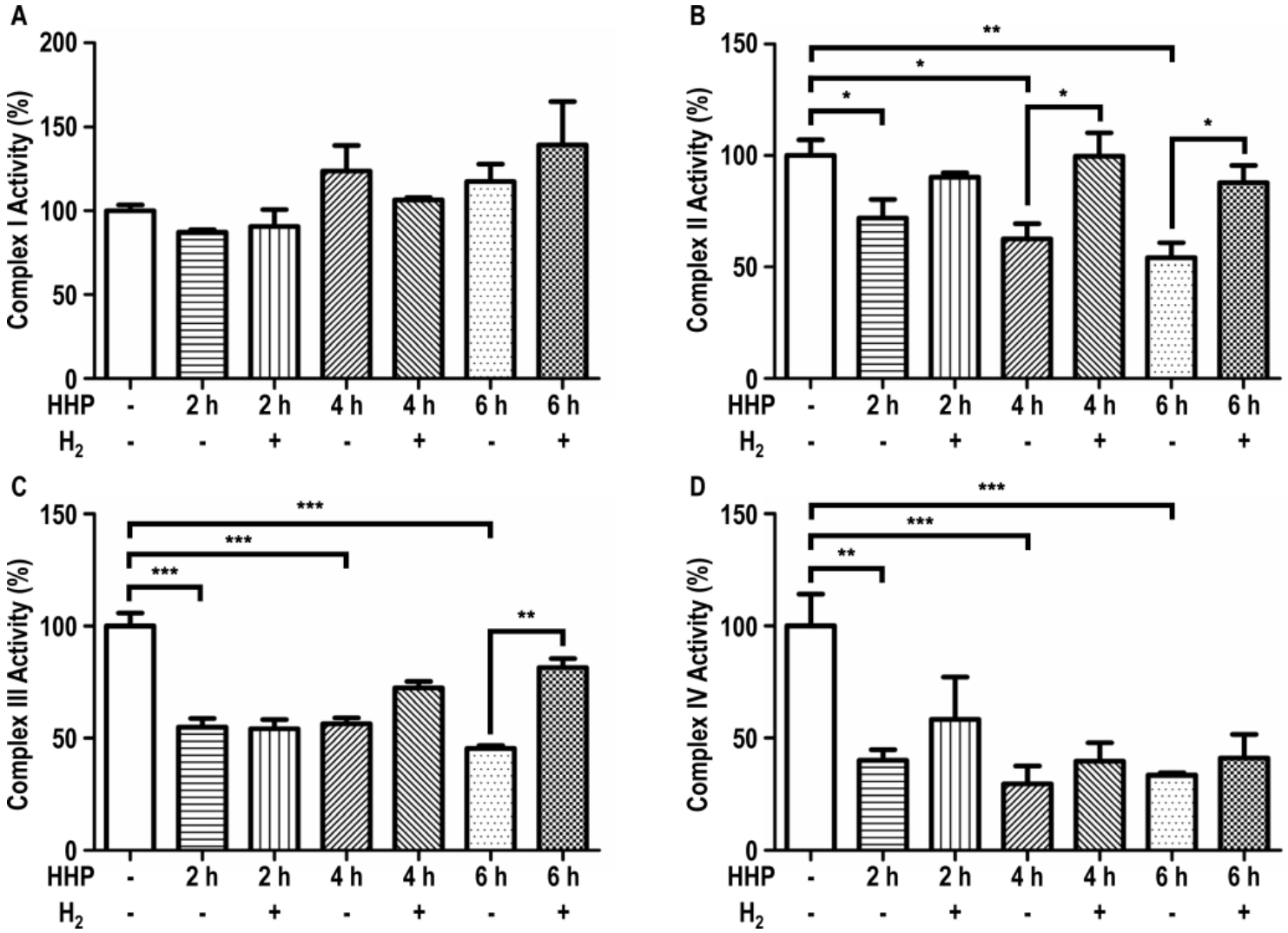

Figure 1

Enzymatic activity of mitochondrial ETC complexes I-IV in mitochondria isolated from SH-SY5Y cells subjected to HHP and H2 treatment. Significance was determined by two-way ANOVA followed by a Bonferroni posttest at 95% confidence. The asterisks indicate significance at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) compared to the control groups. The histograms shown are the averaged data of three independent experiments. All activities are presented as percentages of the raw control values (no HHP or H2 treatment, mean ± SEM).

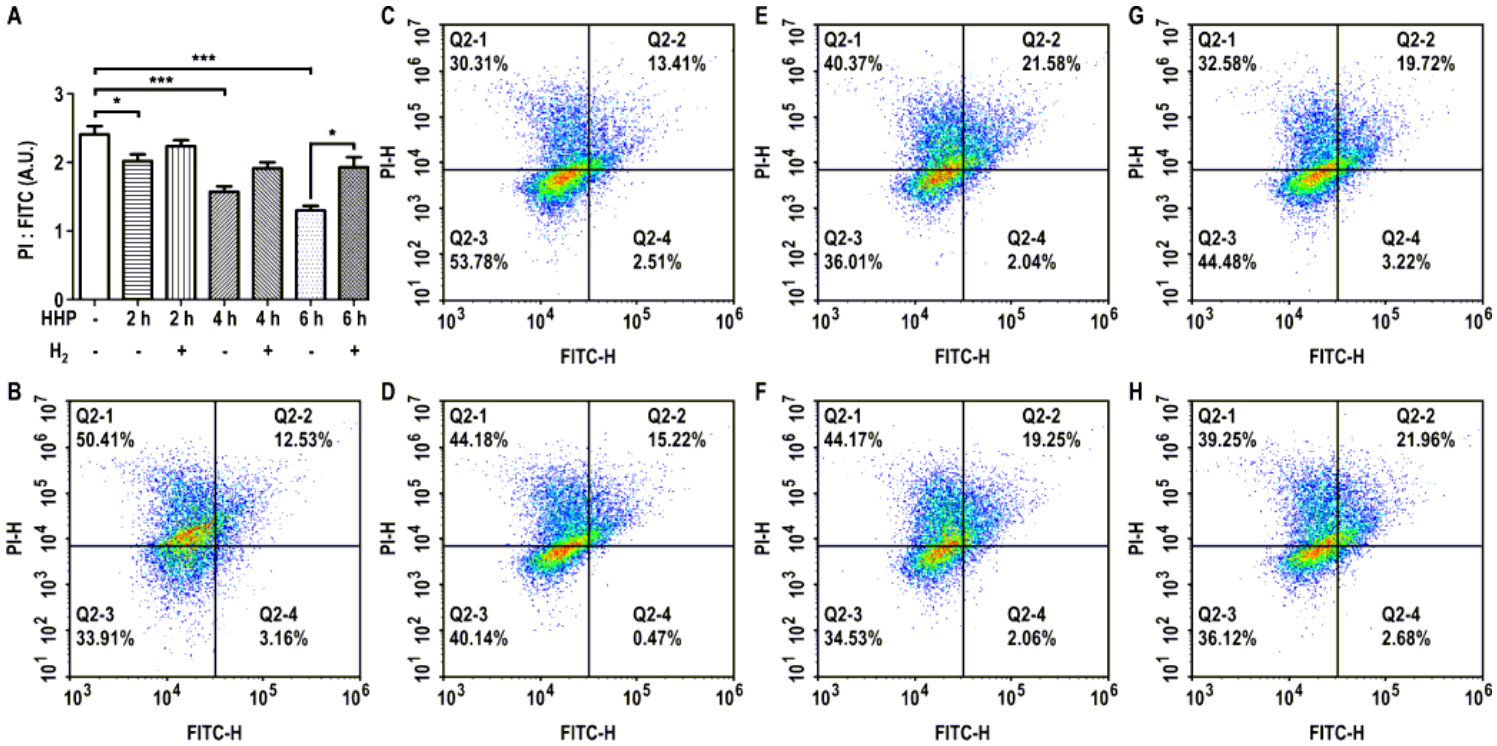

Figure 2

Flow cytometric analysis of MMP in SH-SY5Y cells subjected to HHP and H2 treatment and stained with JC-1. A. The typical histograms are the averaged ratio of PI : FITC fluorescent intensity of three independent experiments and are presented as the mean ± SEM. Differences in MMP were statistically analyzed by two-way ANOVA followed by a Bonferroni posttest at 95% confidence. The asterisks indicate significance at $p < 0.05$ (*) and $p < 0.001$ (***) compared to the control groups. B-H. Typical flow cytometry plots of JC1-stained cells in normal control group (B), 2 h of 50 mmHg HHP treatment group (C), 2 h of 50 mmHg HHP and H2 treatment group (D), 4 h of 50 mmHg HHP treatment group (E), 4 h of 50 mmHg HHP and H2 treatment group (F), 6 h of 50 mmHg HHP treatment group (G), 6 h of 50 mmHg HHP and H2 treatment group (H). The numbers show the percentage of cells in each quadrant.

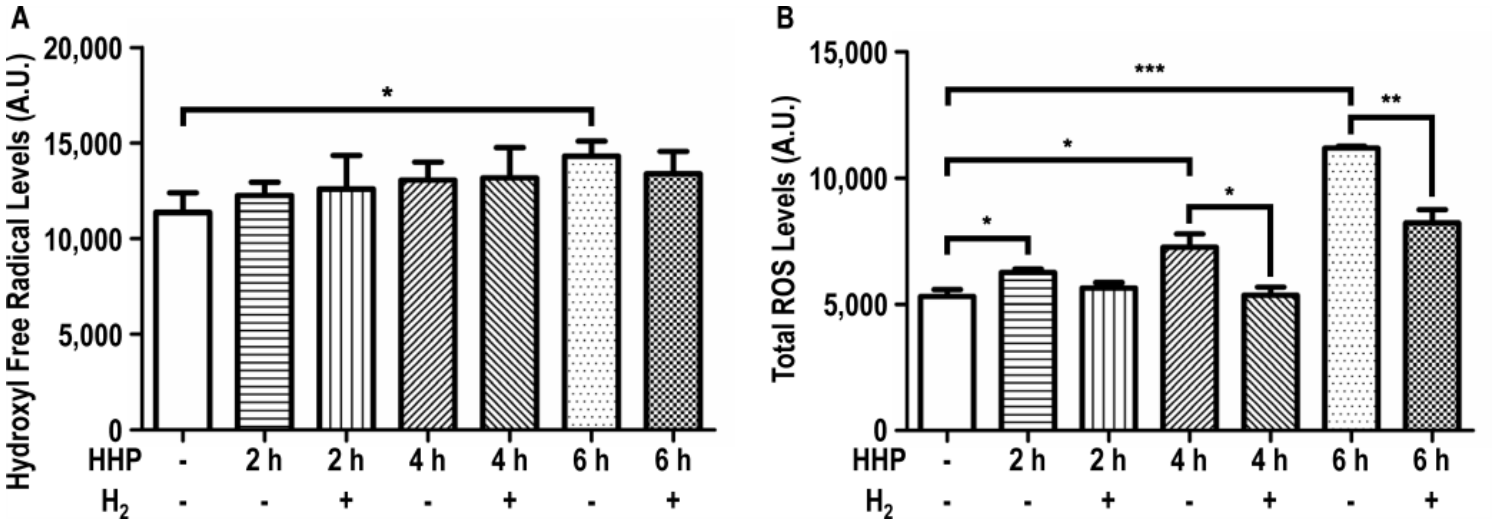

Figure 3

Flow cytometric analysis of intracellular ·OH and total ROS production in SH-SY5Y cells subjected to HHP and H2 treatment and stained with HPF and ROS Brite 670, respectively. The typical histograms are the averaged fluorescent intensity of three independent experiments and are presented as the mean ± SEM. Differences in intracellular ·OH were statistically analyzed by two-way ANOVA at 95% confidence. The asterisks indicate significance at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) compared to the control groups.

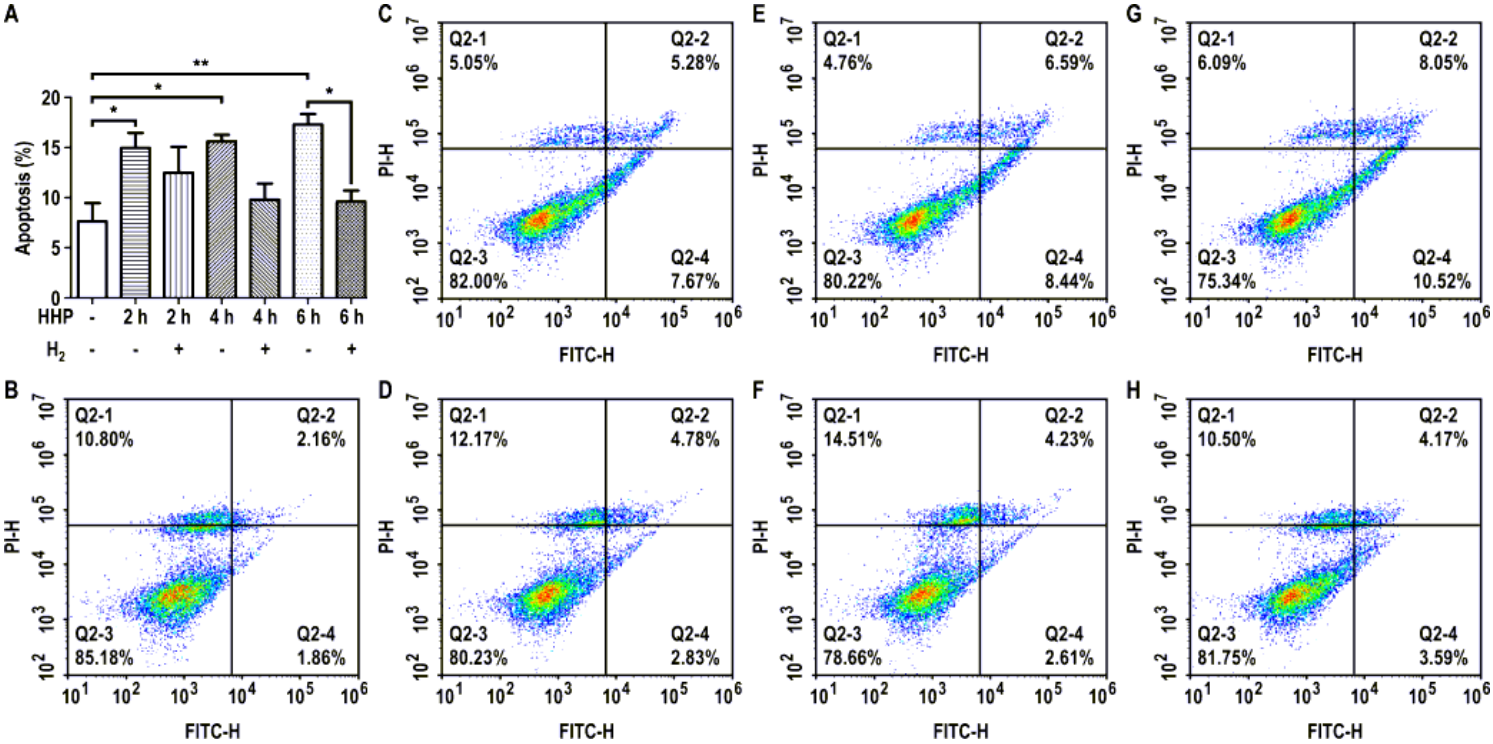

Figure 4

Flow cytometric analysis of apoptotic rates in SH-SY5Y cells subjected to HHP and H2 treatment and stained with Annexin V and PI. A. The typical histograms are the averaged apoptotic rates of three independent experiments and are presented as the mean ± SEM. Differences in apoptotic rates were statistically analyzed by two-way ANOVA followed by a Bonferroni posttest at 95% confidence. The asterisks indicate significance at $p < 0.05$ (*) and $p < 0.01$ (**) compared to the control groups. B-H. Typical flow cytometry plots of Annexin V and PI-stained cells in normal control group (B), 2 h of 50 mmHg HHP treatment group (C), 2 h of 50 mmHg HHP and H2 treatment group (D), 4 h of 50 mmHg HHP treatment group (E), 4 h of 50 mmHg HHP and H2 treatment group (F), 6 h of 50 mmHg HHP treatment group (G), 6 h of 50 mmHg HHP and H2 treatment group (H). The numbers show the percentage of cells in each quadrant.