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Molecular hydrogen ameliorates several characteristics of preeclampsia in the Reduced Uterine Perfusion Pressure (RUPP) rat model

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ABSTRACT

Oxidative stress plays an important role in the pathogenesis of preeclampsia. Recently, molecular hydrogen (H_2) has been shown to have therapeutic potential in various oxidative stress-related diseases. The aim of this study is to investigate the effect of H_2 on preeclampsia. We used the reduced utero-placental perfusion pressure (RUPP) rat model, which has been widely used as a model of preeclampsia. H_2 water (HW) was administered orally ad libitum in RUPP rats from gestational day (GD) 12–19, starting 2 days before RUPP procedure. On GD19, mean arterial pressure (MAP) was measured, and samples were collected. Maternal administration of HW significantly decreased MAP, and increased fetal and placental weight in RUPP rats. The increased levels of soluble fms-like tyrosine kinase-1 (sFlt-1) and diacron reactive oxygen metabolites as a biomarker of reactive oxygen species in maternal blood were decreased by HW administration. However, vascular endothelial growth factor level in maternal blood was increased by HW administration. Proteinuria, and histological findings in kidney were improved by HW administration. In addition, the effects of H_2 on placental villi were examined by using a trophoblast cell line (BeWo) and villous explants from the placental tissue of women with or without preeclampsia. H_2 significantly attenuated hydrogen peroxide-induced sFlt-1 expression, but could not reduce the expression induced by hypoxia in BeWo cells. H_2 significantly attenuated sFlt-1 expression in villous explants from women with preeclampsia, but not affected them from normotensive pregnancy. The prophylactic administration of H_2 attenuated placental ischemia-induced hypertension, angiogenic imbalance, and oxidative stress. These results support the theory that H_2 has a potential benefit in the prevention of preeclampsia.

1. Introduction

Preeclampsia is a pregnancy specific hypertensive syndrome, and affecting approximately 3–5% of all pregnancies worldwide [1]. The World Health Organization estimates that preeclampsia is responsible for over 70,000 maternal deaths and 500,000 neonatal deaths annually [2]. Currently, there are no effective treatments for preeclampsia, except for delivery of babies. Iatrogenic premature delivery often causes severe complications in the neonates.

Over the last two decades, “the two-stage theory” has been widely used to describe the pathogenesis of preeclampsia [3,4]. In the first stage, suppression of trophoblast invasion by immune mechanisms leads to incomplete spiral artery remodeling and poor placental growth, which are related with fetal growth restriction. Poor spiral

Abbreviations: d-ROM, diacron reactive oxygen metabolites; GD, gestational day; H_2, molecular hydrogen; H_2O_2, hydrogen peroxide; HM, hydrogen medium; HW, hydrogen water; NM, normal medium; MAP, mean arterial pressure; 8-OHdG, 8-hydroxy-2′-deoxyguanoisine; ROS, reactive oxygen species; RUPP, reduced utero-placental perfusion pressure; sFlt-1, soluble fms-like tyrosine kinase-1; VEGF, vascular endothelial growth factor

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artery remodeling also evokes ischemia-reperfusion injury, causing oxidative stress in placental villi [5]. At the second stage, placental villi exposed to oxidative stress overproduce anti-angiogenic factors including soluble fms-like tyrosine kinase 1 (sFlt-1) [6], which contribute to maternal endothelial dysfunction [7], and perturb maternal angiogenic balance. The changes encountered in the second stage cause various clinical manifestations in the mother such as hypertension, renal impairment, and proteinuria [7]. Oxidative stress is therefore known to play a central role in the pathogenesis of preeclampsia [8] with placental oxidative stress being the main contributor [3,9].

Hydrogen peroxide (H_2O_2) serum concentration increases in women with preeclampsia [10]. Peroxynitrite is a strong pro-oxidant, which causes lipid peroxidation, tyrosine nitration, and DNA damage, which contribute to endothelial dysfunction [11]. The level of peroxynitrite was reported to increase in the placenta and maternal vasculature of women with preeclampsia [12]. Thus, the preventive or therapeutic potential of antioxidants in preeclampsia has been discussed [8].

Molecular hydrogen (H_2) is a novel antioxidant. It has been first reported that selectively reduces levels of toxic reactive oxygen species (ROS) in cultured cells, including hydroxyl radicals and peroxynitrite, and has cytoprotecitve effects [13]. Thereafter, H_2 has been reported to prevent a variety of diseases associated with oxidative stress in animal models and human diseases [14,15]. We hypothesized that H_2 might have several advantages in preventive application for preeclampsia. Firstly, H_2 selectively reduces peroxynitirite, which has a pathological role on preeclampsia as above mentioned, without deletion of nitric oxide, which is a member of ROS but has a protective role against preeclampsia [16,17]. Secondly, H_2 can rapidly diffuse into tissues, and we previously reported that maternal H_2 administration exhibit antioxidant effects in placenta [18]. Thirdly, no side effect has been reported in animal models and patients [19]. H_2 is also reported to have no cytotoxicity, even at high concentrations, although vitamins caused cytotoxicity at high concentrations [20]. Thus, it is thought to be safe for a prophylactic use.

In this study, we investigated whether administration of H_2 could prevent pathophysiological characteristics related to preeclampsia using the reduced utero-placental perfusion pressure (RUPP) rat model. The rat RUPP has been widely used as an animal model of preeclampsia, which closely mimics several features of preeclampsia, including ischemia-induced hypertension, and angiogenic imbalance [21]. A previous report showed that the rat RUPP model is also a model of oxidative stress observed during preeclampsia [22]. In addition, we investigated the effect of H_2 on the expression of sFlt-1 by using a trophoblast cell line and placental villous explants from women with preeclampsia.

2. Materials and methods

2.1. H_2 water and H_2 medium

H_2 water (HW) and H_2 medium (HM) were formulated at approximately 50% saturation, by dissolving H_2 gas into water or the medium RPMI 1640 (Sigma-Aldrich Japan, Tokyo, Japan) under high pressure using a hydrogen water-producing device, as previously described [23]. The HW and HM used in this study were donated by Blue Mercury Inc. (Tokyo, Japan). The HW and HM were stored in aluminum pouches to prevent hydrogen loss. The H_2 concentration of HW and HM was approximately 0.4 mM. In order to minimize the loss of H_2 while feeding, HW from the aluminum pouch was quickly placed into a special closed glass vessel equipped with an outlet line containing two ball bearings, which prevents H_2 from escaping. The concentration of H_2 remained greater than 0.2 mM after saving in the glass bottle for 24 h [18]. The HW was exchanged every 24 h. HM was maintained below 40 μm after 1 h and below 1 μm after 3 h [24]. The concentration of H_2 in the normal medium (NM) was 0.19 μm [24].

2.2. Experimental protocol in animal models

This study was approved by the institutional animal care and use committee at Nagoya University (approval number: 27025). Time-pregnant female Sprague-Dawley rats (10–12 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). Animals were housed in a temperature-controlled room (23 °C) under a 12 h light/dark cycle and were provided a standard diet and tap water ad libitum.

The pregnant rats were assigned randomly to one of three groups. The control group or the reduced utero-placental perfusion pressure (RUPP) group underwent sham operation or RUPP procedure on gestational day 14 (GD14), respectively, and the HW+RUPP group was given HW ad libitum from GD12 to GD19, starting 2 days before RUPP procedure. The rats in the HW+RUPP group drank approximately 120 mL/kg of HW per day.

On GD14, under anesthesia with 2–3% isoflurane, the control rats underwent a sham surgery, which included midline incision and suture. Pregnant rats entering the RUPP and HW+RUPP groups underwent the following clipping procedure on the same day. After the midline incision, the lower abdominal aorta was isolated, and a plastic clip, AS-1, 40 g/mm^2 (Natsume Seisakusho, Tokyo, Japan) was placed around the aorta above the iliac bifurcation. Plastic clips (AS-1 20 g/mm^2, Natsume Seisakusho, Tokyo, Japan) were also placed on branches of both the right and left ovarian arteries that supply the uterus.

2.3. Measurement of mean arterial pressure (MAP), urinary protein and conceptus

On GD19, all rats were anesthetized via inhalation of isoflurane and a 0.9 Fr optical fiber microcatheter (FISO-LS-PT9, LMS, Tokyo, Japan) was inserted into the right carotid artery. Arterial pressure was monitored and recorded via an attached pressure transducer (FPI-LS-10, LMS, Tokyo, Japan), as previously reported [25]. After measurement of MAP, the pups were delivered via hysterectomy. Urine samples were collected on GD 18 and centrifuged before storing at −80 °C for analysis. Urinary protein concentration was determined using a rat albumin ELISA kit (Nephrat II, Exocell, Philadelphia, PA) according to the manufacturer's instruction. Serum samples were collected for subsequent assays. Placental tissues and pups were excised and weighed, and then stored at −80 °C for later analysis. Resorption rate was shown as percent fetal resorption =(number of absorbed fetuses/total number of numbers) ×100, as previously reported [26].

2.4. Measurement of sFlt-1 and vascular endothelial growth factor (VEGF)

The concentrations of sFlt-1 and VEGF in maternal rat serum on GD19 were determined using a commercially available ELISA kit (E-EL-R0911 and E-EL-R0020, respectively, Elabscience, Wuhan, China) according to the manufacturer's instruction.

2.5. The diacron reactive oxygen metabolites (d-ROMs) test

The d-ROMs test for evaluating oxidative stress, which measures the level of reactive oxygen metabolites (including mainly hydroperoxides), was performed using a free radical automatic analyzer FRAS 4 (Wismerll, Tokyo, Japan) according to the manufacturer's protocol. Briefly, 20 μL of serum sample and 1 mL of acetic acid buffer solution of pH 4.8 to stabilize the hydrogen ion concentration were mixed in a cuvette, and then 10 μL of colorless chromogen (N, N-Diethyl-p-phenylenediamine) was added to the cuvette. After incubation for 5 min at 37 °C, chromogen was oxidized by free radicals and converted to a radical cation with a magenta color. The magenta color was measured using a photometer (505 nm).

2.6. Immunohistochemistry

Immunohistochemistry of the rat placenta and kidney was performed as previously reported [18]. All placenta samples from resorbed embryos were excluded. Briefly, samples were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm-thick sections. For heat-induced epitope retrieval, deparaffinized sections were placed in 10 mM citrate buffer (pH 6.0) and heated for 20 min at 95 °C using microwave equipment MI-77 (Azumaya, Tokyo, Japan). Immunohistochemical staining was performed using the avidin–biotin immunoperoxidase technique with the Histofine SAB-PO kit (Nichirei, Tokyo, Japan) according to the manufacturer's protocol. For placenta, anti-8-hydroxy-2′-deoxyguanoisine (8-OHdG) monoclonal antibody (the Japan Institute for the Control of Aging, Shizuoka, Japan) was used at a concentration of 5 μg/mL. The count of stained cells in the placental images was analyzed using ImageJ software (version 1.48, W.S. Rasband, NIH, Bethesda, MD) according to the manufacturer's protocol.

2.7. Analysis of 3-nitrotyrosine by Western blotting

Placental tissues were smashed under liquid nitrogen and lysed in RIPA Lysis buffer (Merck Millipore Corporation, Darmstadt, Germany) with cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich Japan, Tokyo, Japan). Samples were homogenized using 27 G needle and then centrifuged at 10,000 rpm at 4 °C for 10 min. The protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific K.K., Yokohama, Japan). Immunoblotting was performed with anti-nitrotyrosine antibody (Merck Millipore Corporation) and anti-β-actin antibody (Santa Cruz Biotechnology, Inc., Dallas, TX) at a dilution of 1:500 and 1:5000, respectively. The band intensities were quantified by densitometry, and normalized according to the level of β-actin.

2.8. Cell culture and treatment

The choriocarcinoma cell line BeWo (ATCC® CCL98™) has been widely used as an in vitro model of human trophoblast cells [27,28]. BeWo cells were cultured in RPMI 1640 with 10% fetal calf serum (FCS), penicillin 100 U/mL, streptomycin 100 μg/mL, and amphotericin B 2.5 μg/mL, and maintained in 5% CO_2 at 37 °C. BeWo cells were seeded at a density of approximately 2×10^6 cells in a 6 cm dish. After 18 h, the culture medium was replaced with 2 mL of ordinary RPMI 1640 (NM) or H_2-rich RPMI 1640 (HM). After the first 3 h of incubation, the culture medium was replaced with fresh medium, and H_2O_2 was added to the dishes at a final concentration of 100 μm and 500 μm, which previously been reported to induce oxidative stress in trophoblasts [29].

For hypoxia experiments, BeWo cells were incubated at 1%, 5%, or 20% O_2 in oxygen regulators (9000E, wakenbtech, Kyoto, Japan) for a total of 6 h.

To maintain the concentration of H_2, medium in all groups was replaced every 3 h. After incubation for 6 h under oxidative stress or hypoxia, cells were collected and RNA was extracted.

2.9. Cell proliferation assay

BeWo cells (3×10^3 cells/well in 96-well plates) were cultured in medium with 10% FCS. After seeding for 24 h, the culture medium was replaced with 100 μL of ordinary RPMI 1640 (NM) or H_2-rich RPMI 1640 (HM) with 2% FCS. Cell viability was measured with the modified tetrazolium salt assay using the Cell Titer 96 Aqueous One Solution Proliferation Assay kit (Promega, Madison, WI), after treatment for 24 h, 48 h, and 72 h, according to the manufacturer's instructions. Medium in both groups was replaced every 24 h. The mean values of three independent experiments performed in six wells are shown.

Table 1
Clinical characteristics for human subjects.

	NP (n=6)	PE (n=6)
Age (year)	34.0 (27.0–42.0)	39.5 (26.0–42.0)
Primipara, n (%)	3 (50)	2 (33)
Body mass index	24.3 (22.0–26.7)	24.4 (22.0–30.2)
Systolic blood pressure (mmHg)*	116 (112–128)	166 (165–183)
Diastolic blood pressure (mmHg)*	62 (57–76)	98 (86–110)
Proteinuria, n (%)**	0 (0)	6 (100)
Gestational age at delivery (weeks)**	38.4 (37.0–38.9)	29.9 (25.0–37.0)
Infant's birth weight (gram)*	2925 (2674–3287)	1126 (619–2149)

NP: normal pregnancy, PE: preeclampsia.
Data were presented as median (minimum-maximum) value or number (percentage). Date shown as number of cases and percentage were statistically analyzed by Fisher's exact test.
* $p < 0.05$.
** $p < 0.01$.

2.10. Preparation of cultured villous explants from human placenta

The research protocol was approved by the ethical committee of Nagoya University Hospital (approval number 648). All human placental tissue samples were obtained within 1 h of delivery from women who underwent Cesarean section deliveries at Nagoya University Hospital. Preeclampsia was defined as onset of hypertension (≥140/90 mmHg in two consecutive measurements at least 6 h apart) and proteinuria after 20 weeks of gestation. Proteinuria was defined as ≥300 mg/24 h protein excretion, spot urinary protein/creatinine ratio of ≥0.3 [30] or urine dipstick protein ≥2+ in two random urine samples. Placental tissues obtained from women who met these criteria constituted the preeclamptic placental tissue (PE) used in the study. Normal pregnancy placental tissues (NP) were collected from women undergoing Cesarean sections for breech presentation or previous Cesarean section. Clinical characteristics of patients were summarized and indicated in Table 1.

Placental villous explants were prepared as previously described [31]. Briefly, samples of chorionic villi were randomly dissected from 3 to 5 placental cotyledons and rinsed extensively with sterile physiologic saline. Decidual tissue and large vessels were removed from villous placenta. Samples were placed into 24-well plates, and were cultured in NM (750 μL) or HM (750 μL) with penicillin 100 U/mL and streptomycin 100 μg/mL at 37 °C under a 5% CO_2 atmosphere. The villous explants were collected after 12 h of incubation.

2.11. Real-Time PCR

Total RNA was extracted from BeWo cells and villous explants using the RNeasy mini kit (Qiagen, Tokyo, Japan). Equal amounts of RNA were reverse transcribed to generate cDNA using Rever Tra Ace (Toyobo, Osaka, Japan). Real-Time PCR was performed using the Thermal Cycler Dice Real Time System TP800 and SYBR Premix Ex Taq2 (Takara Bio, Otsu, Japan) and primers, as follows:

sFlt-1: sense 5′-ACAATCAGAGGTGAGCACTGCAA-3′, antisense 5′-TCCGAGCCTGAAAGTTAGCAA-3′.
GAPDH: sense 5′-CGGGAAACTGTGGCGTGAT-3′, antisense 5′-ATGCCAGTGAGCTTCCCGT-3′.

The PCR condition was as follows: denaturation at 95 °C for 30 s, annealing at 95 °C for 5 s and extension at 60 °C for 30 s (40 cycles), dissociation at 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. The expressions of sFlt-1 were normalized according to GAPDH expression.

2.12. Statistical analysis

Statistical analyses were performed using the SPSS 22 software

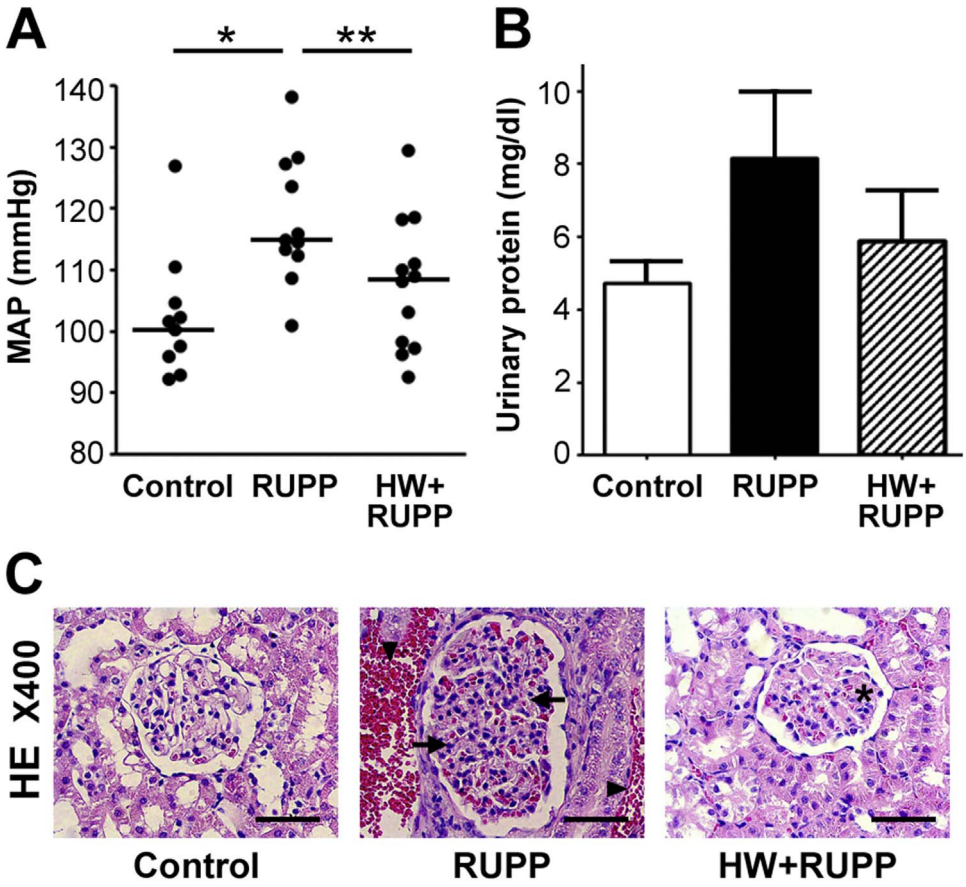

Fig. 1. A. Effect of HW on MAP. MAP was measured via right carotid artery on GD19. Scatter dot plots with median line showed the MAP of control, RUPP and HW+RUPP group (n =10–12). *$p < 0.01$, **$p < 0.05$ according to the Tukey's HSD test (ANOVA). **B.** Effect of HW on urinary protein. Urinary protein was measured on GD18 (n=7–12). Data are shown as the means ± SEM. **C.** Representative images of HE staining of kidney. The occlusion of the capillary loops with increase in cellularity was observed in the enlarged glomerulus (arrows) from RUPP group. Hemorrhage (triangle) was also detected in RUPP. * indicates the occlusion of the capillary loops in HW+RUPP. Scale bars =50 μm. HE, hematoxylin and eosin.

package (SPSS Inc., Chicago, IL). Distributions and variances were evaluated by means of the Shapiro-Wilk test and the Levene test, respectively. To compare the two groups, the Student's *t*-test and Mann-Whitney test were performed with a normal and non-normal distribution, respectively. For comparisons of multiple groups, either one-way ANOVA with the Tukey HSD test or Kruskal-Wallis test was used, with a normal and non-normal distribution, respectively. All data are expressed as mean ± SEM. The findings of capillary occlusion and bleeding in kidney were analyzed with the Fisher's exact test using the R version 3.1.1 software program. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. H_2 attenuated RUPP-induced hypertension, maternal angiogenic imbalance and oxidative stress

MAP was measured on GD19 in the control, RUPP, and HW+RUPP groups. MAP increased significantly from 103 ± 3 mmHg in the control group to 118 ± 3 mmHg in the RUPP group ($p < 0.01$; Fig. 1A). This increase in MAP in the RUPP group was significantly decreased in the HW+RUPP group (108 ± 3 mmHg) ($p < 0.05$; Fig. 1A), which exhibited a level similar to the control group. With respect to the concentration of urinary protein, the trend to increase in the RUPP group and the trend to reduce in the HW+RUPP group were detected, but these differences were not statistically significant (Fig. 1B). The histological examination of kidney revealed that bleeding, increase in cellularity, swelling of glomerulus and capillary occlusion in the RUPP group were observed compared with control group, but these findings except capillary occlusion were attenuated in the HW+RUPP group (Fig. 1C). The percentage of findings of capillary occlusion was 9.5%, 21.3%, and 17.9% in the control (total number of examined glomerulus, n=210), RUPP (n=164) and the HW + RUPP (n=173) groups, respectively ($p < 0.01$). The finding of massive bleeding spread beyond the glomerulus (Fig. 1C) was observed in 22.2% of the RUPP (n=9) kidneys, but were not detected in the control (0.0%, n=9) and HW+RUPP (0.0%, n=9) kidneys, and no significant difference was detected among these groups.

Circulating serum sFlt-1 significantly increased in the RUPP group compared to that in the control group ($p < 0.05$; Fig. 2A), and was unaltered in the HW+RUPP group compared to that in the control group (Fig. 2A). Conversely, serum VEGF decreased in the RUPP group compared to that in the control group ($p < 0.01$; Fig. 2B), and the decrease was significantly attenuated in the HW+RUPP group ($p < 0.05$; Fig. 2B). The concentration of d-ROMs, a biomarker of ROS [32,33], increased in the RUPP group compared with that observed in the control group ($p < 0.05$; Fig. 2C). This affect was attenuated in the HW+RUPP group ($p < 0.05$; Fig. 2C).

3.2. H_2 improved fetal and placental weight

Maternal H_2 administration for control rats had no effect on fetal weight (Control, n=5 vs. HW + Control, n=5; 2.25 ± 0.06 g vs. 2.22 ± 0.07 g, p=0.73) and percent fetal resorption (Control, n=5 vs. HW + Control, n=5; 7.6 ± 2.4% vs. 5.6 ± 2.3%, p=0.57).

The average fetal weight decreased in the RUPP group (1.92 ± 0.04 g) compared to that in the control group (2.48 ± 0.03 g; $p < 0.01$; Fig. 2D). Fetal weight in HW+RUPP group (2.07 ± 0.04 g) significantly

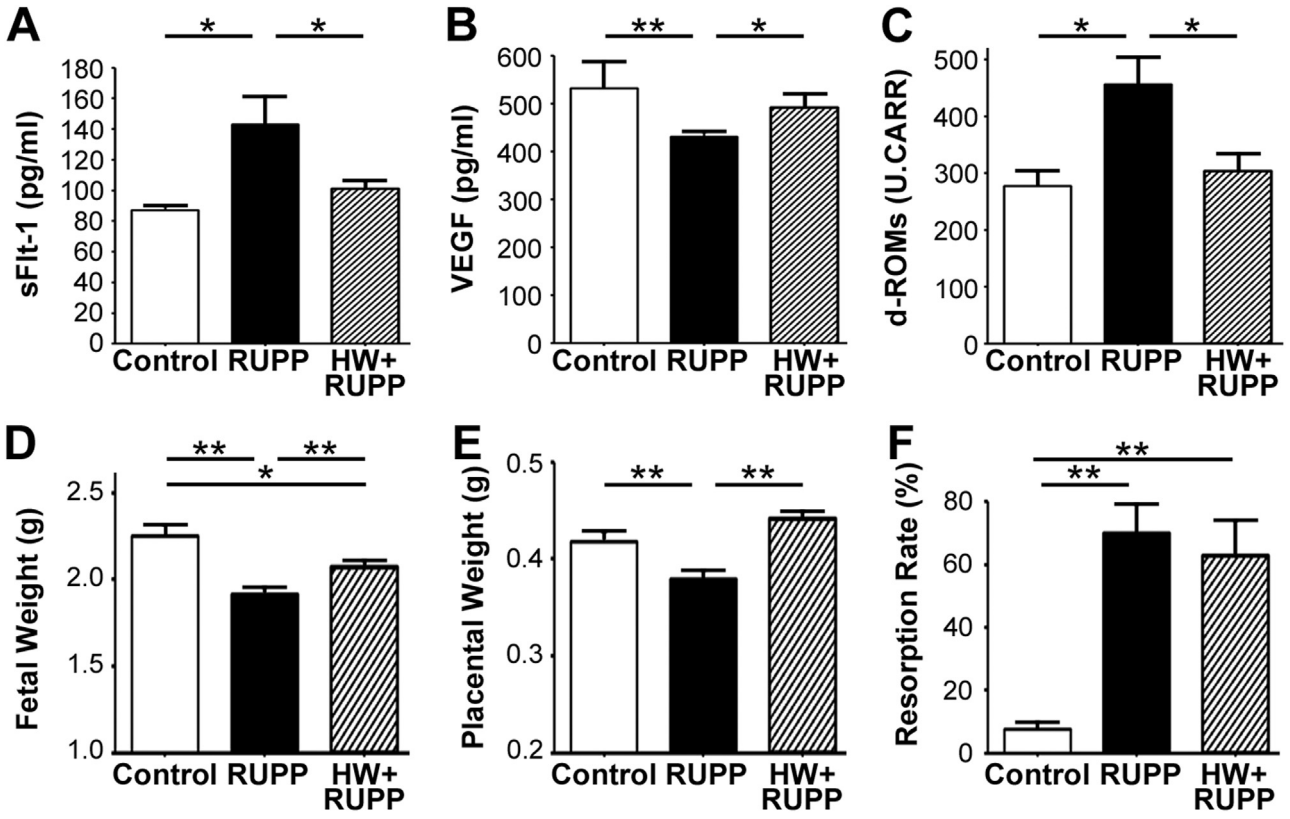

Fig. 2. A-C. Effect of HW on circulating angiogenic factors and maternal oxidative stress on GD19. Serum concentration of sFlt-1 (n=18–23, **A**), VEGF (n=18–23, **B**), and d-ROMs (n=8, **C**). The values of the d-ROMs test are expressed as U.CARR. **D-F.** Effect of HW on fetal weight, placental weight, litter size, and resorption rate on GD19. Fetal weight (n=63–113, D), Placental weight (n =63–113, E), Resorption rate (%) (n=16–25, F). Fetal and placental weight were restored by HW administration. Resorption rate showed no significant difference after treatment with HW. The results are expressed as the means ± SEM. *$p < 0.01$ according to Kruskal-Wallis test.

increased compared to that in the RUPP group ($p < 0.01$; Fig. 2D), but was not comparable to that of the control group. Fig. 2E illustrates that placental weight was lower in the RUPP group (0.379 ± 0.01 g) than that in the control group (0.433 ± 0.01 g; $p < 0.01$). Administration of HW to RUPP-treated rats significantly increased placental weight to that of the control group (0.441 ± 0.01 g; $p < 0.01$; Fig. 2E). Resorption rate increased in the RUPP group (70.0 ± 6.7%) compared to that in the control group (6.5 ± 2.0%, $p < 0.01$; Fig. 2F). There was no difference between the RUPP and the HW+RUPP groups, although the trend to decrease in the HW+RUPP group (63.0 ± 7.9%) was detected (Fig. 2F).

3.3. H_2 attenuated placental oxidative stress

No significant histological differences in labyrinth or junctional zones were observed among the three groups (Fig. 3A, upper panels). 8-OHdG is widely accepted as a sensitive marker of oxidative DNA damage [34], and is produced by various ROS, including hydroxyl radicals. 8-OHdG-positive cells in the placenta were localized to the nuclei of trophoblast cells in the labyrinth and junctional zone (Fig. 3A, middle panels). The results show that 8-OHdG-positive cells in the labyrinth zone increased in the placenta from RUPP group compared to that observed for control group (Fig. 3A, lower panels, $p < 0.01$; Fig. 3B). There was also a significant decrease in 8-OHdG-positive labyrinth cells in HW+RUPP group ($p < 0.01$; Fig. 3B). However, there was no difference among the three groups with regard to the 8-OHdG staining in the junctional zone.

For quantification of oxidative stress induced by peroxynitrite, 3-NT levels in placenta was assessed by Western blot (Fig. 4A). The formation of 3-NT was detected prominently in the RUPP group as the band at around 70 KD indicated by arrow (Fig. 4A), and the intensity of the bands was quantitated using β-actin as a loading control. The trend to increase of 3-NT was detected in the RUPP group, compared with that in the control group (Fig. 4B). The induction of 3-NT in the RUPP group was significantly reduced in HW+RUPP group ($p < 0.05$, Fig. 4B).

3.4. H_2 improved oxidative stress-induced sFlt-1 mRNA expression, but not that induced by hypoxia

We examined the effect of H_2 on cell proliferation of a trophoblastic cell line (BeWo) (Fig. 5A), and increased proliferation by HM treatment was observed for 24–72 h of treatment, but proliferation was significantly induced after only 48 h of HM treatment ($p < 0.01$), and showed a tendency to increase after 72 h (p=0.05).

We investigated whether H_2 can decrease oxidative stress-induced sFlt-1 expression by BeWo. BeWo cells exposed to H_2O_2 and cultured in NM showed a dose-dependent increase in sFlt-1 mRNA ($p < 0.01$; Fig. 5B). BeWo cells exposed to H_2O_2 and cultured in HM showed a significant decrease in sFlt-1 mRNA expression compared to that observed with NM ($p < 0.05$; Fig. 5B). We also examined whether H_2 can reduce hypoxia-induced sFlt-1 expression in BeWo cells. BeWo cells exposed to hypoxia showed a significant increase in sFlt-1 mRNA expression ($p < 0.01$; Fig. 5C). BeWo cells exposed to hypoxia and cultured in HM showed no change in sFlt-1 mRNA expression compared with NM (Fig. 5C). These results suggest that H_2 only affects the oxidative stress-induced increase in sFlt-1 expression. However, without oxidative stress and hypoxia, sFlt-1 expression was slightly increased in HM, although not significant (Fig. 5B and C).

3.5. H_2 attenuated overproduction of sFlt-1 in placental villous explants from women with preeclampsia

In villous explants isolated from normal pregnant and preeclamptic women, the levels of sFlt-1 were evaluated in the presence (HM) or

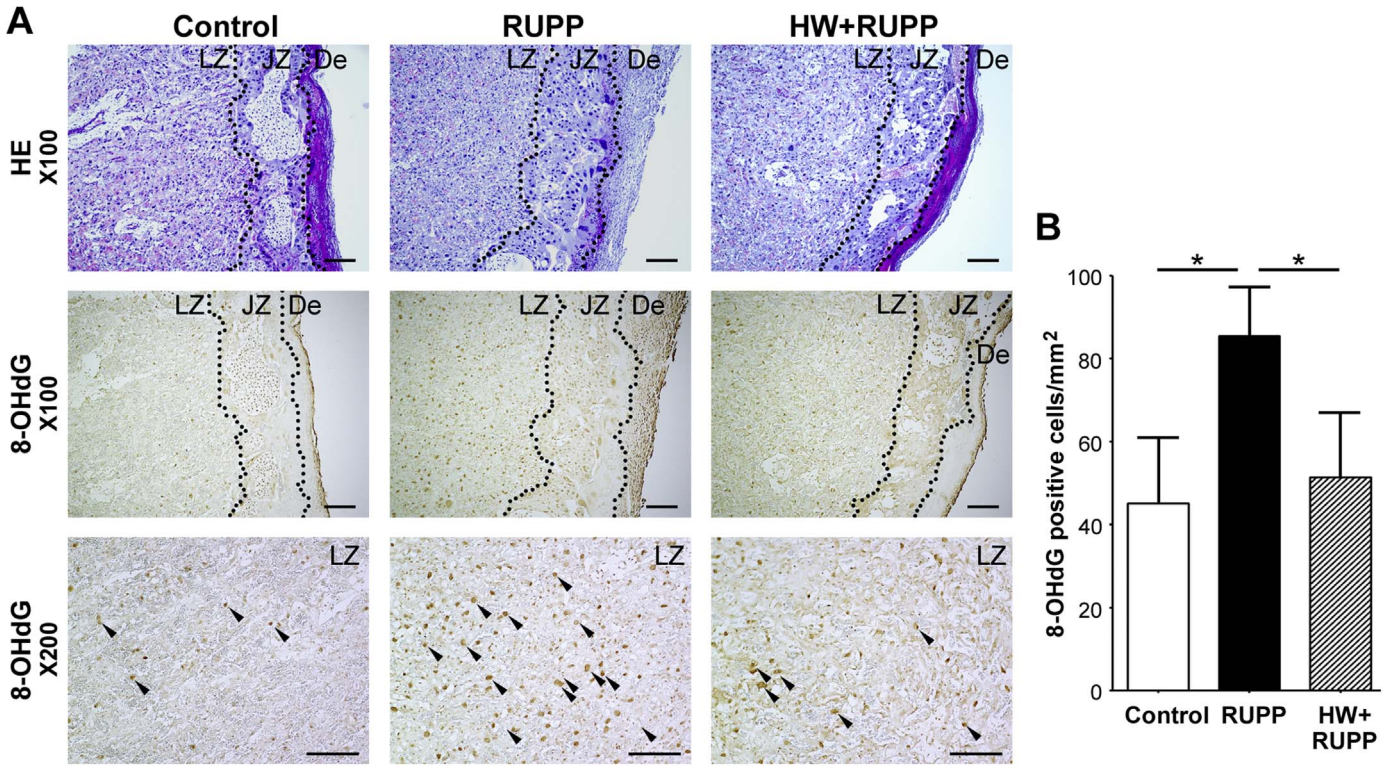

Fig. 3. Effect of HW on placental oxidative stress on GD19. **A.** Representative images of HE staining of placenta (upper panels) and 8-OHdG immunostaining of placenta (middle panels). Pictures of the same microscopic field of the placental subfield were taken. Representative images of 8-OHdG immunostaining of labyrinth zone (lower panels). Arrowheads indicate 8-OHdG immunopositive trophoblast cells in labyrinth zone. Scale bars =50 μm. **B.** Quantification of 8-OHdG-positive cells was performed (n =8). The results are expressed as the means ± SEM. $^*p < 0.05$ according to the Tukey's HSD test (ANOVA). LZ, labyrinth zone; JZ, junctional zone; De, decidua; HE, hematoxylin and eosin.

absence of H_2 (NM). We found that the expression of sFlt-1 was elevated approximately 2.3 fold in placental tissue from patients with preeclampsia compared to that in pregnant women without preeclampsia ($p < 0.05$; Fig. 6A). H_2 significantly reduced sFlt-1 mRNA expression in villous explants from patients with preeclampsia ($p < 0.05$; Fig. 6C). However, there was no difference in sFlt-1 mRNA expression in normotensive placenta after HM treatment (Fig. 6B).

4. Discussion

This study is intended to demonstrate the preventive effect of maternal H_2 administration on several clinical features of preeclampsia in the RUPP rat model. The main findings were that H_2 administration ameliorated RUPP-induced hypertension, angiogenic imbalance, placental oxidative damage, and the level of a biomarker of ROS. These results suggest that placental oxidative damage induced by ischemia-reperfusion injury in the RUPP model led to angiogenic imbalance and ultimately hypertension in the mother.

A previous study showed that positive staining of 8-OHdG significantly increased in placental tissue from women with preeclampsia, and d-ROMs increased in maternal serum of women with preeclampsia [35]; similar findings were observed in our RUPP model. The positive

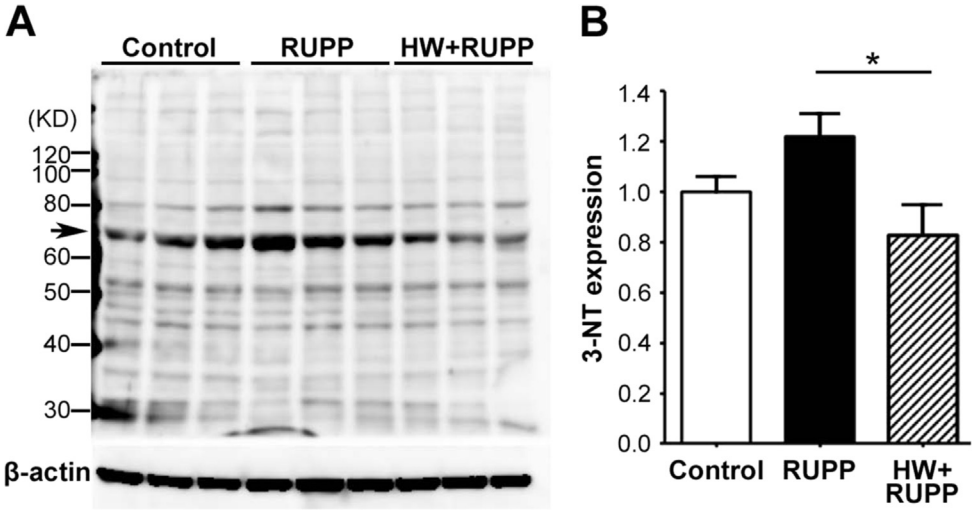

Fig. 4. A. Representative image demonstrated 3-NT bands in placental tissue. **B.** Quantitative density values of 3-NT protein bands relative to those values of β-actin as internal controls are shown. The bands indicated by arrows in **A** were used for comparative analyses. Data are shown as the means ± SEM, n=6 in each group. $^*p < 0.01$ according to the Tukey's HSD test (ANOVA).

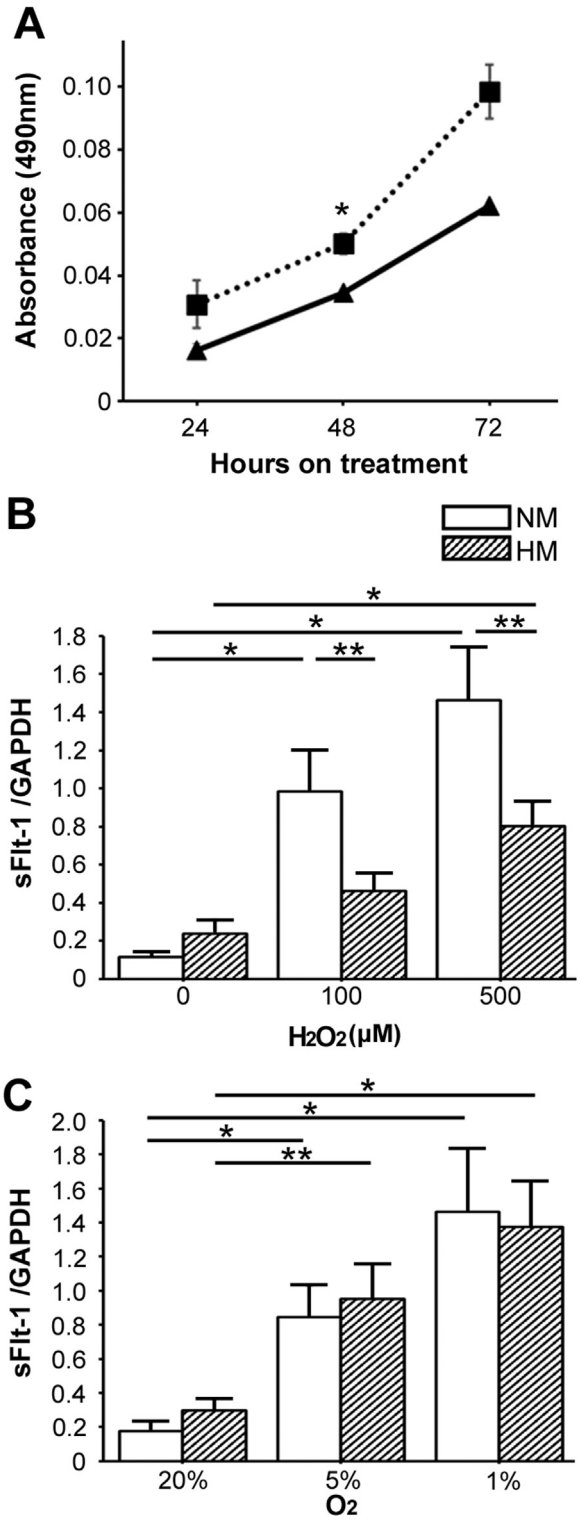

Fig. 5. Effects of HM on cell proliferation, and sFlt-1 expression induced by H_2O_2 or hypoxia in BeWo cells. sFlt-1 mRNA was determined by qRT-PCR at 6 h after exposure to H_2O_2 or hypoxia (n =5–10). **A.** BeWo cells increased proliferation after 24–72 h of incubation with HM. The squares and triangles mean the values incubated with HM and NM, respectively. The mean ± SEM were shown as three independent experiments performed in 6 wells. *$p < 0.05$ according to Mann-Whitney, in comparison between the value of NM and that of HM at 24, 48 or 78 h of incubation. **B.** BeWo cells exposed to H_2O_2 showed significant increase in sFlt-1 mRNA expression in dose-dependent manner. H_2 significantly reduced sFlt-1 mRNA expression compared with that in NM. **C.** BeWo cells exposed to hypoxia showed significant increase in sFlt-1 mRNA expression. However, H_2 had no effect on hypoxia-induced elevation in sFlt-1 mRNA. Data were normalized according to GAPDH expression. The results are expressed as the means ± SEM. *$p < 0.05$, **$p < 0.01$ according to Kruskal-Wallis test and Mann-Whitney.

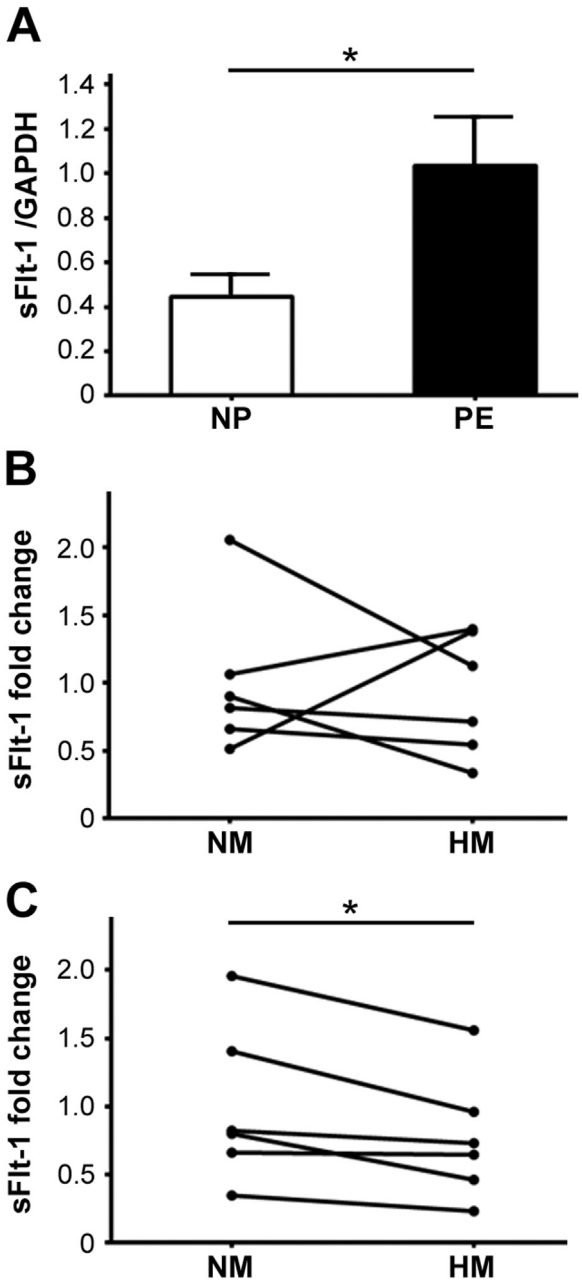

Fig. 6. Effects of HM on sFlt-1 expression in human placenta. sFlt-1 mRNA was determined by qRT-PCR at 12 h after placental villous explant cultured (n =6). **A.** The levels of sFlt-1 mRNA in placenta from women with normal pregnancy (NP) or with preeclampsia (PE). The results are expressed as the means ± SEM.*$p < 0.05$ according to the Student's *t*-test. **B.** H_2 had no effect on sFlt-1 mRNA expression in placenta from women with normal pregnancy. The values were shown as the ratio to the mean value in NM group, which was set to 1.0. **C.** H_2 significantly reduced sFlt-1 mRNA expression in placenta from women with preeclampsia. The values were shown as the ratio to the mean value in NM group, which was set to 1.0. *$p < 0.05$ according to the Student's paired *t*-test.

staining of 8-OHdG in human placenta was mainly detected in the syncytotrophoblast of villous trophoblasts [36]. Trophoblasts in the labyrinth zone of rodent placenta are analogous to villous trophoblasts in human placenta [37], and the staining of 8-OHdG was also observed at the labyrinth zone in our RUPP model. The level of 3-NT in placenta, as a marker of peroxynitrite, was observed the trend to increase in RUPP group, as the increased level of peroxynitrite in preeclamptic placenta was reported [12]. Moreover, the expression of 3-NT was significantly decreased in HW+RUPP group. The result is consistent with the previous report that H_2 reduce peroxynitrite [13]. These

findings support the theory that the RUPP rat model demonstrates features of oxidative stress in preeclampsia. Furthermore, we found that maternal and placental oxidative stress were attenuated by administration of HW.

Therefore, we investigated the effect of H_2 on the production of sFlt-1 in placenta in vitro. H_2 significantly suppressed the expression of sFlt-1 induced by oxidative stress in human trophoblastic BeWo cells, and in villous explants from women with preeclampsia. BeWo cells were established from choriocarcinoma, but a recent review concluded that BeWo cells, as well as fresh villous explants, are one appropriate in vitro model to investigate preventive and therapeutic agents for preeclampsia [27]. In this study, H_2O_2 was treated as a generator of oxidative stress, because it has been already reported that H_2O_2 produces intracellular ROS and induces oxidative damage in the human trophoblast cell line [29]. In this study, sFlt-1 mRNA expression was increased by oxidative stress, which was similar with a previous report [6]. The present study demonstrated that H_2 significantly reduced sFlt-1 mRNA expression. It is well known that hypoxia also increases the secretion of sFlt-1 in human trophoblast cell line and villous explants [31], which is consistent with our results. However, H_2 did not decrease hypoxia-induced sFlt-1 mRNA expression. These results suggest that H_2 might have only a suppressive effect on oxidative stress-induced sFlt-1. Although remodeling failure of the spiral artery is presumed to lead to hypoxia in preeclamptic placenta, it has been recently revealed that placenta with abnormal spiral artery remodeling shows increased oxygen levels [38]. Moreover, our present data demonstrated elevated level of sFlt-1 in villous explants from preeclamptic women cultured in normal oxygen conditions. This result is similar to those of a previous report [39]. These findings suggest that oxidative stress, rather than hypoxia, might be involved in elevating sFlt-1 level in preeclamptic placenta. H_2 had no effect on hypoxia-induced sFlt-1, which might not be disadvantageous in vivo. In addition, H_2 had no effect on sFlt-1 expression in normal placental tissue, but suppressed overproduction of sFlt-1 in preeclamptic placental tissue. In a normal pregnancy, the serum levels of sFlt-1 in pregnant women are higher at later gestational age, and sFlt-1 is markedly elevated in the third trimester compared with that in the first and second trimesters [40]. Thus, it might be meaningful that H_2 does not reduce the physiologic level of sFlt-1 in normal pregnancy.

It is well known that H_2 concentration is increased in blood by oral H_2 administration in both pregnant [24,41] and non-pregnant [42,43] animal models. RUPP model reduces uterine perfusion pressure [21], and it might be difficult that H_2 passes to the placenta and fetus. We failed to show the increased concentration of H_2 in the fetus by HW administration (data not shown). In the present study, the effect of H_2 on the fetus such as fetal weight or present fetal resorption was partial or none, respectively. On the contrary, d-ROM, a biomarker of ROS, in maternal blood was completely reduced in HW + RUPP to the same level of that in control group. These finding suggested that H_2 reduces oxidative stress in maternal blood, which would have an indirect effect on placenta, protects placenta from oxidative damage and suppresses the production of sFlt-1. The direct effect of H_2 on placenta and fetus might be minimal.

There are few reports about the effect of H_2 on hypertension or preeclampsia. H_2 attenuated inflammation, endothelial dysfunction, and reduced oxidative stress for a rat model of spontaneous hypertension [44–46]. In the field of preeclampsia, only one report showed the efficacy of H_2 in a rat model [47]. The study employed N (omega)-nitro-L-arginine methyl ester (L-NAME) to induce preeclampsia. Intraperitoneal administration of hydrogen-rich saline improved endothelial dysfunction and reduced oxidative stress and the mean systolic blood pressure on GD22. The results were similar to our results, although the route and timing of administration of H_2 were different from those employed in our research. In the previous study, the mean systolic blood pressure was reduced only by GD22, and the effect of H_2 on blood pressure was less than that in our report. It should be noted that the model of preeclampsia employed in our study is different from that used in the previous study. The RUPP rat model is known to show no findings of glomerular endotheliosis [21], which are observed in the L-NAME model. In addition, the effect observed in our study might be attributed to prior administration of H_2 before RUPP procedure. These results suggest that H_2 might be more effective in prior administration, although more investigation is required. The present study also reported the new finding that angiogenic imbalance was also improved by HW. Thus, H_2 reduced not only oxidative stress but also level of an anti-angiogenic factor, sFlt-1, which could improve hypertension. Moreover, placental and fetal weights were increased by H_2 administration, compared with those observed in the RUPP group.

The therapeutic potential of statins has been demonstrated [48–50]. A recent study also showed that pravastatin attenuated RUPP-induced hypertension, markers of oxidative stress, and the level of sFlt-1 [48]. The outcome of H_2 treatment in this study is similar to that of pravastatin. All statins are designated Food and Drug Administration pregnancy category X rating: contraindicated at any time during pregnancy, although teratogenic risk has not been reported [51]. Further studies would be required to confirm the safety of exposure to statins *in utero*.

Previous clinical trials using antioxidants failed to reduce the incidence of preeclampsia, mainly because of a detrimental effect on placental function, limitations of barriers to the utilization of exogenous antioxidants, and a narrow window of therapeutic dosage [52]. A recent report also showed that high levels of vitamin C and E inhibited the proliferation ability to trophoblast cell lines and stimulated the secretion of TNF-α, which is a pro-inflammatory cytokine increased in preeclampsia [20]. H_2 even at a high concentration had no detrimental effects on them [20].

However, data on the safety of H_2 to the fetus remains limited. We found no mutagenicity by maternal H_2 administration (data not shown) and no effect on the fetal weight and percent fetal resorption. *In vitro*, H_2 had no adverse effect on cell viability, which is similar with the previous report [20], as above mentioned. The present study showed that H_2 enhanced proliferation of BeWo cells. However, it should be noticed that BeWo cells is derived from choriocarcinoma, which has different potential of proliferation from trophoblast. Increased expression of sFlt-1 in HM was also detected, although not significant. For preventive administration of H_2, it should not be neglected. We are now preparing for the further investigation. There are a few papers that have reported the effects on the fetus following the maternal administration of H_2 [18,24,41,53], but the evidence for long prognosis remains insufficient. Therefore, further accumulation of the evidence is required for clinical use, and we are also planning to evaluate the long-term effects of H_2 on fetuses using an animal model.

The present study has several limitations. The RUPP model in the present study demonstrated mild symptoms of hypertension (15 mmHg) and proteinuria (2-fold increase, but not significant). It suggests that the effect of H_2 on severe preeclampsia remains unknown. Li et al. reviewed the RUPP model and reported that MAP is increased 20–25 mmHg [21], and Alexander et al. reported five-fold increase in urinary protein excretion. In terms of hypertension, both measurement of blood pressure and RUPP procedure in this study might be related with the discrepancy. Firstly, the blood pressure in this study were measured in recovering stage from anesthesia, and the effect of anesthesia on blood pressure cannot be discounted. It is very difficult to measure arterial blood pressure after recover from anesthesia by the instrument used in this study. That might underestimate blood pressure in the RUPP group, although arterial blood pressure under anesthesia was stable and the depth of anesthesia was similar in all three groups. Secondary, the clips cited in the review [21] are unavailable in Japan, and degree of closure of vascular by clips used in this study might be different. The degree of closure of vascular determines uterine perfusion pressure, which influence blood pressure and glomerular function. However, there are also reports that showed

similar increased level as 15 mmHg of MAP in RUPP model [54,55]. In addition, approximately 1.5-fold induction of sFlt-1% and 75% reduction of VEGF were similar with other previous reports [55–57], and these suggested that the RUPP model in this study at least demonstrated angiogenic imbalance, which is central characteristics of preeclampsia. The difference of urinary protein might be dependent on different measurement. In the previous study assessed it with 24 h urine collection [58], but in this study, it was measured as the concentration of spot urine, which might underestimate the value. However, to our knowledge, there is one paper [58] that reported increased urine protein in RUPP rat model. Others suggested that RUPP model shows no findings of glomerular endotheliosis [21], and the level of urine protein might be mild in RUPP model. Moreover, RUPP model resulted in too high percent fetal resorption of approximately 70%, which were consistent with previous reports [26,48,59], but not with clinical characteristics of preeclampsia. Thus, to evaluate the effect of H_2 on preeclampsia precisely, it should be also evaluated using other models of severe preeclampsia.

In the present study, the preventive effect of H_2 was only evaluated by the administration prior to the RUPP procedure, because the previous trials using other antioxidants shown limited effect owing to delayed administration [60]. The therapeutic potential remains unrevealed. For clinical use, we should also determine the timing and dosage of H_2 to be most effective for prevention.

In conclusion, HW administration before onset of preeclampsia relieved hypertension and angiogenic imbalance by reducing oxidative stress in a RUPP rat model of preeclampsia. In the in vitro model, we also found that H_2 reduced sFlt-1 in the trophoblastic BeWo cells and villous explants from preeclamptic women. These results suggest that administration of HW for high-risk patients of preeclampsia could have a potential clinical benefit for mothers and neonates.

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Disclosures

The authors have no conflicts of interest to declare in association with this study.

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