Hydrogen-rich water ameliorates bronchopulmonary dysplasia (BPD) in newborn rats

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ABSTRACT

Bronchopulmonary dysplasia (BPD) is characterized by developmental arrest of the alveolar tissue. Oxidative stress is causally associated with development of BPD. The effects of hydrogen have been reported in a wide range of disease models and human diseases especially caused by oxidative stress. We made a rat model of BPD by injecting lipopolysaccharide (LPS) into the amniotic fluid at E16.5. The mother started drinking hydrogen-rich water from E9.5 and also while feeding milk. Hydrogen normalized LPS-induced abnormal enlargement of alveoli at P7 and P14. LPS increased staining for nitrotyrosine and 8-OHdG of the lungs, and hydrogen attenuated the staining. At P1, LPS treatment decreased expressions of genes for FGFR4, VEGFR2, and HO-1 in the lungs, and hydrogen increased expressions of these genes. In contrast, LPS treatment and hydrogen treatment had no essential effect on the expression of SOD1. Inflammatory marker proteins of TNFα and IL-6 were increased by LPS treatment, and hydrogen suppressed them. Treatment of A549 human lung adenocarcinoma epithelial cells with 10% hydrogen gas for 24 h decreased production of reactive oxygen species in both LPS-treated and untreated cells. Lack of any known adverse effects of hydrogen makes hydrogen a promising therapeutic modality for BPD.

Key words

Bronchopulmonary dysplasia (BPD); molecular hydrogen; lipopolysaccharide (LPS); reactive oxygen species (ROS)

INTRODUCTION

Bronchopulmonary dysplasia (BPD), which is also called chronic lung disease of very preterm infants (CLD), is characterized by impaired pulmonary development resulting from a variety of risk factors of neonates such as prematurity, inflammation, oxygen toxicity, and volutrauma by the mechanical ventilation. Similarly, prenatal risk factors include chorioamnionitis and oxidative stress of the fetus. Infants with BPD are predisposed to retardation of psychomotor development[1].

The lungs develop through the embryonic, pseudoglandular, canalicular, saccular, and alveolar stages. Alveolar septal formation, which starts from the end of the saccular stage, is the key step in alveolarization. The alveolar counts are increased, while the alveolar sizes are decreased, by the secondary septa formed in the transition from the saccular to alveolar stages. Premature infants born at gestation 24 to 28 weeks have susceptibility to BPD, because, at these gestational ages, the lung development switches from the canalicular to saccular stages. Prenatal risk factors such as amnionitis around these periods cause developmental arrest of the alveolar tissue. This is characterized by the decreased saccular branching of the airway, secondary septation, and fewer and larger alveoli, and leads to the reduced surface-to-volume ratio of the alveoli[2,3].

Since the first report of the marked effect of molecular hydrogen on a rat model of cerebral infarction, the beneficial effects of hydrogen have been reported in more than 60 model animals and more than 10 human diseases, especially in oxidative-stress mediated and inflammatory diseases[4]. The therapeutic effects of hydrogen have been reported in lung injuries due to ischemia/reperfusion[5,6]; oxygen[7,8]; ventilation[9,10]; LPS[11-14]; paraquat[15,16]; smoke[17]; and cigarette smoking[18]. The effects of hydrogen have also been reported in lung injuries secondary to intestinal ischemia/reperfusion[19] and extensive skin burn[20]. We and others have recently reported that hydrogen is also effective for pulmonary hypertension[21,22]. The effects of hydrogen have been also reported in perinatal disorders: neonatal cerebral hypoxia[23,24]; LPS-induced fetal lung injury[25]; preeclampsia[26,27] and perinatal brain injury caused by prenatal inflammation[28].

We here report the effect of *per os* administration of hydrogen-rich water to mothers of a rat model of BPD, which was induced by lipopolysaccharide (LPS)-mediated chorioamnionitis.

As no side effects have been reported in hydrogen-rich water, drinking hydrogen-rich water is a possible preventive measure for infants with a high risk of development of BPD.

MATERIALS AND METHODS

A rat model of BPD and ad libitum *administration of supersaturated hydrogen water*

All animal studies were approved by the Animal Care and Use Committee of the Nagoya University Graduate School of Medicine. We used 4 pregnant rats for the control group, 5 for the LPS group, and 5 for LPS with hydrogen group. The numbers of fetuses were 43 for the control group, 60 for the LPS group, and 61 for the LPS with hydrogen group.

Pregnant Sprague-Dawley rats were purchased from Japan SLC. Rats were mated at night and a vaginal plug was confirmed the next morning. As we performed experiments in daytime, the first day after mating was counted as E0.5. Pregnant rats divided into three groups: intra-amniotic injection of saline and drinking dehydrogenized water (control group, $n = 4$); intra-amniotic injection of LPS and drinking dehydrogenized water (LPS group, $n = 5$); intra-amniotic injection of LPS and drinking hydrogen-rich water (LPS + H_2 group, $n = 5$). Supersaturated hydrogen water was freshly prepared every evening using Aquela 7.0, which was kindly provided by MiZ Co. Ltd. We measured the hydrogen concentration by equilibrating hydrogen water with nitrogen gas and by injecting the gas into a gas chromatography connected to a semiconductor gas detector (EAGanalyzer GS-23, SensorTec). The concentration of dissolved hydrogen was 5 to 7 ppm before being transferred to a 50-ml closed glass vessel, whereas the concentration of saturated hydrogen under the standard ambient temperature and pressure (SATP) is 1.6 ppm. The hydrogen concentrations in the glass vessel after 24 h were more than 0.25 ppm. The mother started drinking hydrogenized or dehydrogenized water *ad libitum* at gestational day 9.5 (E9.5) and continued drinking up to the day when the pups were sacrificed. The hydrogen concentration of the milk that remained in the stomach of neonates was ~0.5 ppm. Rats were housed in a controlled temperature (25°C) and light-dark cycle room (12:12h, from 7 to 19 o'clock), and had free access to food and water.

We induced chorioamnionitis by injecting LPS to make a rat model of BPD[29]. LPS isolated from *Escherichia coli* (055:B5) was purchased from Sigma-Aldrich Japan. A pregnant mother at

E16.5 was anesthetized by inhalation of 5.0% isoflurane, which was subsequently reduced to 2.5% for maintenance. The uterus of the mother was exposed by a midline abdominal incision. LPS (5 µl x 0.2 µg/µl) dissolved in saline was injected into each amniotic sac, and the uterus was packed into the abdominal cavity. As a control, we injected saline alone. The abdominal wall was sutured in two layers. The numbers of treated fetuses were 43 for the control group, 60 for the LPS group, and 61 for the LPS with hydrogen group. Pups were born spontaneously. After delivery, pups were kept with their mother until euthanized for experiments. Intrauterine fetal death (IUFD) rate was calculated by the number of stillbirths based on fetus counts at E16.5. The number of surviving pups was counted until postnatal day 14 (P14). The body weights of pups were measured at P7 and P14.

Tissue preparation for histological analysis

Pups were euthanized at P7 or P14 by an intraperitoneal injection of 120 mg/kg (2 ml/kg) of sodium pentobarbital. After raising pups, mothers were euthanized in a similar way. The lungs were inflated and fixed by 4% paraformaldehyde, which was instilled by a cannula inserted from the trachea. The lung tissues were then paraffin-embedded and sliced at 5 µm.

Morphometrical analysis of the lungs

The lungs were stained with hematoxylin and eosin (H.E.). For morphometric analysis of the alveolar size, we used mean linear intercept (Lm)[30]. We drew six random lines on the image of each visual field. Intersections of the drawn line with the alveolar walls were individually marked, and the length of the line segment spanning two neighboring intersections was measured with PhotoShop CS6 (Adobe). In each rat, we measured six lines per field and six fields per section. In each group, 3-6 rats were analyzed to calculate Lm.

Immunofluorescent staining for nitrotyrosine

Nitrotyrosine was used as a marker for protein oxidation. After sections were deparaffinized and blocked by 5% horse serum, the sections were incubated with an anti-nitrotyrosine antibody (2A8.2, Millipore) at a dilution of 1:200 at 4˚C overnight. After

incubating with a secondary antibody (Fluorescein Horse Anti-mouse IgG, Vector Laboratories), fluorescence intensity of the images was quantified by MetaMorph (Molecular Devices).

Immunostaining for 8-hydroxy-2'-depxyguanosine (8-OHdG)

After blocking the sections with 5% horse serum, the sections were incubated with an anti-8OHdG monoclonal antibody (N45.1, Nikken SEIL) at 1:100 at 4°C overnight. Then the sections were incubated with the Biotinylated Horse Anti-mouse IgG antibody (Vector Laboratories) at 1:200, followed by incubation with the avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories) and the ImmPACT DAB substrate (Vector Laboratories). Images of the lung section were quantified by MetaMorph.

Expression analysis of the lungs

Pups were euthanized at P1 by an intraperitoneal injection of sodium pentobarbital. After the thoracic cavity was opened, the lungs were removed and frozen with liquid nitrogen immediately. Total RNA was extracted using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Reverse transcription of RNA into cDNA was carried out using ReverTra Ace (Toyobo). Quantitative real-time PCR using SYBR Premix Ex Taq II (Takara Bio) was performed with LightCycler 480 (Roche Applied Science). Primers for *B2m* encoding β2 microglobulin were 5'-CGAGACCGATGTATATGCTTGC-3' and 5'-GTCCAGATGATTCAGAGCTCCA -3'. Primers for *Fgfr4* were 5'-GTTGGCACGCAGCTCCTT-3' and 5'- GCAGGACCTTGTCCAGAGCTT-3'. Primers for *Kdr* encoding VEGFR2 were 5'- CCACCCCAGAAATGTACCAAAC-3' and 5'-AAAACGCGGGTCTCTGGTT-3'. Primers for *Hmox1* encoding hemeoxygenase-1 (HO-1) were 5'-CAGAGAATGCTGAGTTCATGAG-3' and 5'-AGCTCTTCTGGGAAGTAGACAG-3'. Primers for *Sod1* were 5'-GCAGAAGGCAAGCGGTGAAC-3' and 5'-TAGCAGGACAGCAGATGAGT-3'.

Protein expression analysis of the placenta

The uterus was eviscerated from rats under anesthesia with isoflurane at E17.5, which was

24 h after intra-amniotic injection of LPS. The placentas were removed and frozen with liquid nitrogen immediately. Placentas were homogenized with an extraction buffer [100 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and protease inhibitors of 1 μg/ml leupeptin (Sigma Aldrich), 10 μg/ml aprotinin (Sigma Aldrich), and 10 μg/ml pepstatin (Sigma Aldrich)], and then centrifuged for 20 min at 13,000 x rpm at 4°C to remove debris. The amounts of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) were quantified using the ELISA kits (LF-EK028 and LF-EK224, respectively) from AbFrontier.

Cell culture and quantification of ROS

Human lung adenocarcinoma epithelial cell line, A549, were obtained from the JCRB Cell Bank and were cultured with Eagle's minimum essential medium (MEM) with 0.1 mM non-essential amino acids and 10% fetal bovine serum (Gibco). Cells were treated with either 20% O_2/5% CO_2/75% N_2 (Control) or 10% H_2/20% O_2/5% CO_2/65% N_2 (Hydrogen) for 24 h before being exposed to 0 or 10 μg/ml LPS for 1 h. To reduce a risk of combustion, the flow rate of 10% hydrogen was reduced to 66 ml/min; overflown hydrogen was continuously exhausted outside the building through a ventilated draft; and the room was equipped with a hydrogen alarm. The amount of ROS was fluorescently quantified using oxidative stress indicator, CM-H2DCFDA (Life Technologies) with PowerScan 4 (BioTek). Cells were analyzed in triplicate, and the experiments were performed twice.

Statistical analysis

Statistical analysis was performed with Prism 6.0f (GraphPad). All data were analyzed using one-way analysis of variance (ANOVA) with the Tukey multiple comparison test.

RESULTS

Oral intake of hydrogen water ameliorates LPS-induced BPD in rats

We generated a rat model of BPD by injecting 1 μg LPS into each amniotic sac at E16.5. The pregnant rats started taking hydrogen-rich water *ad libitum* from E9.5 up to the day when the pups were sacrificed. The hydrogen-treated BPD model rats are referred to as the LPS + H_2 group

in this communication. Disease controls (the LPS group) were injected with 1 μg LPS but took dehydrogenized water. Negative controls (the control group) were injected with saline and took dehydrogenized water. The intrauterine fatal death (IUFD) rates were 3/43 (7%) in the control group, 31/60 (52%) in the LPS group, and 27/61 (44%) in the LPS + H_2 group (Supplementary Table 1). No statistical difference was observed between LPS and LPS + H_2 groups. Similarly, survival rates of pups observed up to P14 were not different between the LPS and LPS + H_2 groups (Fig. 1A). In addition, body weights of pups at P7 and P14 were not different between the two groups (Fig. 1B).

LPS treatment abnormally increased the alveolar sizes and hydrogen reduced the alveolar sizes (Fig. 2A). Quantitatively, the mean linear intercept (Lm), which represents the mean alveolar size, was increased by LPS, and was normalized by hydrogen (Fig. 2BC).

Hydrogen reduces LPS-induced oxidative stress production in vivo

We next examined protein oxidation by immunostaining of nitrotyrosine of the lung tissues. The signal intensities of nitrotyrosine were increased by LPS and were suppressed by hydrogen at P7 and P14 (Fig. 2DEF). We similarly examined DNA oxidation by immunostaining of 8-OHdG. The signal intensities of 8-OHdG were increased by LPS and were markedly suppressed by hydrogen at P7 and P14 (Fig. 2GHI). Taken together, hydrogen treatment reduced oxidative stress in the lungs of the BPD model rats.

Hydrogen ameliorates LPS-induced suppression of genes encoding FGFR4, VEGFR2, and HO-1, as well as LPS-induced overexpression of TNFα and IL-6 proteins

Quantitative RT-PCR showed that LPS treatment markedly decreased expressions of genes for FGFR4, VEGFR2, and HO-1 in the lungs (Fig. 3A). Hydrogen ameliorated the decreased gene expressions. In contrast, LPS treatment and hydrogen treatment had no essential effect on the gene expression of SOD1 (Fig. 3A). Among inflammatory marker proteins, TNFα was minimally increased and IL-6 was markedly increased by LPS treatment. Hydrogen was able to suppress the induced expressions of these proteins (Fig. 3B).

Hydrogen reduces the amount of ROS in A549 pulmonary epithelial cells

We simulated BPD *in cellulo* using human lung adenocarcinoma epithelial cells, A549, as described elsewhere[25,31-34]. A549 cells were treated with either control air or 10% hydrogen air for 24 h. Cells were then exposed to 10 μg/ml LPS for 1 h. LPS treatment increased ROS production (Fig. 4). Hydrogen decreased ROS production in both LPS-treated and untreated cells.

DISCUSSION

Intra-amniotic administration of LPS at E16.5 induced enlargement of alveoli modeling for human BPD. In addition, LPS increased protein oxidation estimated by nitrotyrosine staining of the lungs, as well as DNA oxidation estimated by immunostaining for 8-OHdG. When the mother started drinking supersaturated hydrogen water (5 to 7 ppm) *ad libitum* from E9.5, abnormal alveolar development and abnormal oxidation of protein and DNA were efficiently ameliorated. IUFD, however, were not improved by hydrogen. One possible explanation for lack of effect on IUFD is that some pups might have been severely affected by intra-amniotic injection of LPS, and hydrogen was not effective for the severely affected pups.

Vascular endothelial growth factor receptor 2 (VEGFR2) is important for the maintenance of alveolar structures. Reduced activation of VEGFR2 is associated with the arrest of lung development and increased apoptosis of endothelial and mesenchymal cells[35-38]. FGFR4 is responsible for secondary septation of alveoli[39]. Intra-amniotic injection of LPS suppressed gene expressions for VEGFR2 and FGFR4 of the lungs at P1, which likely have led to abnormal increase of the alveolar sizes of the lungs at P7 and P14. Hydrogen-rich water ameliorated abnormal suppression of genes encoding VEGFR2 and FGFR4, and improved histological findings.

The effect of hydrogen on a rodent model of cerebral infarction was first attributed to specific scavenging activity of hydroxyl radicals and peroxynitrite[40]. Hydrogen is the smallest molecule and is able to be easily dissipated throughout the body and the cells. Indeed, when pregnant mice drink hydrogen-rich water, the hydrogen concentrations of the embryos are increased[24]. We also observed that hydrogen that the mother took was transferred to the milk. However, the high diffusion rate of hydrogen also makes hydrogen easily dissipated through the

lungs, and the half-lives of hydrogen in human[41] and mouse[42] bodies are as short as 30 and 20 min, respectively. Thus, continuous and plentiful production of hydroxyl radical is unlikely to be sufficiently scavenged by hydrogen in drinking water. We previously reported that pulsatile inhalation of hydrogen, but not continuous inhalation of hydrogen, is effective for a rat model of Parkinson's disease, although the total amount of hydrogen taken into the body is estimated to be ~100 times higher with inhalation of hydrogen gas[41]. Similarly, we reported that hydrogen prevents degranulation of mast cells in type I allergy not by scavenging hydroxyl radicals but by modulating FcεRI-mediated signal transduction[43]. Hydrogen in drinking water increases gastric secretion of ghrelin, and inhibition of ghrelin secretion nullifies the effect of hydrogen on a mouse model of Parkinson's disease[44]. Involvement of oxidative stress is implicated in human BPD[45]. We have shown that 24-hr continuous exposure to 10% hydrogen gas decreases ROS production in A549 pulmonary epithelial cells. Similarly, hydrogen tends to increase gene expressions for HO-1 and SOD1. The effect of hydrogen on a rat model of BPD is thus likely to be partly mediated by the radical scavenging activity of hydrogen and also by signal modulating activity.

Hydrogen has been reported to be effective in more than 60 disease models and 10 human diseases[4]. No adverse effects of hydrogen have been reported in animal models or humans. Only in recent years have we begun to apply hydrogen to human diseases. We further need to confirm the lack of adverse effects of hydrogen in these diseases. We also have to prove molecular mechanisms underlying its diverse effects on a variety of diseases, especially the inflammatory and ROS-mediated variety. Safety is the highest priority in treating pregnant mothers and infants. Hydrogen will be accordingly applied to BPD later than other adult diseases, but will be a promising therapeutic option in the near future.

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Conflict of Interest

The Miz Co. Ltd. played no role in collection, management, analysis, or interpretation of the data; or preparation of the manuscript. The authors have no competing interests.

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Figure Legends

Figure 1. (A) Kaplan-Meier survival curves of pups from P0 to P14 (n = 40 for Control, 29 for LPS, and 34 for LPS + H_2). No significant difference is observed in any pair by the logrank test. LPS, pups from LPS-treated mothers; LPS + H_2, pups from LPS- and H_2-treated rats. **(B)** Body weights of pups at P7 (n = 12 for Control, 15 for LPS, and 16 for LPS + H_2) and P14 (n = 6 for Control, 9 for LPS, and 12 for LPS + H_2). No significant difference is observed in any pair by one-way ANOVA.

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Figure 2. Lung histologies at P7 and P14. (**A**) Hematoxylin and eosin staining of lung tissues. (**B, C**) Mean linear intercept (Lm) is calculated by drawing random lines and measuring a line segments spanning crossings with the alveolar walls (see Methods) at P7 (**B**) and P14 (**C**). Mean and SD (n = 3-6) are indicated. (**D**) Immunostaining of nitrotyrosine of lung tissues at P7 and P14. (**E, F**) Fluorescent intensity for nitrotyrosine is measured with the MetaMorph software at P7 (**E**) and P14 (**F**). Mean and SD (n = 3-6) are indicated. (**G**) Immunostaining of 8-OHdG of lung tissues at P7 and P14. (**H, I**) Signal intensity for 8-OHdG in nuclei is measured with the MetaMorph software at P7 (**H**) and P14 (**I**), and is divided by the area of alveolar cells. Mean and SD (n = 3-4) are indicated. Bar = 50 μm. *p < 0.05 and **p < 0.01 by one-way ANOVA and the post-hoc Tukey test. n.s., not significant.

Figure 3. Expression analysis of the lungs and placentas. (A) Real-time RT-PCR analysis of lung tissues at P1. **(B)** Enzyme-linked immunosorbent assay (ELISA) of the placentas at E17.5. Mean and SD (n = 3) are indicated. *p < 0.05 by one-way ANOVA and the post-hoc Tukey test.

Figure 4. Reactive oxygen species in A549 cells. Cells were treated with either 20%O_2/5%CO_2/75%N_2 (Control) or 10%H2/20%O_2/5%CO_2/65%N_2 (Hydrogen) for 24 h before exposed to 0 or 10 μg/ml LPS for 1 h. The amount of ROS is fluorescently quantified by CM-H2DCFDA. n = 6, *p < 0.05 and **p < 0.01 by one-way ANOVA and the post-hoc Tukey test.

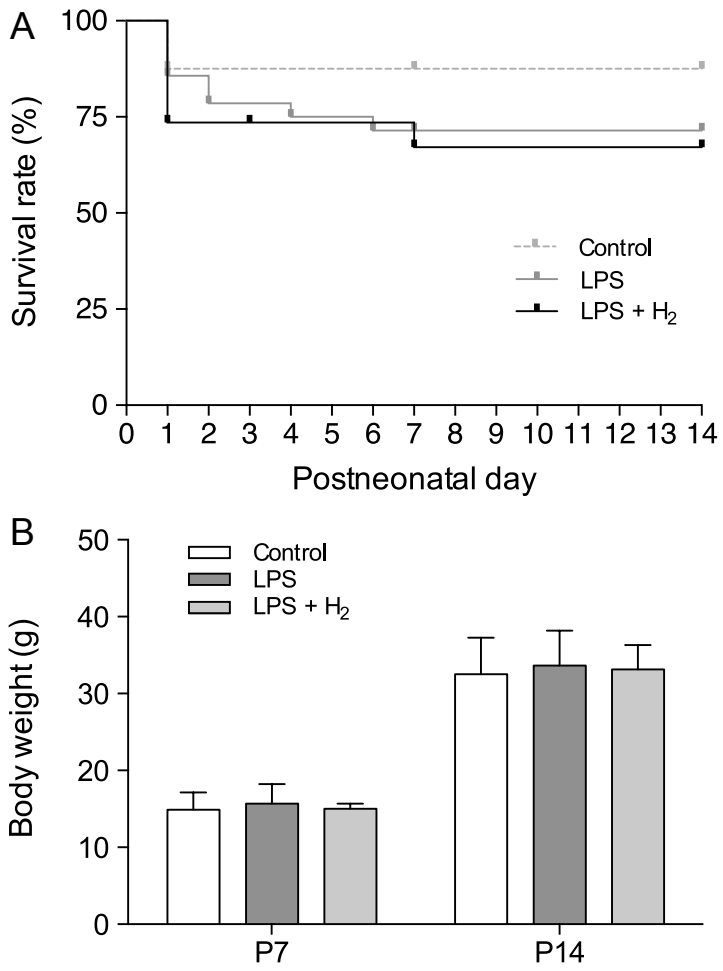
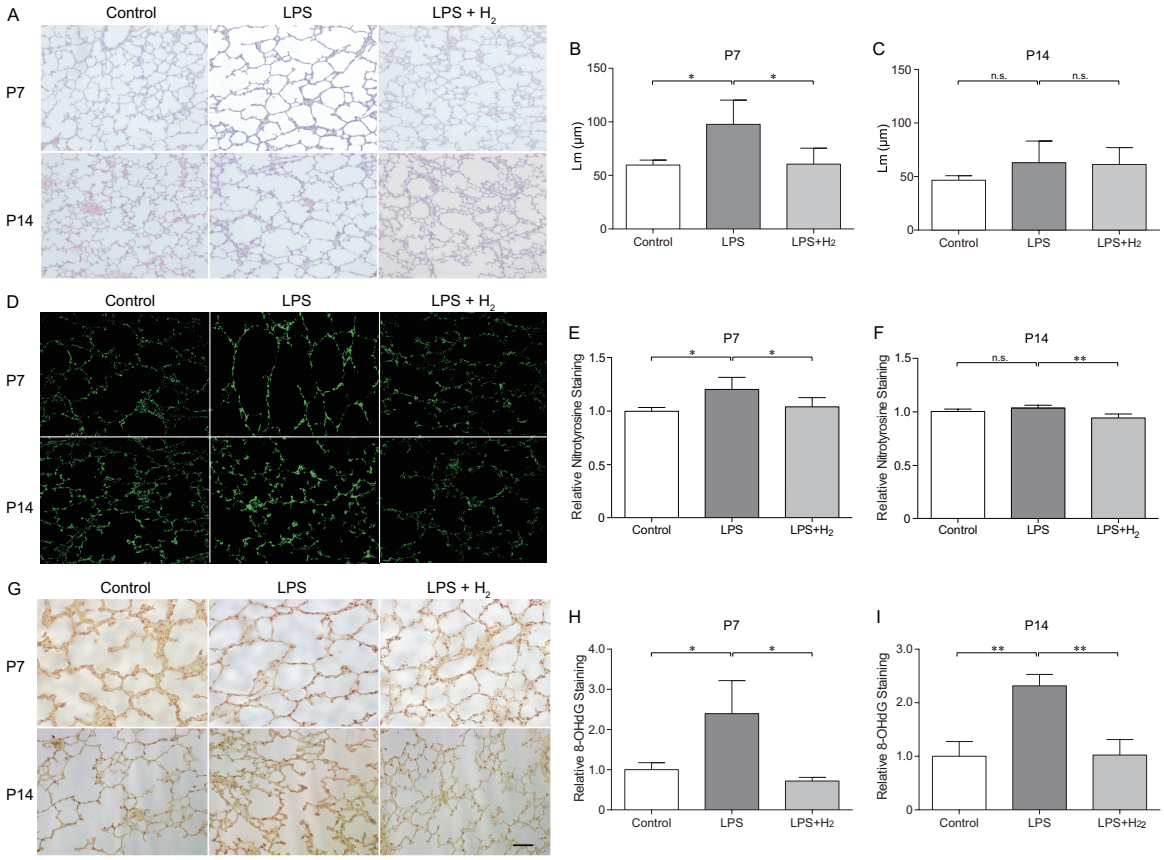


Figure 1. (A) Kaplan-Meier survival curves of pups from P0 to P14 (n = 40 for Control, 29 for LPS, and 34 for LPS + H_2). No significant difference is observed in any pair by the logrank test. LPS, pups from LPS-treated mothers; LPS + H_2, pups from LPS- and H_2-treated rats. **(B)** Body weights of pups at P7 (n = 12 for Control, 15 for LPS, and 16 for LPS + H_2) and P14 (n = 6 for Control, 9 for LPS, and 12 for LPS + H_2). No significant difference is observed in any pair by one-way ANOVA.



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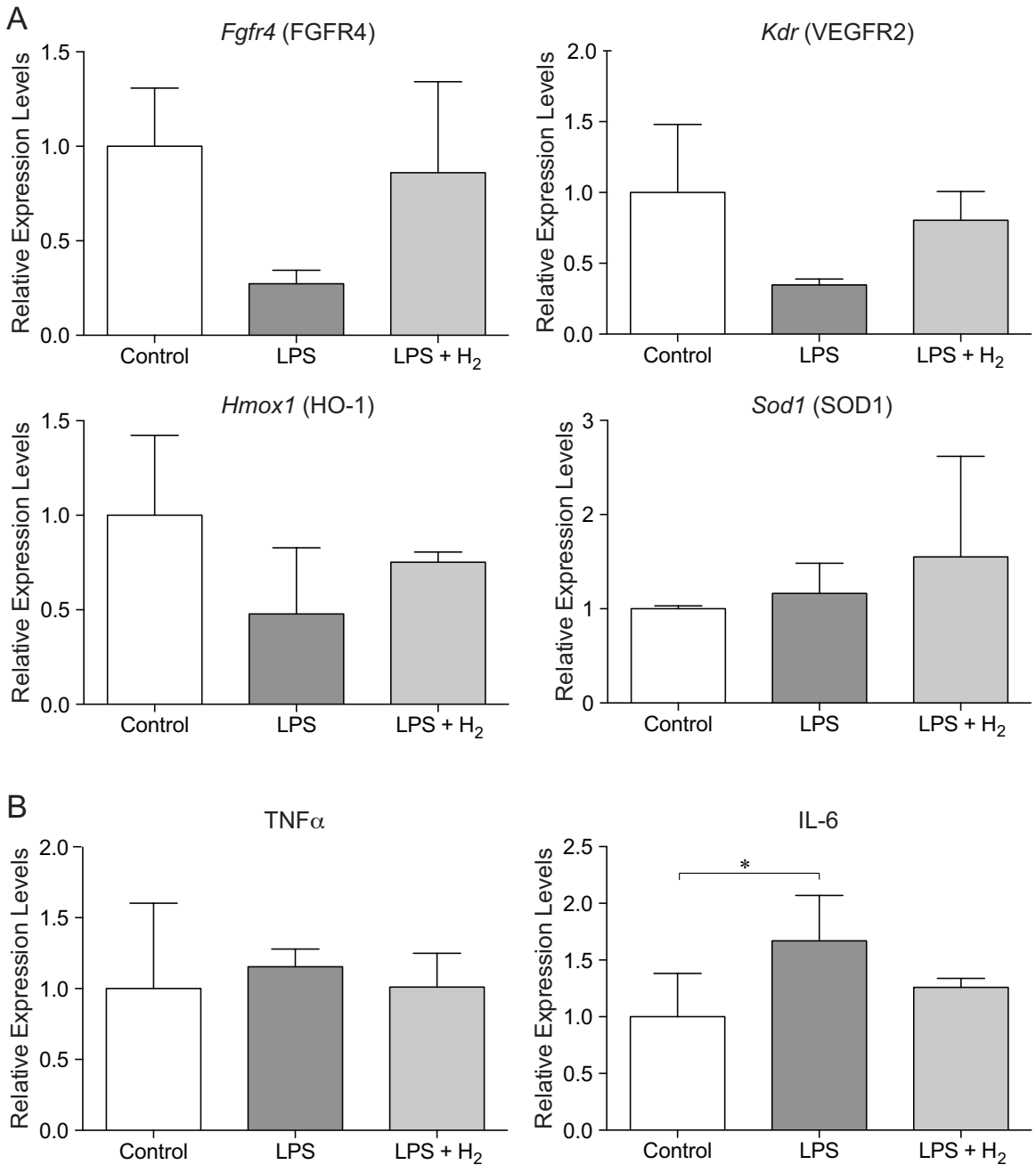

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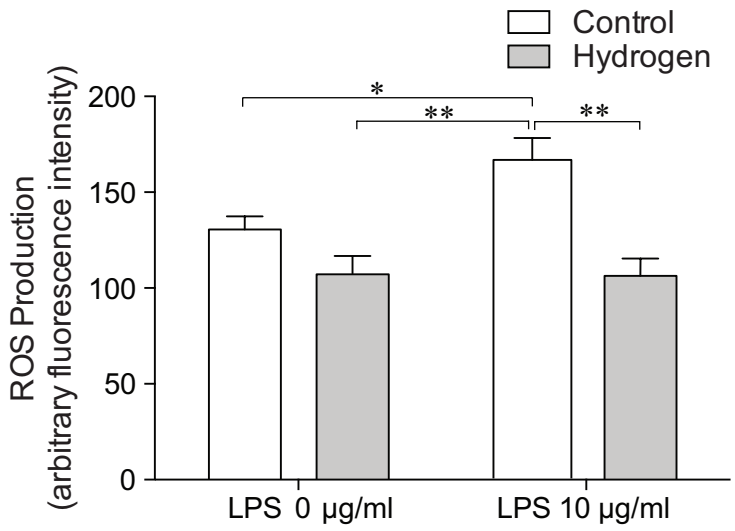

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Supplementary Table S1. The numbers of intrauterine fetal death (IUFD)

	Control	LPS	LPS + H_2
IUFD	3 (7%)	31 (52%)	27 (44%)
Live birth	40 (93%)	29 (48%)	34 (56%)

No statistical difference between LPS and LPS + H_2 by Fisher's exact test.