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Original Research

Hydrogen inhibits cytotrophoblast cells apoptosis in hypertensive disorders complicating pregnancy

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Abstract: Hypertensive disorders complicating pregnancy (HDCP) is one of the most serious medical disorders during pregnancy. Hydrogen is a therapeutic antioxidant and used to treat HDCP effectively. However, the molecular mechanism about the effect of hydrogen on HDCP still remains unclear. In this study, we found ROS content in HDCP group was significantly higher than that in the control and was reduced markedly in the presence of 100μmol/L hydrogen. IL6, Caspase3, Bax1, P-JAK2, P-Stat3 and P-p38 expression was much higher than the control, and was notably decreasedby the application of 100μmol/L hydrogen. Bcl2 expression in HDCP group was notably lower than the control and was increased by 100 μmol/L hydrogen. The apoptosis rate of cytotrophoblast cells was decreased, andratio of cytotrophoblast cells at G1 and G2 phase was increased and decreased by hydrogen, respectively. All those data indicated a potential molecular mechanism of hydrogen-mediated treatment in HDCP.

Key words: Hypertensive disorders complicating pregnancy, Reactive oxygen species, Hydrogen, MAPK pathway.

Introduction

Hypertensive disorders complicating pregnancy (HDCP) is taken as one of the most serious medical disorders during pregnancy (1). It forms the deadly triad along with hemorrhage and infection that greatly increases maternal morbidity and mortality rates (2). It causes many complications such as preterm delivery, fetal growth restriction and placental abruption (3).Despite researches on HDCP have been conducting, its etiology and pathogenesis is still unclear, it may be caused by multiple genes and affected by various environmental factors.

At present, the pathological physiology change of placenta is taken as a central culprit in HDCP (4). Placenta provides an appropriate womb growth environment for fetus. In normal, cytotrophoblast cells invade the uterine spiral arteries, replacing the endothelial layers of these vessels, which result in vascular cavity expansion, blood resistance drop and blood volume increase (5). The form and function change of placenta would result in adverse pregnancy reaction. The key pathogenic mechanism of HDCP is thought to be the lack of cytotrophoblast cells invasion and damage of vessel endothelial layers (6). When placental vascular remodeling obstacle happens, the oxidative stress (OS) of placenta is deepened and a large number of reactive oxygen species (ROS) was produced (7, 8). ROS influences many physiological processes including host defense, hormone biosynthesis, fertilization, and cellular signaling (9). Moreover, mounting evidence reports a physiological function for ROS as a "second messenger" in intracellular signaling cascades that control cell growth, proliferation, migration, and apoptosis (10). It can activate mitogen-activated protein kinases (MAPKs) pathways (11). MAPKs are serine-threonine protein kinases that play important role in signal transduction from the cell surface to the nucleus (12). It consists of growth factor-regulated extracellular signal-related kinases (ERKs), the stress-activated MAPKs, c-jun NH2-terminal kinases (JNKs) and p38 MAPKs. P38 MAPKs are the most widely distributed and the main system that mediates intracellular signal transduction in the cell (13). JNK and p38 MAPKs function in a cell context-specific and cell type-specific manner to integrate signals that affect proliferation, differentiation, survival and migration (14).

As OS is one of the most key factors resulting in HDCP, so reducing OS is an important measure to inhibit HDCP. There are many antioxidants have been used including vitamin C, vitamin E, hydrogen peroxide (15-17). Although those antioxidants have some curative effect, the results are not good enough. When vitamin C or vitamin E concentration is higher compared with the normal, the production of human chorionic gonadotrophin (HCG) decrease and tumor necrosis factor-α (TNF-α) concentration increase observably (18). Therefore, the research on more effective and safe antioxidants is of great clinical significant. Studies showed hydrogen could act as a therapeutic antioxidant in reducing cytotoxic oxygen radicals (19). It is not only permeable to cell membranes and can target organelles, but also specifically quenches exclusively detrimental ROS, such as $^{\cdot}OH$ and peroxynitrite ($ONOO^{-}$) (20). Moreover, it has strong specificity and not affect the other normal

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physiological function of free radicals (21). Therefore, hydrogen may be used as a selective antioxidant and play key role in the prevention and treatment of HDCP.

In this study, we determined the effect of hydrogen on ROS content, protein expression of key factors in the MAPK pathway, cytotrophoblast apoptosis and cell cycle alteration.

Materials and Methods

Cell culture

Twenty HDPC patients were selected from Ruijin Hospital Shanghai Jiao Tong University School of Medicine. Cytotrophoblast cells were taken from the center of maternal placenta after the delivery of placenta during delivery or cesarean. Percoll density gradient centrifugation was used to separate and purify the cytotrophoblast cells. Substrate of DMEM/Ham's F12 (Gibco BRL, Grand Island, NJ, USA) was added to suspend cells and then cells were cultivated on 6-wells plate with 1×10^6 cells/mL. After culturing cells at 37ºC and 5%$CO_2$for 24 h, Hank's solution was used to clean the non-adherent cells.

MTT assays

Cytotrophoblast cells were grown in 96-well plates, incubated with different hydrogen concentration at 0, 25, 50, 100, 200 μmol/L.The cells were treated with hydrogen for 4 hours. After a 48h incubation of placenta cytotrophoblast cells in 100 μl RPMI-1640 substrate supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, 20 μL of a 5 mg/ml MTT solution in PBS was added, and cells were incubated at 37°C, 5% CO2-humidified atmosphere for 4 h. The cells were then lysed by addition of 100 μl per well extraction buffer DMSO. After overnight incubation with extraction buffer, the optical density (OD) at 490 nm was measured.

ROS measurement by Reactive Oxygen Species Assay Kit

The experiment was divided into 6 groups and they were: the control (cells collected from health pregnant woman), HDCP, hydrogen concentration of 25, 50 and 100 μmol/L, N-Acetyl-L-cysteine (NAC) group. The ROS content in those groups was determined by Reactive Oxygen Species Assay Kit (Pierce, Rockford, IL, USA) with the DCFDA fluorescence method, as described previously(22). Cells were incubated at 37°C for 20 min in RPMI with 10 μmol/L DCFDA. At the end of the incubation, the substrate containing DCFDA was aspirated, and the cells were washed thrice in RPMI. For induced activation, cells were simultaneously cultured, along with DCFDA, with 30 ng/ml phorbol 12-myristate-13-acetate (PMA) (Sigma, St. Louis, MO, USA) for 30 min. Rosup was used as a positive control. The fluorescence of oxidized DCFDA in cell lysates was determined on an Aminco Bowman series-2 spectrofluorometer (Spectronic Instruments, Rochester, NY, USA) at 490/530-nm excitation/emission, using appropriate blanks.

Western blot assay

To compare the differences of related enzyme expression between the 6 groups, western bolt assay was used. Substrate was discarded and attached cells were washed thrice with PBS. Then cells were pelleted by centrifugation at 3000 r/min for 10 min, at 4°C and resuspended in a lysis buffer (25 mM HEPES; 2,5 mM EDTA; 0.1% Triton X-100, 1 mM PMSF, 5 μg/ml leupeptin). Samples were sonicated for 30 s at 1.5 mA and lysates were clarified by centrifugation at 4000 rpm for 10 min. After protein concentration analysis of cell lysates, by using the protein assay kit (Bio-Rad, Richmond, CA, USA), 75–100 μg protein/condition were separated by SDS-12% polyacrylamide gel electrophoresis. Bands of proteins were then transferred to a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad). The PVDF membrane was blocked by PBS containing 5% milk overnight at 4 °C and then incubated with antibodies against IL6, Caspase3, Bax1, P-JAK2, JAK2, P-Stat3, Stat3, P-p38, p38 Bcl2and β-actinfor 3 h at room temperature. All the antibodies were purchased from Cell Signaling Technology. After additional 1h incubation with horseradish peroxidase-conjugated secondary antibodies, the binding of antibodies to the PVDF membrane was detected with an enhanced chemiluminescence (ECL) analysis.

Apoptosis and cell cycle analysis

The cells were placed in a 96-well plate at a density of 2×10^3 cells/well in supplemented RPMI 1640 and incubated for 16 h before the cells were subjected to treatment in triplicate wells. After treatment, the cells were washed twice in phosphate-buffered saline (PBS) (2.68 mMKCl, 1.47M KH2PO4, 8 mMNa2KPO4, 136.75 mMNaCl) and counted. Fifty to one hundred thousand cells were selected and centrifuged 5 min at 1000r/min. Annexin V-FITC mixed liquor of 195 μL was added to resuspendcytotrophoblast cells and 5 μL was added to mix. Centrifugation at 1000r/min for 5 min was performed after cultivation 10 min. Sample was obtained after discarding supernatant and 10μL propidium iodide (PI) was added. Afterwards, the sample was stilled in dark for 30 min. Finally, the apoptosis and cell cycle were detected using flow cytometry (FCM) on the Moflo (DakoCytomation, Glostrup, Denmark).

Data analysis

Mean ± standard deviation (SD) was employed to convey the statistics from three separate experiments. Density of western blot bands was calculated by Image J software. The differences between groups were analyzed using SPSS 10.0 software and considered statistically significant at p value less than 0.05.

Results

MTT assays determined the optimal hydrogen concentration

In this study, DMEM substrate with different hydrogen concentration of 0, 25, 50, 100 and 200 μmol/L was used to incubate cytotrophoblast cells. The cell proliferation in different groups was determined by MTT assays and the result was shown in Figure 1. The cytotrophoblast cells vitality of HDPC increased with the increase of hydrogen concentration. It reached to the maximum with hydrogen concentration of 100 and 200 μmol/L, which was significantly higher than that in the

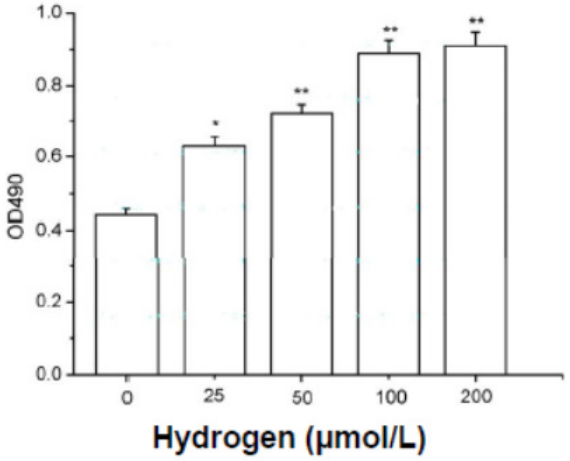

Figure 1. The MTT assay showed that hydrogen could induce the apoptosis of Cytotrophoblast cells, tested through the OD value at 490 nm. ##p<0.01.

0 μmol/L group (p<0.01). However, there was no significant difference of cells vitality between the groups with hydrogen concentration of 100 and 200 μmol/L. Therefore, hydrogen concentration of 25, 50 and 100 μmol/L was selected for further study.

ROS measurement by Reactive Oxygen Species Assay Kit

To study the influence of hydrogen on HDCP, the ROS content in 6 groups was determined and compared due to the key role of ROS on leading to HDCP. In this study, ROS content was determined by Reactive Oxygen Species Assay Kit which used fluorescent probe DCFHDA to detect ROS content. ROS content in the control was significantly lower than that in other groups (p<0.05). It decreased with the increasing concentration of hydrogen and was notably lower compared with that in HDCP group (Fig.2).

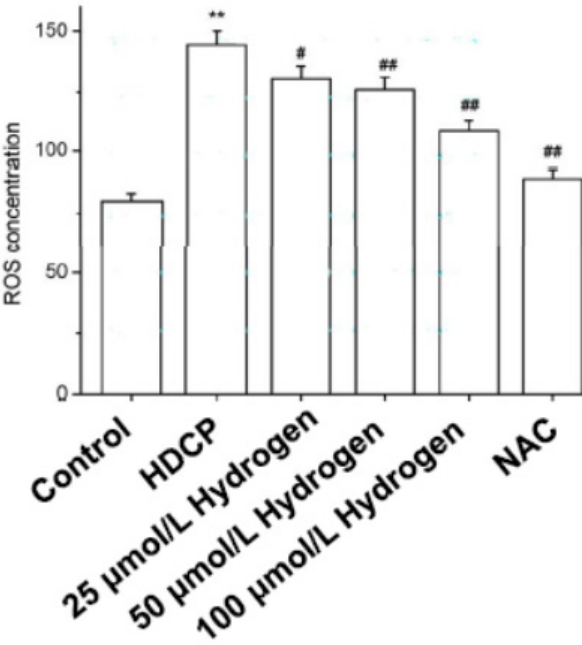

Figure 2. The increasing ROS concentrationinduced by HDCP was counteractedin the presence of hydrogen. #p<0.05, **p<0.01, ##p<0.01.

The effect of hydrogen on HDCP was mediated through MAPK pathway

To study how hydrogen, play as an antioxidant role in HDCP, the expression of IL6, Caspase3, Bax1, P-JAK2, JAK2, P-Stat3, Stat3, P-p38, p38 and Bcl2 was detected. The results indicated that the expression of IL6, Caspase3, Bax1, P-JAK2, P-Stat3 and P-p38 in HDCP group was notably higher than that in the control (Fig.3). Their expression value decreased distinctly with increasing hydrogen concentration, and the expression in the group with 100 μmol/L hydrogen was also similar with that in the control. Bcl2 expression in HDCP group was notably lower than that in the control. It increased obviously with the increase of hydrogen concentration in substrate and reached the maximum with 100 μmol/L hydrogen. All the statistical analysis data was shown in the supplementary figure 1.

Apoptosis and cell cycle analysis

To study the mechanism how hydrogen act as an antioxidant, Flow Cytometry was used to analyze the apoptosis and cell cycle. The effect of hydrogen on the apoptosis of HDCP placenta cytotrophoblast cells was shown in Figure 4. The apoptosis rate of cytotrophoblast cells in HDCP group was much higher compared to the control and decreased with hydrogen concentration increased from 25 μmol/L to 100 μmol/L. The apoptotic rate of cytotrophoblast cells in NAC group was the lowest (Fig.4).

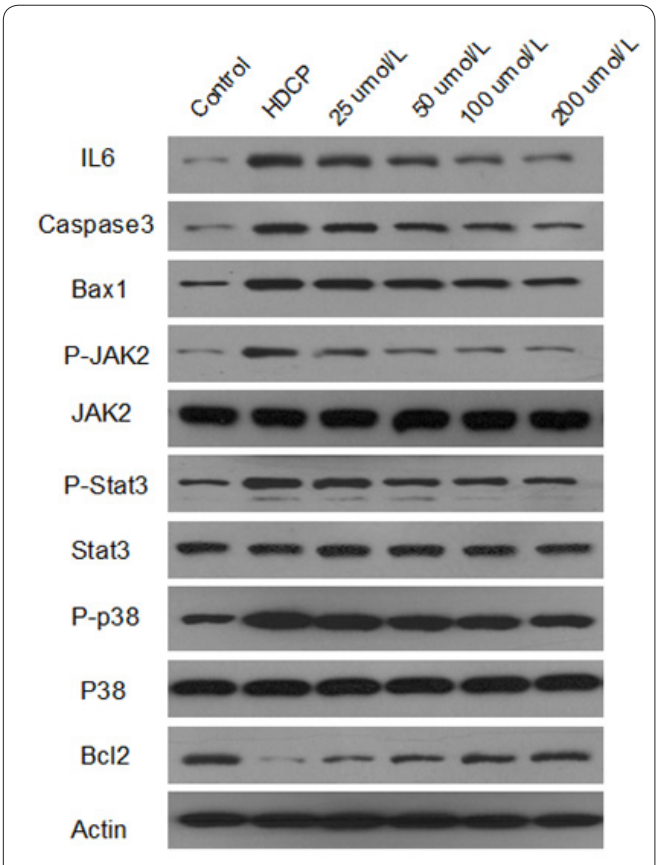

Figure 3. Application of hydrogen affected the expression level of MAPK signaling proteins, such as IL6, Caspase3, Bax1, P-JAK2, JAK2, P-Stat3, Stat3, P-p38, P38 and Bcl2.

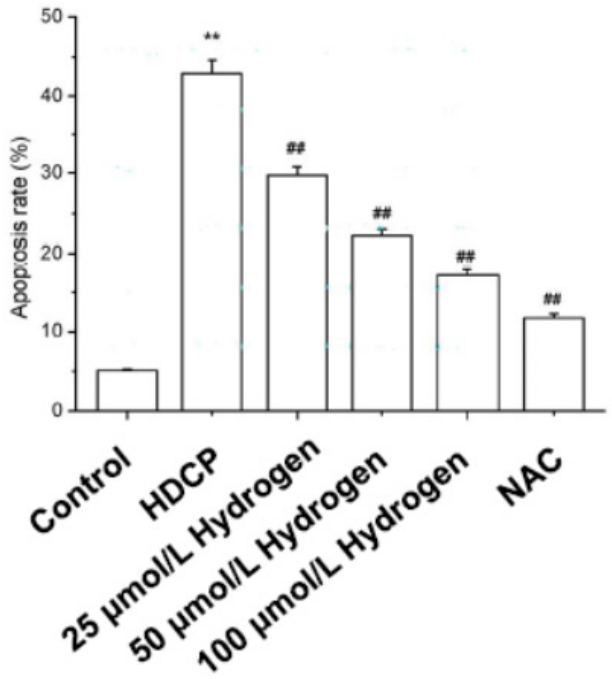

Figure 4. Hydrogen caused apoptosis ofcytotrophoblast cells at concentrations of 25, 50 and 100 μmol/L.*p<0.05, **p<0.01.

As shown in Fig 5, G1 content in the control was markedly higher than that in HDCP, substrate with hydrogen concentration of 25, 50 and 100 μmol/L group (p<0.05), and slightly higher than the NAC group. The value increased with hydrogen concentration increased from 25 μmol/L to 100 μmol/L. G2 content in the control was markedly lower than that in the other groups (P<0.05) and decreased gradually with hydrogen concentration increase (Fig.5).

Discussion

HDCP are the common and significant cause of maternal morbidity and mortality especially in developing countries (23). Various theories about the etiology of HDCP have been put forward including abnormal trophoblastic invasion, oxidative stress, genetic factors, and maternal maladaptation to vascular and inflammatory changes. In HDCP, the abnormal cytotrophoblasts invasion fails to replace tunica media, resulting in the vasoconstriction of mostly intact arterioles (24). Previous study reported hemoglobin-induced oxidative stress is a pathogenic factor in HDCP (25). There were many antioxidants used to treat HDCP including N-Acetyl-L-cysteine (NAC), Vitamin C, Vitamin E and carbon monoxide. NAC is a precursor of glutathione and has immunomodulatory properties (26, 27). Although those antioxidants are effective sometimes in HDCP, there exist many problems such as the removal disorder of oxygen free radical, significant toxicity and narrow therapeutic window (28, 29). Hydrogen has been used as a therapeutic antioxidant in treating HDCP. However, the pathogenesis still remains unclear. Therefore, we study the effect of hydrogen of HDCP in molecular mechanism in this study. ROS are constantly formed in the human body and removed by antioxidant defenses (30). It plays an important role in apoptosis induction under both physiologic and pathologic conditions (31). The overproduction of ROS are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates and DNA which ultimately results in OS (32). Moreover, ROS was reported activate multiple intracellular proteins and enzymes, including the epidermal growth factor receptor, c-Src, p38 MAPK, Ras, and Akt/protein kinase B (33). IL-6 is an important inducer of acute phase proteins at the transcriptional level (34). Caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins (35). It is required for DNA fragmentation and some of the typical morphological changes of cells undergoing apoptosis (36). Bax1 is the potent apoptosis inducer (37). During apoptosis, oxidative stress could result in Bax1 activation (38). JAK2 is reported the substrate of protein-tyrosine phosphatase 1B (39). STATs belong to a family of transcription factors activated in response to cytokines and growth factors (40). The activation of Stat3 has been found in many tumour-derived cell lines, as well as in tumor specimens from human cancers (41). P-Stat3 is a key signaling molecule in the regulation of growth and malignant transformation (42). P38 signaling transduction pathway, a MAPK pathway, plays a key role in regulating many cellular processes including inflammation, cell differentiation, cell growth and death (43). Moreover, P38 MAPK can be activated by OS (44). Bcl-2 functions in an antioxidant pathway to prevent apoptosis (45). The result indicated hydrogen can reduce IL6, Caspase3, Bax1, P-JAK2, P-Stat3 and P-p38 content which were activated by OS. Therefore, the apoptosis of cytotrophoblast cells decreased, the cell viability increased and the inflammation alleviated. Furthermore, based on the result of cell cycle analysis, we concluded the use of hydrogen could increase protein synthesis and the cytotrophoblast cells proliferation, which decreased the OS level and reduce the burden of HDCP.

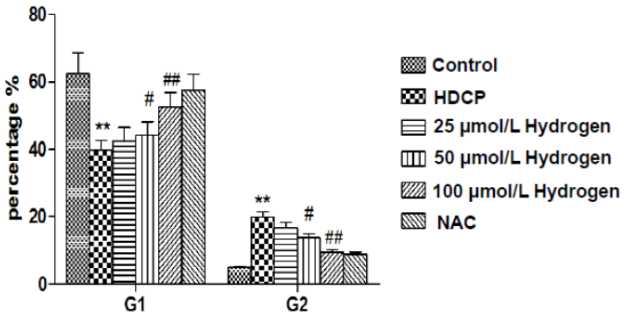

Figure 5. The ratio of G1cells was decreased in the HDCP, while it was increased in the presence of hydrogen. By contrast, the ratio of G2 cells showed the opposite tendency to G1 cells. #p<0.05, **p<0.01, ##p<0.01.

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