Hydrogen-rich water ameliorates rat placental stress induced by water restriction

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Abstract

Dehydration is one of the intrauterine abnormalities that could lead to fetal growth retardation and to increase the risk of a variety of adult diseases later in life. This study were to determine the impact of hydrogen-rich water (HRW) supplementation on placental angiotensin II type 1 receptor and placental oxidative stress induced by water restriction. Pregnant Wistar rat were randomly assigned to one of the three groups (n = 12 per group). In control group, pure water and food were supplied ad libitum. Water restriction group and HRW group were respectively given pure water and HRW with free access to food, excepting only one hour was available for drinking from day 7 to day 17 of pregnancy. The placental damages and biomarkers of stress were detected by histopathology, immunohistochemistry and western blot, as well as serological test were performed. We demonstrated that maternal water restriction resulted in reduced urine volume and increased serum osmotic pressure, along with decreased fetus weight and crown-rump length. Although placental weight and the number of fetuses had no significant difference among groups, the placental efficiency significantly increased after the oral administration of HRW to the mothers. Meanwhile, the serological derivatives of reactive oxygen metabolites decreased, a significant improvement of placental microstructure with more developed junctional zone and denser labyrinth was manifested, the upregulated expression of angiotensin II type 1 receptor, nuclear factorκB, malondialdehyde, 8-hydroxydeoxyguanosine, p38, e-Jun N-terminal kinase and down-regulation of superoxide dismutase were revealed in the placenta. Collectively, HRW administration is able to effectively attenuate placental stress induced by water restriction.

Key words: hydrogen-rich water; dehydration; oxidative stress; AT1R; growth retardation; fetus; placenta; rat

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INTRODUCTION

Fifty to eighty percent of women during early pregnancy are vulnerable to nausea and vomiting,1,2 in 22% of the cases, the symptoms might be severe (or hyperemesis gravidarum) and continue until delivery, that may contribute to dehydration. Dehydration is one of the intrauterine abnormalities that could lead to fetal growth retardation (FGR) and the ensuing risk of a variety of adult diseases later in life. This study were to determine the impact of hydrogen-rich water (HRW) supplementation on placental angiotensin II type 1 receptor and placental oxidative stress induced by water restriction. Pregnant Wistar rat were randomly assigned to one of the three groups (n = 12 per group). In control group, pure water and food were supplied ad libitum. Water restriction group and HRW group were respectively given pure water and HRW with free access to food, excepting only one hour was available for drinking from day 7 to day 17 of pregnancy. The placental damages and biomarkers of stress were detected by histopathology, immunohistochemistry and western blot, as well as serological test were performed. We demonstrated that maternal water restriction resulted in reduced urine volume and increased serum osmotic pressure, along with decreased fetus weight and crown-rump length. Although placental weight and the number of fetuses had no significant difference among groups, the placental efficiency significantly increased after the oral administration of HRW to the mothers. Meanwhile, the serological derivatives of reactive oxygen metabolites decreased, a significant improvement of placental microstructure with more developed junctional zone and denser labyrinth was manifested, the upregulated expression of angiotensin II type 1 receptor, nuclear factorκB, malondialdehyde, 8-hydroxydeoxyguanosine, p38, e-Jun N-terminal kinase and down-regulation of superoxide dismutase were revealed in the placenta. Collectively, HRW administration is able to effectively attenuate placental stress induced by water restriction.

Materials and Methods

Animals and protocol

The study was approved by the Ethics Committee of Taishan Medical University, China (approved No. 2014007). Forty female and ten male Wistar rats aged 7 weeks were purchased from the Experimental Animal Center of the Lukang Company, Jinan, Shandong Province, China (SCXK 2014007). After acclimation for 1 week, the female rats in estrus stage were put together with the male for mating overnight. The estrus cycle or pregnancy was decided by vaginal smears. Day 1 was defined as the following morning if spermatozoa were seen in the vaginal...
The pregnant rats were put into the metabolic cage and randomly assigned to one of the three groups ($n=12$ per group).

In control group (CG), water and food were supplied ad libitum. Water restriction group (WR) was given pure water ad libitum from D1 to D6, but 1 hour a day was available to drink from D7 to D17 with free access to food. HRW group (HW) was supplied HRW at 80°C concentration; 10–800 µg/L, HWP-200WWD, SEEEMS Bionics Inc., Wonju, Korea) twice a day from D1 to D6, but 1 hour a day was available to drink from D7 to D17 with free access to food. To prevent hydrogen degassing as well as air refill, a glass bottle with a ball bearing at the outlet was used.

All rats were sacrificed under isoflurane (Sigma, USA) anaesthesia from 9:00 a.m. to 11:00 a.m. on D17. After collecting blood from the heart, acesarean section was carried out, then number of fetus was counted, individual fetus and placenta were weighed and measured. The middle one third of the placenta, including decidua and mesometrial triangle were fixed in 10% buffered formalin solution (pH 7.4) for 1 day for histological and immunohistochemical analysis. The other part of placenta were snap-frozen in liquid nitrogen and stored at −20°C for Western blotting. Placental sufficiency was represented as the ratio of fetal to placental weight, indicating the ability of the placenta to transport nutrients to the fetal side.

Measurement of cyclic oxidative stress and osmolality

Serum was separated by centrifugation at 1500 × g for 15 minutes (4°C) and stored at −80°C. The derivatives of reactive oxygen metabolites (dROMs) (whole oxidant capacity of serum against N,N-diethylparaphenylendiamine in acidic buer) in serum was determined using the free radical elec-

triculator (Diacon International, Grosseto, Italy). The measurement unit was Carratelli Unit (CARR U). One unit of CARR corresponded to 0.08 mg/dL hydrogen peroxide. Serum osmotic pressure (OsmP) was determined using a standard freezing-point depression osmometer (Genotec, Germany).

Immunohistochemistry

The slices were processed through protocols of immunohis-
tochemistry with minor modifications. Briefly, following 3% H$_2$O$_2$ quenching endogenous peroxidase, the slices were microwaved to further expose nuclear antigens. Primary antibodies against 8-hydroxydeoxyguanosine (8-OHdG, 1:200, Chemicon, USA), malondialdehyde (MDA, 1:100, ab6463, Abcam), NFkB (1:500, ZS-109), AT1R (1:500, ab9391, Abcam), p38 (1:500, Cell Signaling Technology, Beverly, MA, USA), and c-Jun N-terminal kinase (JNK, 1:500, Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. The specificity of the immune response was detected without adding the first antibodies. After hybridization with a secondary antibody (1:2000), the target proteins were finally detected using ECL Western Blotting Detection Reagents (Thermo Scientific Pierce, Rockford, IL, USA).

Statistical analysis

Data are presented as the mean ± SEM. For multiple compa-

risons, repeated-measures analysis of variance (ANOVA) was per-
formed. When the overall F ratio was significant, the Dunnet’s test was used to locate differences with the appli-
cation of SPSS 13.0 (SPSS Inc., Chicago, IL, USA). A $P$ value of less than 0.05 was considered to be statistically significant.

RESULTS

HRW alleviated cyclic oxidative stress and placental efficiency of placental stress rat induced by water restriction

As shown in Table 1, water deprivation resulted in a decrease in urine volume, as well as an increase in serum osmotic pressure and dROMs. Either the average weight or the crown-rump length of fetuses in the WR and HW group was significantly lower than that of the CG group ($P<0.05$). No significant difference was found between WR and HW group regardless of the average number of fetus ($P>0.05$). Interestingly, although the fetus weight had no significant difference among groups, placental efficiency showed a trend of CG > HW > WR.

HRW restored placental histopathological changes of placental stress rat induced by water restriction

In the WR group, the amnion epithelium was detached from the surface of chorionic plate (Figure 1). Furthermore, the invasion of cytotrophoblasts into the endometrium was inadequate, even a separation between endometrium and junctional zone (JZ) could be observed (a condition known as placental abruption that can affect both the mother and fetus), as well as abnormal vasculogenesis in the labyrinth (the main compartment for maternal-fetal hematocrit exchange) was seen.

Western blot assay

Homogenized placental samples were lysed in RIPA buffer with protease and phosphatase inhibitors. Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, USA). A quantity of 30–40 µg total protein per lane was separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, USA). Blocked membranes were incubated with primary antibodies such as β-actin (1:1500, Sigma), MDA (1:400, ab6463, Abcam), NFkB (1:500, ZS-109), AT1R (1:500, ab9391, Abcam), p38 (1:500, Cell Signaling Technology, Beverly, MA, USA), and c-Jun N-terminal kinase (JNK, 1:500, Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. The specificity of the immune response was detected without adding the first antibodies. After hybridization with a secondary antibody (1:2000), the target proteins were finally detected using ECL Western Blotting Detection Reagents (Thermo Scientific Pierce, Rockford, IL, USA).

Contrast slices could not be stained. Stained slices randomly chosen from each specimen were photographed in random visual fields. The images were then analysed with Image Pro-Plus 4.5 (Media Cybernetics, Inc, Rockville, MD, USA), the average optical density (AOD) of positive area was obtained to reflect the quantity of target antigen. At least ten specimens from each of six animals were examined for all investigations.
HWF intake significantly improved placental pathological damages (Figure 1).

HRW reduced water restriction-induced stress protein in placenta of placental stress rats induced by water restriction

Immunohistochemistry revealed that SOD was mainly expressed in the cytotrophoblasts and the villous stromal cells (Figure 2A). AT1R was mainly localized in the decidual cells, especially around the spiral arteries in the mesometrial triangle. Sometimes light shadings in the cytotrophoblasts of JZ were observed. NFκB and 8-OHdG positive staining was seen in the decidual cells, cytotrophoblasts, syncytiotrophoblasts, and stromal cells of placental villi in the JZ. However, much weaker and discontinuous staining of MDA was observed in the cytotrophoblasts, syncytiotrophoblasts, and stromal cells of placental villi among different groups (Figure 2A). Quantitative analysis showed HRW supplementation significantly decreased the immunopositivities of AT1R, NFxB, MDA and 8-OHdG, up-regulated the immunopositivities of SOD as compared with WR group (P < 0.05; Figure 2B).

HRW improved the stress protein expression in placenta of placental stress rat induced by water restriction

The Western blot detection signal of the specific protein in the placenta of different experimental groups revealed AT1R, MDA, NFxB, p38and JUK (Figure 3A). The results indicated that HRW inhibited the expression of AT1R, NFxB, MDA, p38 and JUKas compared with WR group (P < 0.05; Figure 3B).

**DISCUSSION**

Dehydration during pregnancy may be harmful for the mother as well as the fetus.22 In the rat, the fertilized embryo reaches the uterus on D4 and implantation occurs at approximately D5 to D6.52,53 Maternal models of water-restricted/food-reduced were usually established from D7 to D17 with free access to food. Placental sufficiency was represented as the ratio of fetal weight to placental weight. Data are shown as the mean ± SEM, and analyzed by repeated measures analysis of variance. *P < 0.05, vs. CG group; #P < 0.05, vs. WR group. h: Hours.

**Table 1: Effects of hydrogen-rich water on fetus growth and fluid circulation of placental stress rats induced by water restriction**

| Item                                  | CG       | WR       | HW       |
|----------------------------------------|----------|----------|----------|
| Water intake (mL/24 h)                 | 42.29±5.53 | 34.67±7.16* | 35.52±5.19* |
| Urine volume (mL/24 h)                 | 15.71±7.50 | 10.12±1.73* | 11.45±1.03* |
| Average number of fetuses              | 12.24±3.43 | 12.83±2.04 | 12.57±2.91 |
| Body weight (g)                        | 287.61±11.04 | 262.12±16.44* | 265.32±10.55* |
| Fetus weight (g)                       | 0.68±0.13 | 0.50±0.14* | 0.55±0.08* |
| Placenta weight (g)                    | 0.32±0.10 | 0.30±0.18 | 0.29±0.15 |
| Placental sufficiency                  | 2.12±0.17 | 1.67±0.04* | 1.96±0.19* |
| Crown-rump length of fetuses (cm)      | 1.67±0.11 | 1.38±0.12* | 1.39±0.14* |
| Osmotic pressure (mOsm/kg)             | 280.81±2.15 | 296.07±7.90* | 291.35±3.77* |
| d-ROMs (CARR U)                        | 353.61±24.31 | 463.55±19.52* | 400.22±31.02* |

Note: In control group (CG), water and food were supplied ad libitum. Water restriction group (WR) was given pure water, but 1 hour a day was available to drink from day 7 of pregnancy (D7) to D17 with free access to food. Hydrogen-rich water group (HW) was supplied hydrogen-rich water HRW twice a day from D1 to D6, but 1 hour a day was available to drink from D7 to D17 with free access to food. Placental sufficiency was represented as the ratio of fetal weight to placental weight. Data are shown as the mean ± SEM, and analyzed by repeated measures analysis of variance. *P < 0.05, vs. CG group; #P < 0.05, vs. WR group. h: Hours.

![Figure 1: Effect of hydrogen-rich-water (HRW) on the placental histopathological changes of placental stress rats induced by water restriction (hematoxylin and eosin staining).](image)
water restriction resulted in rat FGR, reduced urine volume and increased serum osmotic pressure during D7 to D17 of pregnancy, which was consistent with those in another study by Desai et al. In addition, a significant improvement of placental microstructure with more developed junctional zone and denser labyrinth was manifested after the oral administration of HRW to the mothers. Molecular H₂ could be incorporated into the body by drinking and peak at 5 to 15 minutes after oral HRW administration in rat tissue. H₂ is assumed to penetrate the placental barrier and diffuse into the cytosol, mitochondria, and nucleus. We found H₂ administration might markedly profit placentation by decreasing cyclic d-ROMs and down-regulating placental oxidative insult, including decreased the expression of biomarkers, such as 8-OHdG (an indicator of oxidative DNA damage), MDA (a marker of oxidative lipid damage), NFkB (oxidative stress sensitive transcription fac-
Hydrogen plays an essential role in the process of placental pathophysiology. In our present study, expression of AT1R was validated in placenta by immunohistochemistry and Western blot assay. AT1R plays an essential role in the process of placental pathophysiology. In our present study, expression of AT1R was validated in placenta by immunohistochemistry and Western blot assay.

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In conclusion, this study provided the evidence supporting that HRW ameliorates placental damage and dysfunction induced by water restriction through ameliorating placental stress. This strategy could have a potential clinical benefit for prophylaxis and treatment of pregnant dehydration and related complications.

**Author contributions**
SQ, GS and YS designed this study; HQ and SJ established the animal model; YS, HJ and SJ conducted detections; YS and HJ conducted statistical analysis.

**Conflicts of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Data sharing statement**
Datasets analyzed during the current study are available from the corresponding author on reasonable request.

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