Research Square

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Protective effect of hydrogen against ischemia-reperfusion induced spinal nerve cell apoptosis

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Research

Keywords: Hydrogen, Spinal ischemia-repurfusioninjury, OGSD/R · PC12, Apoptotic inhibition

Posted Date: March 3rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-258588/v1

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Abstract

Background: This study aims to explore the protective effect of hydrogen against oxygen-glucose-serum deprivation/restoration (OGSD/R)-induced PC12 cell apoptosis in vitro and the possible underlying mechanism.

Methods: A normal control (NC) group was set where PC12 cells were cultured normal, while a positive control (PC) group, where PC12 cells were exposed to OGSD 12h/R1h without intervention, and a hydrogen intervention (HI) group, where PC12 cells were exposed to OGSD 12h/R1h plus HI, were conducted at the same time. At OGSD 12h/R 1h, cells were DAPI stained to detect viability and changes in the expression of apoptosis-associated proteins caspase-3, caspase-12 and CHOP/GADD153, and the endoplasmic reticulum-related signaling pathway protein PERK-eIF2α-ATF4. At the same time, the effect of HI was observed.

Results: The result revealed that compared with NC group, cell apoptosis was more severe and cell viability was reduced significantly in PC group, while cell apoptosis was ameliorated and cell viability was increased significantly in HI as compared with PC group. In addition, the content of caspase-3 and caspase-12 in HI group was decreased significantly as compared with that in PC group. During this process, the endoplasmic reticulum-related signaling pathway protein PERK-eIF2α-ATF4 was activated. In HI group, the expression of this protein was decreased and cell viability was increased significantly as compared with those in PC group.

Conclusions: Hydrogen was able to inhibit OGSD/R-induced PC12 cell apoptosis and exert a protective effect against ischemia-repurfusion injury (IRI) to nerve cells, probably through inhibiting the endoplasmic reticulum-related signaling pathway protein.

Introduction

The development and progression of spinal ischemia-repurfusion injury (IRI) on the basis of the primary injury to the spinal cord will further accelerate apoptosis of spinal nerve cells and deteriorate the neural function. Therefore, how to prevent the occurrence of spinal IRI effectively, explore the pathogenic mechanism of nerve cell apoptosis following spinal IRI and implement early intervention has become the crux in the treatment of spinal injuries.

Cell apoptosis is mainly mediated through three pathways: the death receptor activation pathway (1), the mitochondrial damage pathway (2) and the ER pathway (3). The ER possesses important physio-biochemical functions and participates in synthesis and transportation of multiple proteins. It is also the main site for calcium ion storage in the body, as well as the site for synthesis of cholesterols, steroids and many lipids. Either accumulation of large amounts of unfolded and mal-folded proteins in the ER or destruction of intra-cellular calcium ion homeostasis could cause injury to the normal physiological function of the ER, and this phenomenon is known as ER stress (ERS). ERS can activate unfolded protein response (UPR), which is a protective response to restore the ER function through reducing accumulation

of unfolded proteins (4). UPR can activate three transcriptional factors: inositol required enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (5). When the stimulation is too strong or exists persistently, UPR is not strong enough to restore and maintain ER homeostasis, followed with programmed death, which leads to cell apoptosis. When ERS occurs, phosphorylation takes place in PERK intrinsically, and subsequently activates its downstream molecule ***eIF2a/ATF4*** to directly participate in cell apoptosis induced by UPR (6), which means that eIF2a/ATF4 can be an observation index to estimate whether cell apoptosis is mediated by UPR.

CHOP (C/EBP homologous protein), also known as growth arrest and DNA damage-inducible 153 (GADD153) and a member of the CCAAT/enhancer-binding protein (C/EBP) family, is associated with cell proliferation, differentiation and expression, as well as cellular energy metabolism (7, 8). It is one of the apoptosis pathways initiated upon ERS, which is mainly induced by genetoxic stress and growth inhibition signals. CHOP can induce apoptosis through inhibiting the expression of anti-apoptosis protein Bcl-2. The Bcl-2 family has 25 members whose molecules contain four conserved sequences known as Bcl-2 homologous domain 1-4 (BH1-4). These family members are mainly divided into two categories: pro-apoptotic proteins BAX, Bak and Bok, and anti-apoptotic proteins Bcl-2, Bcl-X and Bcl-W. Upon ERS, ***CHOP*** downregulates the expression of ***Bcl-2***(9), eventually leading to cell apoptosis.

Hydrogen is a new-type medical gas, which only selectively react with strong oxidants to exert its protective effect on the body by regulating oxidative stress response, inflammatory response and cell apoptosis (10-12). Hydrogen molecules are electrically neutral and easily penetrate the cell membrane to selectively scavenge oxygen free radicals (OFRs)(13). It was found in our prior animal study that hydrogen saline could inhibit motor neuronal apoptosis resulting from spinal IRI in rabbits, showing both anti-inflammatory and anti-oxidative effects (14). In the establishment and optimization of the neuronal IRI model in that study, we found that cell apoptotis peaked at OGSR 1h, during which the expression of ER-related apoptosis protein caspase-12 was enhanced, whose trend of expression was basically parallel to the expression of apoptosis-associated protein caspase-3. Therefore, we started HI at OGSR 1h and the result was compared with that of PC group to see whether HI could inhibit PC12 cell apoptosis and explore the underlying mechanism.

To undertake an in vitro simulation study on the mechanism of spinal IRI, the research group used PC12 cells to study the physio-biochemical properties of neurons in vitro, knowing that PC12 cells are a cell line cloned from rat adrenal medullary pheochromocytoma with typical characteristics of human neuroendocrine cells. In our prior study, we had established and optimized an oxygen-glucose-serum deprivation/restoration (OGSD/R)-induced cell injury model and clarified the law of change in apoptosis of neurons in vitro following IRI, which laid an experimental basis for the timing of intervention in the present study.

Materials And Methods

Preparation of the medium containing hydrogen

Before initiation of the experiment, high-glucose DMEM was injected into an aluminium bag and stored in the refrigerator at 4℃. Using a high-pressure ventilation apparatus, hydrogen was infiltrated at 0.4MPa for 6 h until it dissolved completely to a saturation state in the medium with a concentration >0.6mmol/L. hydrogen was supplemented every three days to maintain the saturation concentration. The prepared hydrogen medium was stored in the refrigerator at 4℃.

Experimental grouping

PC12 cells in logarithmic growth phase were spread to a 24-well plate at a density of 1×10^4 cells/well. Confluent cells were divided into a normal control (NC) group, in which cells were normally cultured in 10% FBS+DMEM under 37℃and 5%CO2 conditions; a positive control (PC) group, in which cells were deprived with OGS for 12 h and restored with OGS for 1 h without intervention; and a hydrogen intervention (HI) group, in which cells were exposed to OGS for 12 h and restored in hydrogen-based medium for 1 h.

DAPI staining

The prepared cells in the three groups were washed with 1 x phosphate buffered saline (PBS) twice, fixed in 4% paraformaldehyde for 10 min, washed with 1×PBS twice, membrane-ruptured with 0.1% Triton X-100 for 10min, washed with PBS twice, added with 10ng/ml DAPI, cultured away from light at room temperature for 20 min, washed with PBS twice, observed in 6 randomly selected visual fields under a fluorescence microscope and photographed through the ultraviolet channel at 30ms of exposure time, whose magnification was x 400. Six pictures were selected from each group to calculate the cell apoptosis rate by counting the total number of cells and apoptotic cells.

Cell viability assay

PC12 cells in logarithmic growth phase were digested in 0.25% pancreatin. After centrifugation and counting, cells were spread to a 96-well plate at a density of 5×10^3 cells /well and cultured in a 37℃ 5%CO_2 incubator for 24 h. After cells were treated as designated in each group, 10uL CCK8 was added to each well and cultured at 37℃ for 1 h. Optical density (OD) of each well was measured at 450nm to calculate the relative cell viability.

Detection of reactive oxygen species (ROS)

PC12 cells in logarithmic growth phase were digested in 0.25% pancreatin. After centrifugation and counting, cells were spread to a 6-well plate at a density of 2.5×10^5 cells /well and cultured in a 37℃ 5%CO_2 incubator for 24 h. Cells in each group were harvested, added with H2DCF-DA probes and cultured away from light at 37℃ for 30 min. After removing the H2DCF-DA probe diluent, cells were washed with PBS, digested in 0.25% pancreatin and centrifuged at 500xg for 3min. Probes that were not mounted to the cells were washed off with serum-free medium thoroughly. Cells were re-suspended with serum-free medium, and fluorescence was collected and detected through the FL1-A channel of the flow cytometer.

Changes in endoplasmic reticulum (ER)-related signaling pathway proteins p-PERK, p-eIF2a, ATF4 and apoptosis-associated proteins

After activation of the ER apoptosis pathway protein PERK-eIF2a-ATF4, the protein expression levels of p-PERK, p-eIF2a and ATF4 were detected by Western-blot to compare differences in protein content between the three groups. Simultaneously, the relative content of caspase-12, cleaved-caspase-3 and CHOP/GADD153 in cells was compared between the three groups.

Statistical methods

All data are expressed as the mean ± standard deviation (±SD). Data treatment and statistical analyses were performed using SPSS 13.0 Software. Statistical treatment was performed using One-way ANOVA. Values of $P<0.05$/$P<0.01$ were considered statistically significant.

Results

The effect of hydrogen on PC12 cell morphology after OGSD/R-induced apoptosis by DAPI staining

After 12 h OGSD, cells were cultured in high-glucose DMEM and hydrogen-based DMEM and stained by DAPI at 1 h OGSR. The result showed that cells in NC group were morphologically intact with few apoptotic cells. In PC group, large numbers of apoptotic cells were observed, the nuclei were condensed and dissolved, and the morphology was destroyed seriously. In HI group, the cell morphology was relatively intact, and there were fewer apoptotic cells as compared with PC group, indicating that hydrogen could to some extent to inhibit PC12 cell apoptosis induced by OGSD/R (**Figure 1 and 2**).

Cell viability assay by CCK8

After OGSD 12h/R 1 h, cell viability was detected with CCK8 assay kit and the result showed that after induction of PC12 cell apoptosis by OGSD/R, cell viability was decreased in both PC and HI groups, showing significant differences with that in NC group ($P<0.01$). Cell viability in HI group was superior to that in PC group ($P<0.01$), indicating that hydrogen inhibited OGSD/R-induced PC12 cell apoptosis, increased the cell viability, and exhibited a protective effect against IRI to neurons to some extent (**Figure 3**).

The effect of hydrogen on ROS in OGSD/R-induced apoptotic PC12 cells

The ROS content in the three groups was detected by H2DCF-DA assay and change in ROS of each group was detected by flow cytometry. The results showed that ROS content in HI group was significantly lower than that in PC group ($P<0.01$), indicating that hydrogen could inhibit ROS production in OGSD/R-induced PC12 cells, showing an anti-oxidative effect. Using the ROS value in NC group, the results obtained from three experiments of each group were summed up and compared as follows (**Figure 4**).

The effect of hydrogen on the expression of caspase-12 and cleaved-caspase-3 in OGSD/R-induced apoptotic PC12 cells

Western blot detection of the expression of caspase-12 and cleaved-caspase-3 in OGSD/R-induced apoptotic PC12 cells showed that their expressions in PC control and HI groups were significantly higher than those in NC group ($P<0.010$), and the expression of these protein in HI group was significantly lower than that in PC group ($P<0.01$) (**Figure 5**).

The effect of hydrogen on the expression of p-PERK, p-eIF2a and ATF4 in OGSD/R-induced apoptotic PC12 cells

The expression of p-PERK, p-eIF2a and ATF4 after activation of ER-related apoptosis factors was detected by Western blot. The result sowed that their expressions at OGSD 12h/R 1h in PC and HI groups were higher than those in NC group ($P<0.01$), and their expressions in HI group were significantly lower than those in PC group ($P<0.05$) (**Figure 6**).

The effect of hydrogen on the expression of CHOP/GADD153 in OGSD/R-induced apoptotic PC12 cells

ER-associated apoptosis protein CHOP in each group was detected by Western blot at OGSD 12h/R 1h, and the result showed that CHOP expression at OGSD 12h/R was increased in HI and PC groups as compared with that in NC group ($P<0.05$), and CHOP expression in HI group was significantly lower than that in PC group ($P<0.05$) (**Figure 7**).

Discussion

HI has been proved effective in brain ischemia injury, which arouses our interest in its therapeutic effect in spine IRI. The brain tissue is sensitive to oxidative stress induced by ischemia and hypoxia(15), and oxidative stress is one of the important reasons for cerebral ischemia and hypoxia(16) . Cellular oxidative stress occurs when the balance between the generation of free radicals and the ability of cells to scavenge them is broken down(17, 18)It was found that hydrogen saline can provide some protective effect against cerebral injury by inhibiting oxidative stress(19). In their rat cerebral ischemia and hyoxia experiment, Cai found that inhalation of 2% hydrogen for 2 h could effectively reduce the expression of caspase-3 and caspase-12 in the cerebral cortex and hippocampus(20). Du reported that hydrogen water could provide a protective effect against severe pulmonary injury from hemorrhagic shock by inhibiting the release of inflammatory factors and ameliorating oxidative stress-induced injury(21). Hydrogen not only has anti-inflammatory activity but can provide a protective effect on cerebral neurons by protecting the mitochondrial function(22). The mitochondrium is related to redox reaction, indicating that hydrogen has the function of resisting oxidative response of the body. To a great extent, Spine IRI, causing nerve injury by oxidative stress, inflammatory as well as final apoptosis, share a great possibility in HI sensitivity, which is verified by our experimental data. In spite of that, HI's therapeutic target remains unknown.

Up till now, there is no report about whether the protective effect of hydrogen against cord cell apoptosis is through attenuating ERS response and inhibiting the expression of ERS-related apoptosis proteins, while there have been reports revealing the mechanism of ERS in other organs. Zhao found that hydrogen saline could provide a protective effect against myocardial IRI by inhibiting ERS, and the mechanism may be related to its effect of reducing the expression of apoptosis proteins GRP78, caspase-12 and Bax and increasing the expression of the anti-apoptosis factor Bcl-2 in the myocardial tissue(23). Cai demonstrated that Bcl-2 expression in the myocardiocytes of mice with complete knockout of CHOP gene was significantly higher than that in those without CHOP knockout during ERS-induced cell apoptosis, while the expression level of Bax was significantly lower than that in the control group(24, 25), indicating that CHOP, Bax and Bcl-2 work together to regulate cell apoptosis during ERS, which is in line with our result, confirming that HI could decrease IRI through CHOP path.

Due to funding restriction and limited technology, a lot of related molecules haven't been proved yet. For instance, studies demonstrated that Bcl-2 related gene Bag5 (athanogene 5) was highly expressed in prostate cancer and inhibited ERS-induced apoptosis. Bag5 could reduce both CHOP and Bax expressions, and increase Bcl-2 expression(26). Free radical NO plays a key role in the pathophysiology of cerebral ischemia. It was found in the primarily cultured nerve cells that NO could reduce the content of calcium ions in the ER and promote CHOP gene transcription(27). Our study indicates the possibility of future clinic treatment in IRI, but its safety, side effect now attracts little concern. More work need done to ensure biosafety as well as its detailed mechanism.

Conclusions

It was found in the present study, hydrogen could to some extent inhibit OGSD/R-induced PC12 cell apoptosis. During the process of hydrogen action, the expression of ERS-related apoptosis protein caspase-12 was reduced and the ERS-related pathway protein PERK-eIF2a-ATF4 was inhibited, indicating that hydrogen could exert a protective effect against cell apoptosis by inhibiting the expression of ERS-related apoptosis pathway proteins. In addition, we measured the ROS content in the experimental cells and found that hydrogen could not only inhibit cell apoptosis but resist the effect of cellular oxidative stress response to reduce the production of active oxygen species within cells. ROS production is related to the impairment of the intracellular mitochondrial function, which is consistent with the finding of previous studies.

Abbreviations

OGSD/R	oxygen-glucose-serum deprivation/restoration
NC	normal control
PC	positive control
HI	hydrogen intervention
IRI	ischemia-reperfusion injury
PBS	phosphate buffered saline
OD	Optical density
ROS	reactive oxygen species
ER	endoplasmic reticulum
ERS	endoplasmic reticulum stress
UPR	unfold protein response
IRE1	inositol required enzyme1
PERK	PKR-like ER kinase
ATF6	activating transcription factor 6
CHOP	C/EBP homologous protein
OFR	Oxygen free radicals

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Availability of data and materials

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

Competing interests

The authors declare that they have no conflict of interest.

Funding

This work wasn't funded by any foundation.

Authors' contributions

SYQ, SW, WZW, ZSY, ZY, YB, CXS participated in the study design. SYQ, SW, WZW, YB performed the experiment. SYQ, WZW, ZSY, ZY were involved in data collection and data interpretation. WZW and YB participated in the statistical analyses. SYQ and SW wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

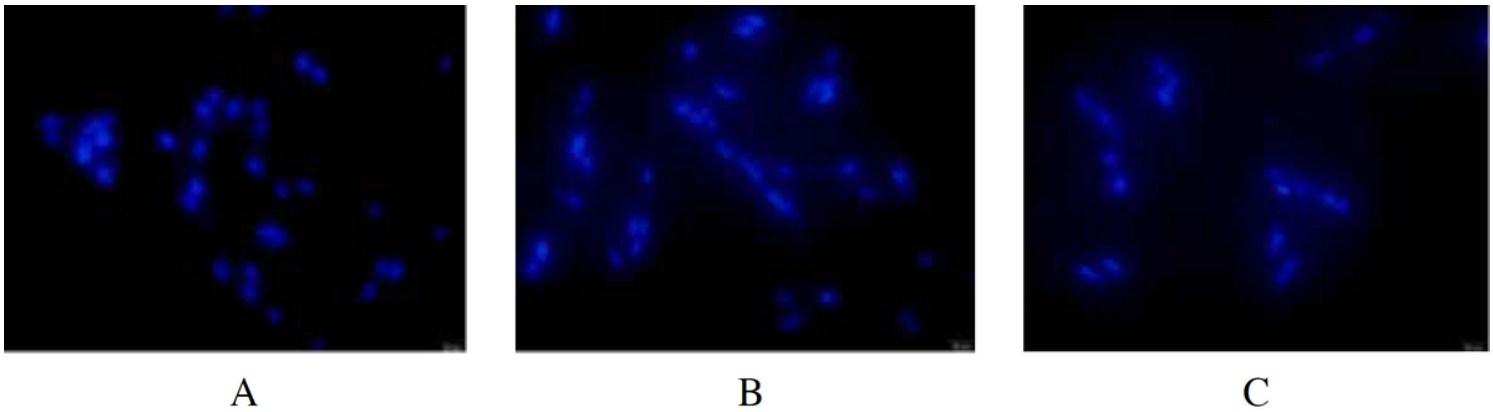

Figure 1

A.Cells in NC group are morphologically intact with uniform cytoplasm and few apoptotic cells. B. Nuclei in PC group are condensed and ruptured. C.A small number of apoptotic cells are seen in HI group.

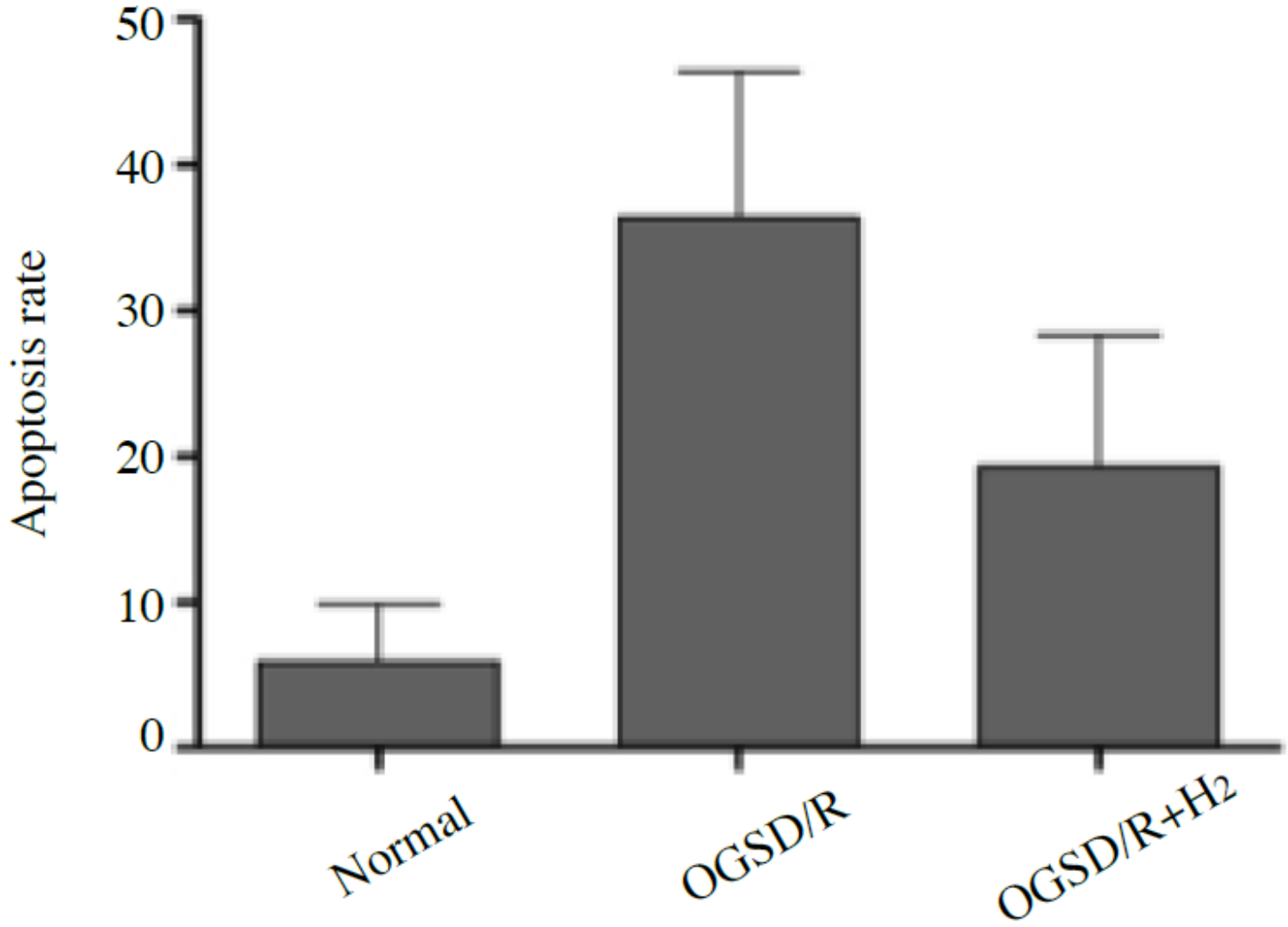

Figure 2

The number of apoptotic cells at OGSD 12h/R 1h in both positive control and hydrogen intervention groups was higher than that in normal control group ($P < 0.05$), and the number of apoptotic cells in hydrogen intervention group was significantly lower than that in positive control group ($P < 0.01$).

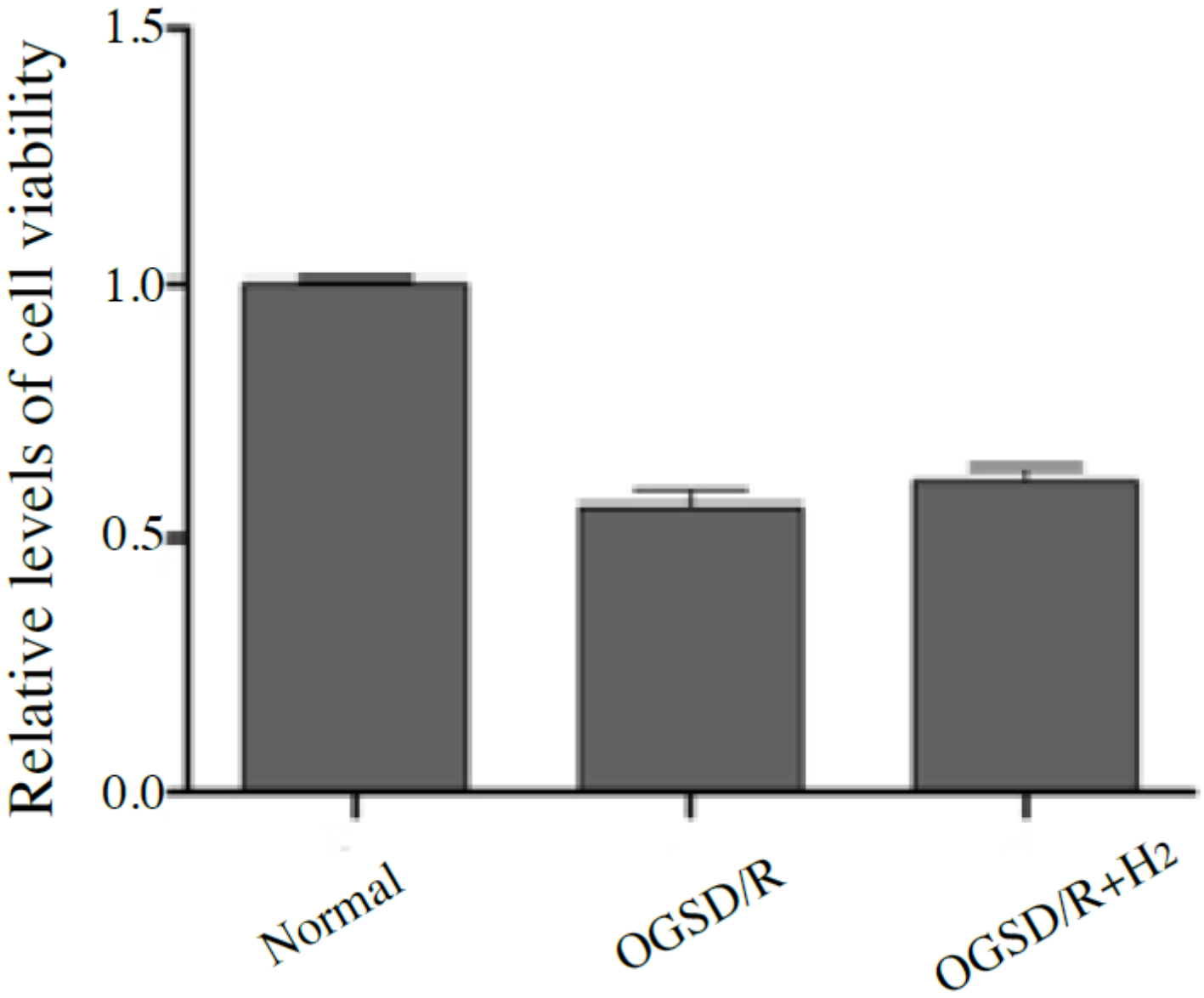

Figure 3

Cell viability was decreased in OGSD 12h/R 1h positive control group and hydrogen intervention group, showing significant differences with that in normal control group (P<0.01). Cell viability in hydrogen intervention group was significantly higher than that in OGSD 12h/R 1h positive control group (P<0.01).

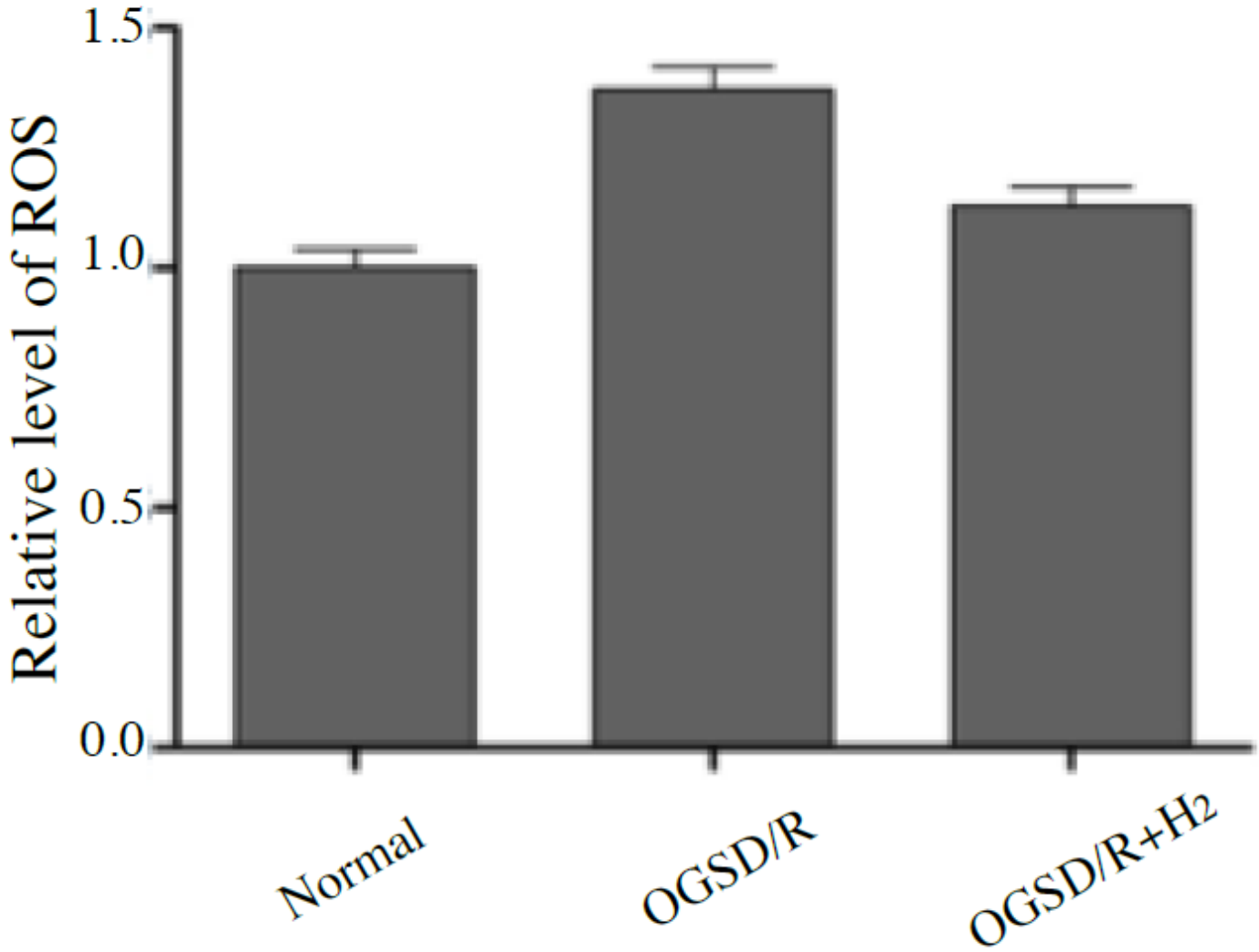

Figure 4

Compared with normal control group, ROS content in positive control group and hydrogen intervention group was both decreased after OGSD 12h/ R 1 h, and ROS content in hydrogen intervention group was significantly lower than that in positive control group ($P<0.01$).

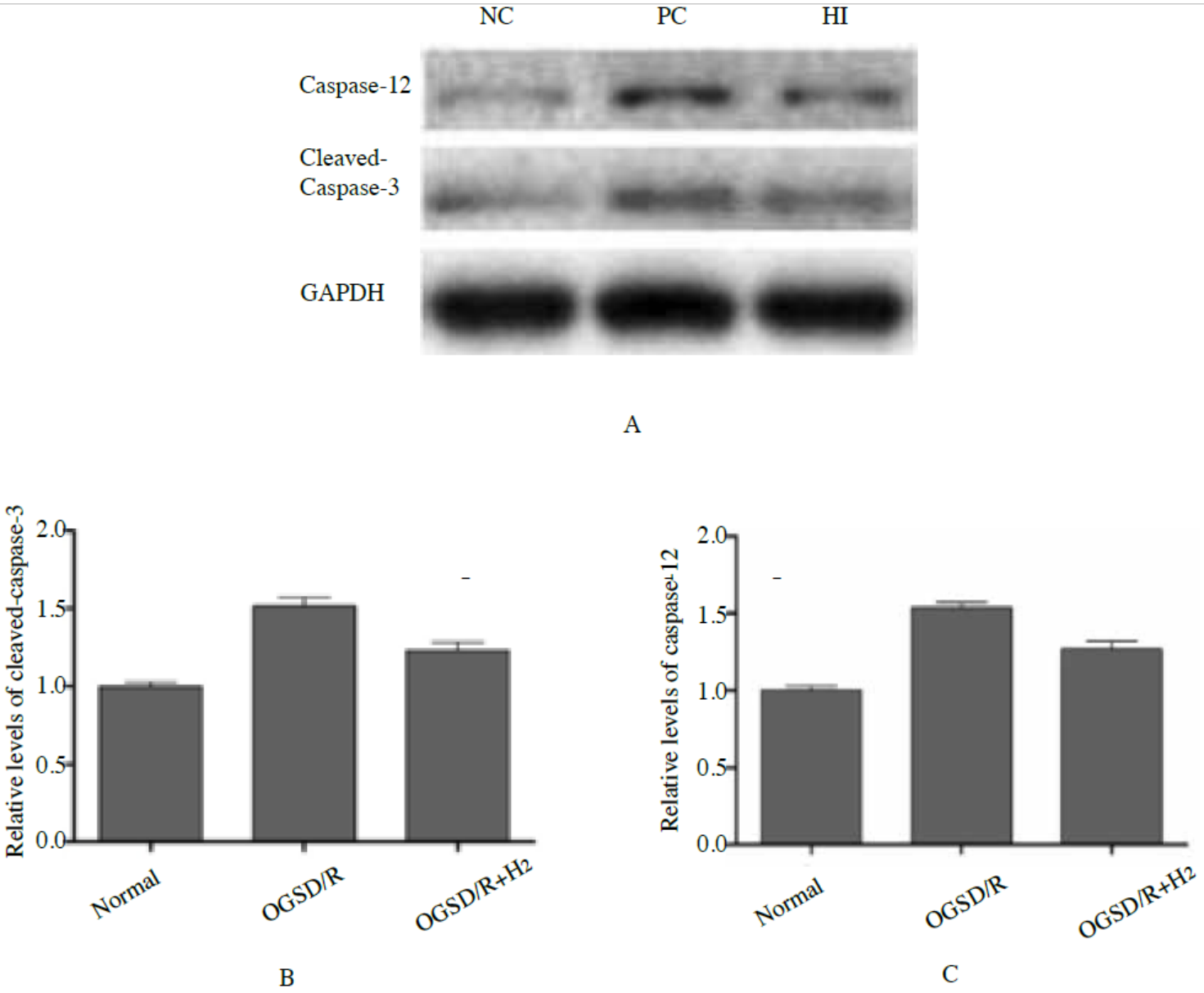

Figure 5

A.Comparison of the expression of caspase-12 and cleaved-caspase-3 in cells of each group after OGSD 12h/R 1h showed that the expression of these proteins in both positive control group and hydrogen intervention group was significantly higher than that in normal control group ($p<0.01$). B/C.After hydrogen intervention, cleaved-caspase-3 and caspase-12 decreased significantly as compared with positive control group ($p<0.01$).

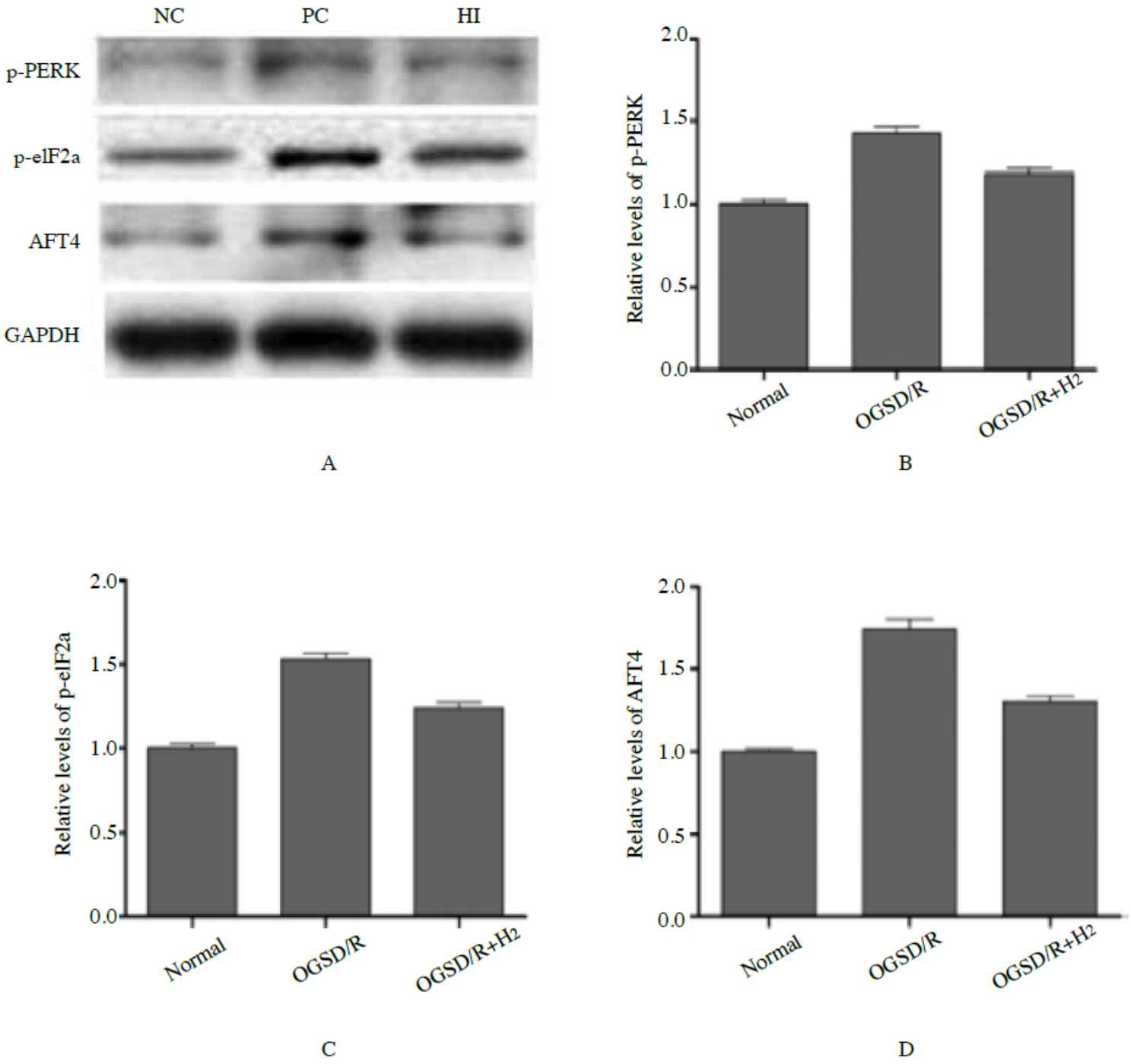

Figure 6

A.The ER-related apoptosis pathway PERK-eIF2a-ATF4 was activated at OGSD 12h/R 1 h. B/C/D.The expressions of the activated p-PERK, p-eIF2a and ATF4 in each group.

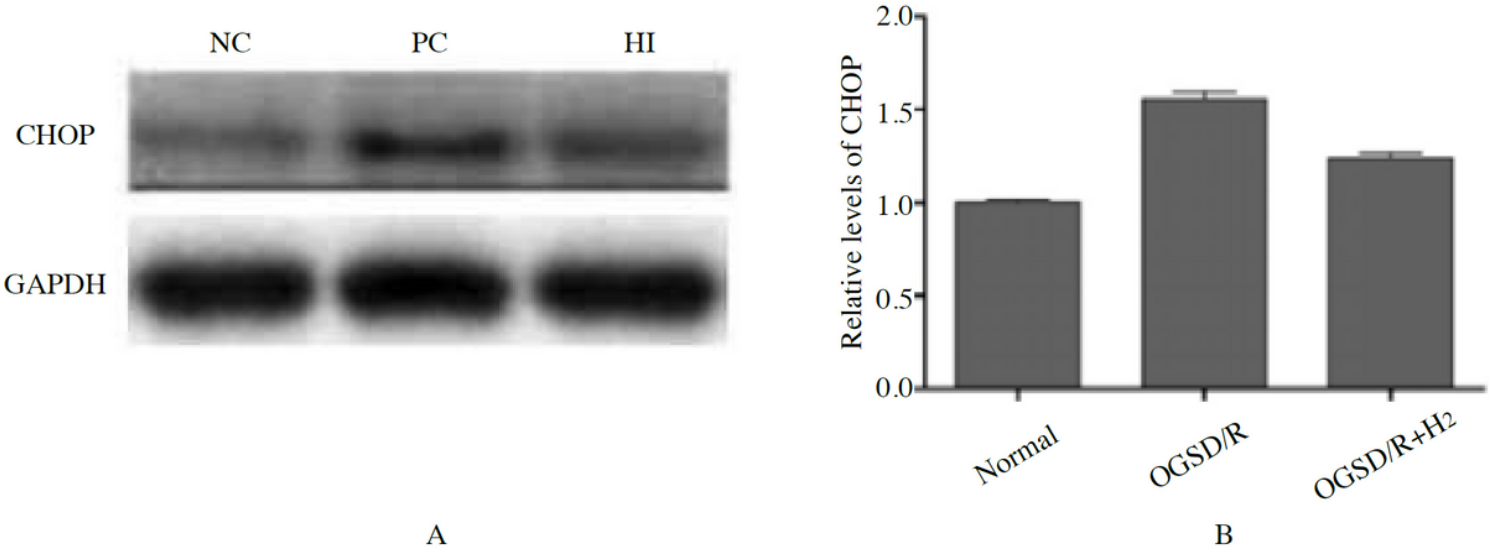

Figure 7

A/B.CHOP expression at OGSD 12h/R 1h was descread in HI group compared with PC group.