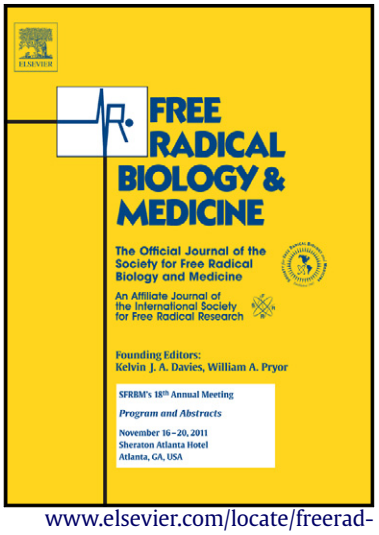

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Maternal molecular hydrogen administration ameliorates rat fetal hippocampal damage by *in utero* ischemia-reperfusion

Yukio Mano[1,2], Tomomi Kotani[2], Mikako Ito[3], Taku Nagai[4], Yuko Ichinohashi[5], Kiyofumi Yamada[4], Kinji Ohno[3], Fumitaka Kikkawa[2] and Shinya Toyokuni[1]

[1]Departments of Pathology and Biological Responses, [2]Gynecology and Obstetrics, [3]Division of Neurogenetics, Center for Neurological Diseases and Cancer, and [4]Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

[5]Department of Neonatology, Center for Maternal-Neonatal Care, Nagoya University Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

Conflict of interest: Approximately 50%-saturated hydrogen water was a kind gift from Blue Mercury Inc. (Tokyo, Japan). The authors have no conflict of interest to disclose except this.

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Correspondence and reprint requests: Shinya Toyokuni, M.D., Ph.D.; Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel: +81-52-744-2086; Fax: +81-52-744-2091; E-mail: toyokuni@med.nagoya-u.ac.jp

Abstract

Molecular hydrogen (H_2) scavenges hydroxyl radicals. Recently, H_2 has been reported to prevent a variety of diseases associated with oxidative stress in model systems and in humans. Here, we studied the effects of H_2 on rat fetal hippocampal damage by ischemia and reperfusion (IR) on day 16 of pregnancy with the transient occlusion of the bilateral utero-ovarian arteries. Starting 2 days prior to the operation, we provided the mothers with hydrogen-saturated water *ad libitum* until vaginal delivery. We observed a significant increase in the concentration of H_2 in the placenta after the oral administration of hydrogen-saturated water to the mothers, with less placental oxidative damage after IR in the presence of H_2. Neonatal growth retardation was observed in the IR group, which was alleviated by the H_2 administration. We analyzed the neuronal cell damage in the CA1 and CA3 areas of the hippocampus at day 7 after birth by immunohistochemical analysis of the 8-oxo-7,8-dihydro-2'-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins. Both oxidative stress markers were significantly increased in the IR group, which was again ameliorated by the H_2 intake. Lastly, 8-week-old rats were subjected to a Morris water maze test. Maternal H_2 administration improved the reference memory of the offspring to the sham level after IR injury during pregnancy. Overall, the present results support the idea that maternal H_2 intake helps prevent the hippocampal impairment of offspring induced by IR during pregnancy.
(229 words)

Keywords: ischemia-reperfusion; in utero; neuronal damage; molecular hydrogen

Introduction

Cerebral hypoxia-ischemia (asphyxia) occurring in the fetus and newborn infant is a major cause of acute mortality and chronic disability in survivors [1]. Certain forms of newborn brain injury, such as stroke, have an incidence as high as 1 in 4,000 live births [2]. More than 95% of infants who have a stroke survive to adulthood, and many have residual motor or cognitive disabilities. Stroke and other forms of brain damage cause considerable consequences to surviving babies, their families and society [3]. Therefore, the development of preventive and therapeutic measures for fetal and neonatal brain injury is eagerly awaited.

Molecular hydrogen (H_2) can reduce hydroxyl radicals ($^{\cdot}OH$) and peroxynitrite but not superoxide (O_2^-), hydrogen peroxide (H_2O_2) or nitric oxide ($^{\cdot}NO$), indicating that H_2 can antagonize damaging species without affecting physiologically important signaling molecules. Furthermore, to date, H_2 has no known side effects, including mutagenicity in rodents or humans [4]. Prompted by these unique characteristics, studies on H_2 for oxidative stress-associated diseases have flourished the past few years. H_2 in the form of gas reduces the cerebral infarction volume in rats [5, 6], suppresses hepatic ischemia/reperfusion (IR) injury in mice [7], reduces the infarct size of myocardial IR injury [8] and cardiac cold IR injury [9] in rats, and reduces apoptosis in neonatal hypoxic brain injury in rats [10]. It also mitigates small intestine transplantation-induced inflammation in rats [11] and decreases the hippocampal neuronal injury in mouse cerebral infarction model [12]. H_2 dissolved in drinking water or saline similarly prevents stress-induced learning impairment in mice [13], protects against 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of Parkinson's disease [14], improves lipid and glucose metabolism in type-2 diabetes [15], and reduces oxidative stress, inflammatory cytokines and apoptosis in a rabbit model of spinal cord ischemia-reperfusion injury [16].

Memory and learning are among the highest functions of the central nervous system, and the hippocampus, which is responsible for memory and learning [17], has been established as being highly sensitive to ischemia [18]. Accordingly, we focused on this area in the present

preclinical study. To examine a neuroprotective effect of H_2 for neonatal cognitive and memory function, an intrauterine IR rat model was given free access to ~50%-saturated hydrogen water (HW) starting 2 days before the transient ligation of the bilateral utero-ovarian arteries [19]. Pathological and behavioral studies have demonstrated that molecular oxygen efficiently prevents both the developmental failure and deterioration of the hippocampal function in rats after birth.

Materials and Methods

Reagents

The following antibodies were used: mouse monoclonal antibody against 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) (N45.1) [20]; monoclonal antibody against 4-hydroxy-2-nonenal (HNE)-modified proteins (HNEJ-2) [21, 22] (Nikken Seil Co., Ltd., Shizuoka, Japan); mouse monoclonal antibody against NeuN [1B7] (Abcam, Tokyo, Japan). Approximately 50%-saturated hydrogen water (HW) prepared by dissolving H_2 gas in water under a pressure of 0.4 MPa as previously described [23] was a kind gift from Blue Mercury Inc. (Tokyo, Japan). HW (more than 0.4 mM) was stored in an aluminum bag and aliquoted every 24 h to glass drinking bottles for rats with two ball bearings at the outlet, which avoids hydrogen degassing as well as air refill. With this glass bottle, the hydrogen concentration remained more than 0.2 mM after 24 h. In IR+HW group, the rats drank HW *ad libitum* from day 14 of pregnancy to vaginal delivery (day 22 of pregnancy). The pregnant rats drank approximately 60 mL/kg of regular water or HW per day. Chemical agents used were of analytical quality.

Animal model

The animal experiment committee of Nagoya University Graduate School of Medicine approved this study. Pregnant Wistar/ST rats (Japan SLC, Shizuoka, Japan) were purchased and housed in plastic cages. They received a standard chow (F2) and water *ad libitum*, and were maintained on a 12-h light/12-h dark cycle (lights on at 9 am, off at 9 pm). The pregnant

rats were assigned randomly to the following three groups of 5 dams each: sham group that underwent laparotomy on day 16 of pregnancy without IR procedure; IR group, in which IR operation was performed on day 16 of pregnancy; IR+HW group, in which pregnant rats were given ~50%-saturation hydrogen water (HW), starting from 2 days prior to operation and thereafter till vaginal birth with IR operation on day 16 of pregnancy. Each pregnant rat gave birth of 5-10 offspring without apparent sex difference (N=42 for sham, N=40 for IR group and N=38 for IR+HW).

Determination of hydrogen concentration

Previous studies reported that the hydrogen concentration in rat tissue peaked 5 to 15 min after oral HW administration and returned to the basal level after 25 to 30 min [15, 24]. Cesarean section was performed under anesthesia 5 min after 4 mL of HW administration orally by gavage, and amniotic fluid, placenta and fetuses were collected (HW group). The fetuses were decapitated immediately; the fetal head and body were examined separately. Pure air (100 mL) was equilibrated either with amniotic fluid or homogenized tissue in an aluminum bag, and the hydrogen concentration in the air was measured with a gas chromatograph connected to a semiconductor gas detector (EAGanalyzer GS-23, SensorTec Co. Ltd., Shiga, Japan). The pregnant rats without HW administration served as the control group (N=3-5 of fetuses for each group, consisting of 6 dams).

Ischemia-reperfusion operation

IR operation was performed as previously reported [25] with a minor modification. Briefly, the pregnant rats were operated on day 16 of pregnancy after injection of ketamine (30 mg/kg, ip) and xylazine (10 mg/kg, ip). To induce fetal ischemia, we occluded bilateral utero-ovarian arteries for 30 min by using forceps covered with soft polyvinyl tube. After 30-min occlusion, the clamp was released to restore circulation for 30 min and reperfusion was achieved. The operation, which omitted the ischemia-reperfusion procedure, was considered as sham operation. Investigators involved in the following experiments were blinded to the operation procedure.

Neonatal growth evaluation and histological analysis of neonatal brain

After vaginal delivery, each pup was weighed on postnatal day 1, 3, 5 and 7. Then, each pup (N=10 for each group with at least one pup from each dam) was decapitated under anesthesia with ice and each head portion was fixed in 10% phosphate-buffered formalin. Each specimen was embedded in paraffin, and frontal plane sections were cut at a 4-μm thickness. Neonatal hippocampal damage was observed with hematoxylin and eosin (HE) staining and quantified as the ratio of degenerated to total hippocampal pyramidal cell number under x400 magnification at one field for each section. The average of total cell counts in one field was 78 cells for CA1 and 45 cells for CA3 region, respectively. The hippocampal pyramidal cells were confirmed as neuronal cells by immunohistochemistry of NeuN, a specific neuronal cell marker. The cells, of which nucleus showed pyknotic change, were defined as degenerated cells.

Immunohistochemistry

Immunohistochemistry was performed as previously described [26] with minor modifications. For antigen retrieval, deparaffinized sections were heated in immunosaver (Nisshin EM Corporation, Tokyo, Japan) for 45 min at 98 °C. Immunohistochemical staining was performed employing the avidin-biotin immunoperoxidase technique using the Histofine SAB-PO kit (Nichirei, Tokyo, Japan) according to the manufacturer's protocol. The concentration of the primary antibody was 10 g/mL for N45.1, 40 g/mL for HNEJ-2 and 5 g/mL for NeuN [1B7].

Quantification of immunostaining

Densitometric analysis of fetal brain images was performed with ImageJ freeware (version 1.45, WS Rasband, NIH, Bethesda, MD). TIFF files were opened, which were split into each RBG channel. After setting a proper threshold, density and area in red channel over threshold were integrated and quantified. The levels of staining in the placental images were analyzed using Hybrid cell count BZ-H2C software (Keyence, Osaka, Japan) according to the manufacturer's protocol.

Morris water maze test

After vaginal delivery, pups were housed with the dam for three weeks, thereafter weaned and maintained separately from the dam and according to their sex until 8-week old. Sham group contained 17 pups from 2 dams; IR group contained 26 pups from 4 dams, and IR+HW group contained 38 pups from 4 dams. The Morris water maze task [27] was performed as previously described [28] with a minor modification at 8-week old. Briefly, a circular water tank of 140-cm diameter consisted of four equally spaced quadrants. A transparent platform of 10-cm diameter was fixed at a quadrant of the tank (platform quadrant) and hidden 2 cm below the surface of the water for the reference memory task. The pool was located in a large room, in which there was a cue on each wall. The positions of these cues were left unchanged throughout the task. Each trial lasted until either the rats had arrived the hidden platform or for a maximum of 90 s. If the rat found the platform, it was allowed to remain there for 15 s and was then returned to its home cage. If the rat could not find the platform within 90 s, the trial was terminated and the animal was put on the platform for 15 s. The time of swimming until the rats reached the platform was assigned using the Ethovision automated tracking program (Neuroscience Idea Co. Ltd.). The task was conducted twice a day at an interval of at least 1 h for 5 consecutive days. Entry points were selected randomly. After "the reference memory task", the platform was removed and "the probe task" was performed. The ratio of the time spent in the platform quadrant was determined and retention of the spatial memory was evaluated. At the end of all tests, the visual task in which platform was marked and visible was performed for confirmation.

Statistical analysis

Statistical analyses were performed using the SPSS 19.0 software package (SPSS Inc., Chicago, IL). Distributions and variances were evaluated by means of the Shapiro-Wilk test and the Levene's test, respectively. Then, for comparisons of the two groups, either Student's *t*-test or Mann-Whitney *U*-test was performed where applicable. For comparisons of multiple groups, ANOVA with the Tukey HSD test was used. Differences between the groups were

considered significant at $P < 0.05$.

Results

HW restored neonatal growth

Significant neonatal growth retardation was observed in IR group compared with sham group at postnatal day 5 and 7. Maternal administration of HW, which was given freely starting from 2 days prior to IR till delivery, significantly improved neonatal growth in weight (**Figure 1**, $P < 0.01$). The still birth rate and the neonatal mortality rate to postnatal day 7 were not different among the three groups (data not shown).

HW alleviated the damage of hippocampal pyramidal cells

We next evaluated the IR-induced brain damage of neonates at postnatal day 7. As previously reported [19], the degeneration of hippocampal pyramidal cells was observed in IR group. In CA1 and CA3 regions of hippocampus, 34.5% and 33.6% of pyramidal cells were degenerated in IR group, respectively. In IR+HW group, pyknotic cells of hippocampus were significantly reduced, compared with IR group (**Figure 2**, $P < 0.01$).

HW reduced IR-induced oxidative stress in hippocampus

To study whether maternal intake of HW leads to reducing IR-induced oxidative stress in the fetal brain, immunohistochemistry of 8-oxodG and HNE-modified proteins were performed. 8-OxodG was strongly immunostained in the hippocampal pyramidal cells of CA1 and CA3 regions in IR group. The staining intensity of 8-oxodG in hippocampal CA1 and CA3 regions was significantly attenuated with HW intake, compared with IR group (**Figure 3**, $P < 0.05$ and $P < 0.01$, respectively). IR significantly increased HNE-modified proteins in neonatal hippocampus. However, HW decreased them without statistically significant difference (**Figure 3**).

Maternal administration of HW improved reference memory of offspring

To evaluate the hippocampal damage, a Morris water maze test was carried out at 8-week old of age. In reference memory task, the escape latency was significantly different between

IR group (46.7±4.4 s) and IR+HW group (32.9±3.1 s) at day 4 (**Figure 4**; N=17 for Sham, N=26 for IR and N=38 for IR+HW; means±SEM; **P* < 0.05 IR *vs* IR+HW, ***P* < 0.05 Sham *vs* IR). The difference was not significant among the three groups in probe task and visual task (data not shown).

Maternal administration of HW relieved oxidative damage of the placenta

We studied whether maternal administration of HW increases hydrogen concentration in the fetus. Placenta, amniotic fluid, fetal head and body were collected by cesarean section 5 min after 4-mL HW administration by gavage. Hydrogen concentration was significantly higher in the placental tissue, fetal head and body after HW administration as compared to control (**Figure 5A**, **P* < 0.01, ***P* < 0.05). To analyze the effect of HW administration in the placenta, the placentae were collected by cesarean section at day 20 of pregnancy; placental oxidative stress was assessed by immunohistochemistry of 8-oxodG. The staining intensity of 8-oxodG in the giant cells, which is equivalent of extravillous trophoblast in humans was increased in the IR group and significantly decreased by HW administration (**Figure 5B and 5C**).

Discussion

The present study, for the first time, demonstrates that H_2 administered in drinking water to pregnant rats works to ameliorate the neonatal complications induced by fetal pathogenic events. Here, we studied the influence of H_2 on the memory and learning function of the offspring using an IR model of hippocampal damage by the transient occlusion of the maternal bilateral utero-ovarian arteries at day 16 of pregnancy. As we can recognize from the results in **Figure 4**, the intellectual disturbance of this model was subtle but recognizable with a Morris water maze test, which is indeed relevant to human situations. The H_2 administration not only improved the reference memory of the offspring (**Figure 4**) but also prevented the growth retardation associated with this oxidative insult (**Figure 1**). Since it is assumed that the neonatal brain damage is related to difficulties in breast feeding, the reason

for improvement in neonatal growth by H_2 administration is considered as the result of alleviated brain damage at least partially.

H_2 is a gas and thus is assumed to penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus [5]. It can even leak through plastic bottles. Thus, we have taken precautions to avoid the leakage of H_2 using aluminum packages for storage and glass drinking bottles with two ball bearings at the outlet for the rats. We determined the H_2 concentration in the placenta, amniotic fluid, fetal head and body 5 min after the oral administration of 4 mL of ~50% saturated hydrogen water (HW) and found that it was significantly increased in the placenta, fetal head and body (**Figure 5**). The hydrogen concentration in the amniotic fluid was not increased by hydrogen administration. Amniotic fluid is produced by fetal and placental cells, and thus is not in direct contact with maternal circulation. This may be the reason of no significant alteration in H_2 concentration after maternal oral intake.

A previous study suggested that H_2 protects neural cells and tissues by scavenging hydroxyl radicals against strong oxidative stress, such as in cerebral infarction [5] and Parkinson disease [14]. Initially, we evaluated the present pregnancy-associated model using morphological and immunohistochemical analyses. 8-oxodG [29] and HNE-modified proteins [30] are established oxidative modifications caused by the generation of hydroxyl radicals, peroxynitrite or its equivalents [31]. We used monoclonal antibodies that our laboratory had previously produced [20, 21]. The hippocampal CA1 and CA3 areas of 7-day-old pups showed significantly higher immunostaining of both modifications, indicating that the fetal hippocampal damage persisted and progressed even after birth with neural development. This outcome was alleviated by the HW intake of the dams, whereas decrease in HNE-modified proteins was not significant (**Figures 2 and 3**). Reperfusion injury is considered to start in the cytoplasm or at the plasma membrane due to xanthine oxidase activation or accumulating inflammatory cells. 8-oxodG is mainly generated in the nucleus, which is most far from the generation site of reactive species, which may be responsible for

the difference in results between 8-oxodG and HNE-modified proteins. This rat model is a model of relatively mild brain damage during pregnancy. Importantly, the morphological and immunohistochemical alterations in the neonates were reflected in the memory and learning function of the adults evaluated by the Morris water maze test **(Figure 4).**

Here, we discuss the molecular function of H_2 in the present model. The results suggest that H_2 spread through maternal-fetal interface and blood-brain barrier, and exerted its antioxidative effect both in placenta and fetal brain. Furthermore, the placenta is an organ full of villous structures comprising vessels and trophoblasts and is attached to the endometrial cavity of the uterus, thus working as an exchange apparatus for O_2, CO_2, nutrients and metabolites between the dam and pups. H_2 may have protected against oxidative damage in the placental circulation during the transient occlusion of the utero-ovarian arteries, which also affected the circulation within the hippocampus. The precise mechanism requires further investigation.

To prevent perinatal brain damage, we may propose two strategies for the use of HW in pregnant women. The pregnant women, who are high risk at preterm labor, fetal growth restriction and preeclampsia, may receive HW from early stage of pregnancy. Alternatively, since perinatal asphyxia is known to cause hypoxic-ischemic encephalopathy, which is associated with oxidative stress [32]. HW may be used during delivery if the repeated fetal heart rate decelerations by distress are observed.

In conclusion, we found that the maternal administration of HW relieves fetal brain damage by reducing oxidative stress in an *in utero* IR model. This strategy could have a potential benefit for the prevention of cerebral injury during pregnancy as a novel intra-uterine therapy.

Conclusion

Maternal intake of hydrogen water relieved fetal hippocampal damage by reducing oxidative stress in a rat *in utero* ischemia-reperfusion model during pregnancy. Here, memory

deterioration was significantly improved with administration of hydrogen water. This simple procedure for mothers could have a potential clinical benefit for the prevention of cerebral damage of their babies caused by a variety of etiology during pregnancy and birth.

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List of Abbreviations

ANOVA, an analysis of variance

HE, hematoxylin and eosin

HNE, 4-hydroxy-2-nonenal

HW, ~50%-saturation hydrogen water

IR, ischemia and reperfusion

8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine

Figure legends

Figure 1. Neonatal growth in body weight. Neonatal growth was significantly retarded in IR group compared with sham group at postnatal day 5 and 7. In IR+HW group, maternal administration of hydrogen water restored neonatal growth to the control level (N=30-42; means±SEM; *IR group *vs* sham and #IR+HW group vs IR group, $P < 0.01$ by both ANOVA according to the Tukey HSD test and Student's *t*-test). HW, ~50% saturated hydrogen water; IR, ischemia-reperfusion. Refer to text for details.

Figure 2. Morphological analysis of neonatal hippocampus. (A) Hematoxylin and eosin staining of hippocampal CA1 and CA3 region in 7-day old neonatal rat brain sections. Representative slides were shown (Arrowheads, degenerated cells). Inset a and d: Immunohistochemistry of NeuN, a specific neuronal cell marker. The hippocampal pyramidal cells were confirmed as neuronal cells. Inset b and e: Normal cells observed in each panel. Inset c and f: Degenerated cells observed in each panel (bar=50 μm in a and d, and 25 μm in b, c, e and f). **(B)** Hippocampal neuronal damage was calculated as the ratio of degenerated to total pyramidal cell number (number of degenerated cells/number of total cells) in the hippocampal CA1 and 3 region (N=10; means±SEM; * $P < 0.01$ by both ANOVA according to the Tukey HSD test and Mann-Whitney *U*-test).

Figure 3. Immunohistochemical analysis of neonatal hippocampus. (A) 8-oxodG and HNE immunostaining of hippocampus in brain sections of 7-dayold neonatal rat. Representative pictures are shown (arrowheads, hippocampal pyramidal cells; 8-oxodG, bar=50 μm; HNE, bar=100 μm). **(B)** Quantitive immunohistochemical analysis of 8-oxodG in the hippocampal CA1 and 3 region and HNE in the hippocampus. Amounts of 8-oxodG and HNE were analyzed by ImageJ software (N=10; means±SEM; *$P < 0.01$, **$P < 0.05$ by both ANOVA according to the Tukey HSD test and Student's *t*-test).

Figure 4. Morris water maze test at 8-week after birth. IR+HW group improved the escape latency to the hidden platform significantly faster at day 4 (N=17 for Sham, N=26 for IR and N=38 for IR+HW; means±SEM. *$P < 0.05$ IR *vs* IR+HW and **$P < 0.05$ IR *vs* Sham by both ANOVA according to the Tukey HSD test and Student's *t*-test).

Figure 5. Hydrogen concentration and immunohistochemical analysis of the placenta. (A) Placentae, amniotic fluid, fetal head and body were taken 5 min after HW administration of 4 mL to the pregnant rats by gavage (HW group). In the control group, HW administration

was omitted. Maternal administration of HW significantly increased hydrogen concentration in placental tissue, fetal head and body (N=3-5, means ± SEM; **P* < 0.01 and ***P* < 0.05, HW *vs* control with Student's *t* test). **(B)** Hematoxylin and eosin (HE) staining and 8-oxodG immunostaining of the placental serial sections. Representative pictures are shown (arrowheads, 8-oxodG immnopositive trophoblast giant cells; bar=50 μm). **(C)** Quantitive immunohistochemical analysis of 8-oxodG in the placenta of day 20 of pregnancy. The levels of 8-oxodG staining were analyzed by Hybrid cell count BZ-H2C software (N=3; means ± SEM; **P* < 0.01, ***P* < 0.05 by both ANOVA according to the Tukey HSD test and Student's *t*-test).

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- Maternal H_2 intake relieved fetal hippocampal damage in a rat *in utero* IR model.
- Memory deterioration was significantly improved with H_2 administration.
- This simple method for mothers could have a potential clinical benefit for babies.

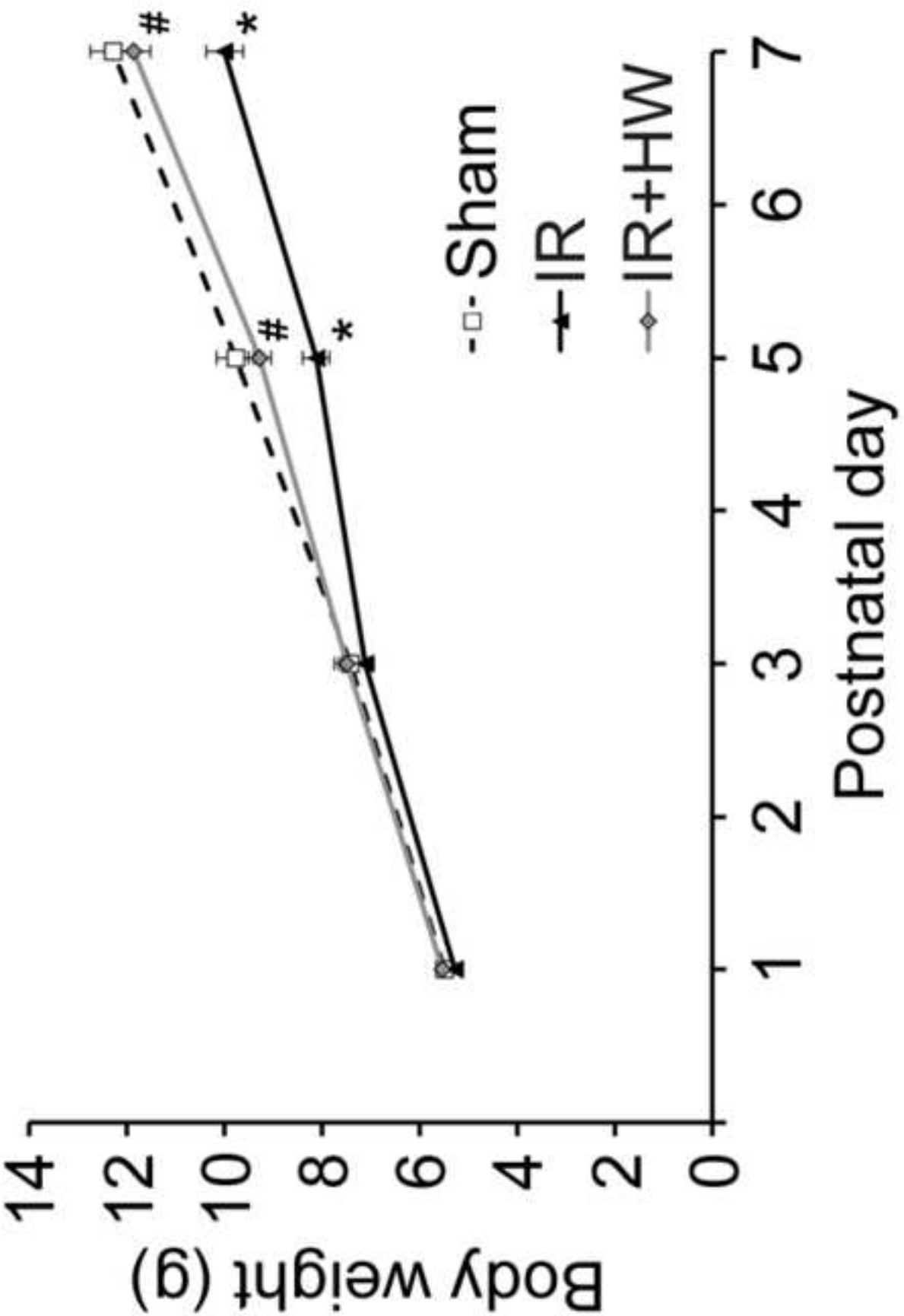


Figure 1

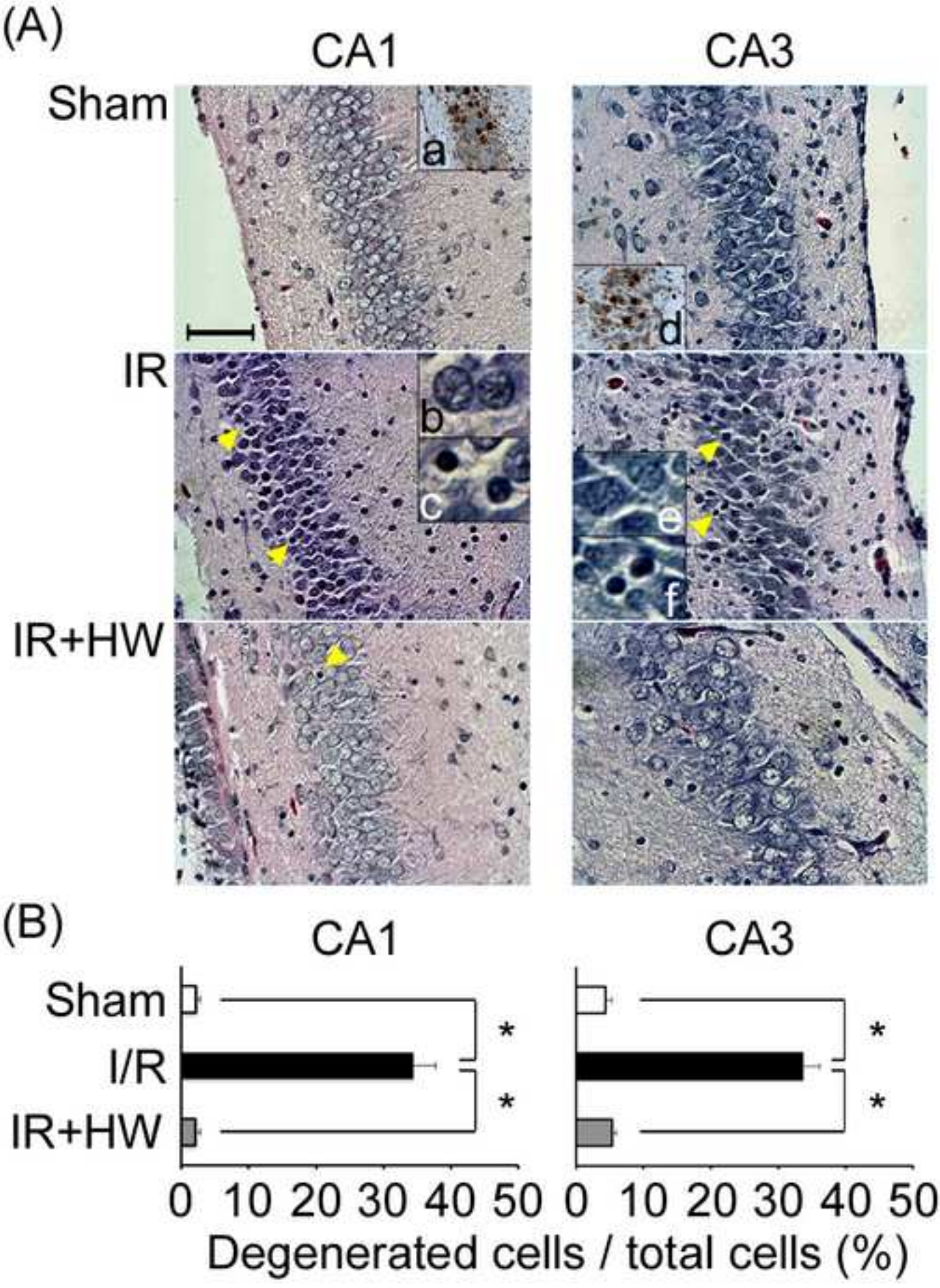
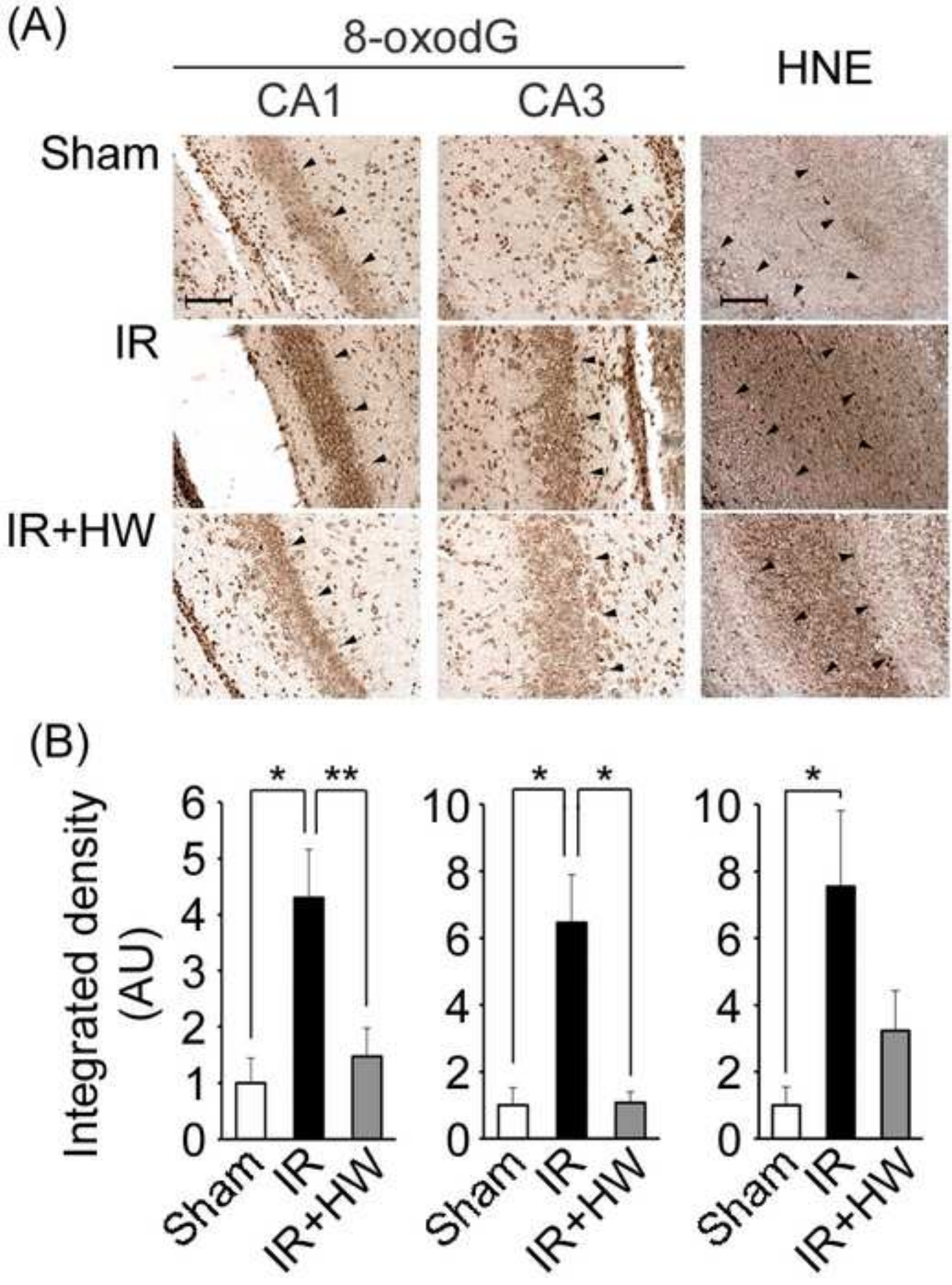


Figure 3



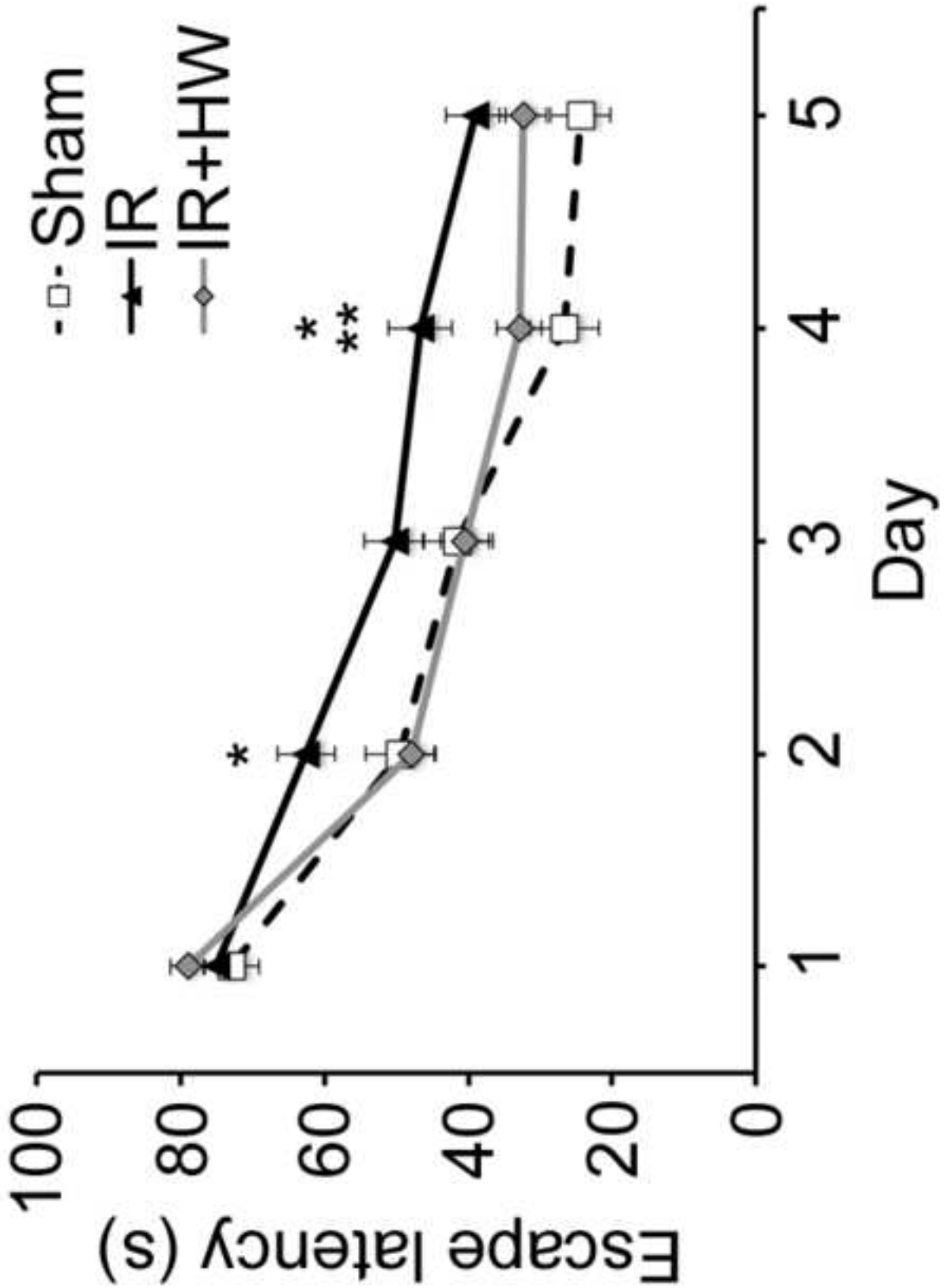
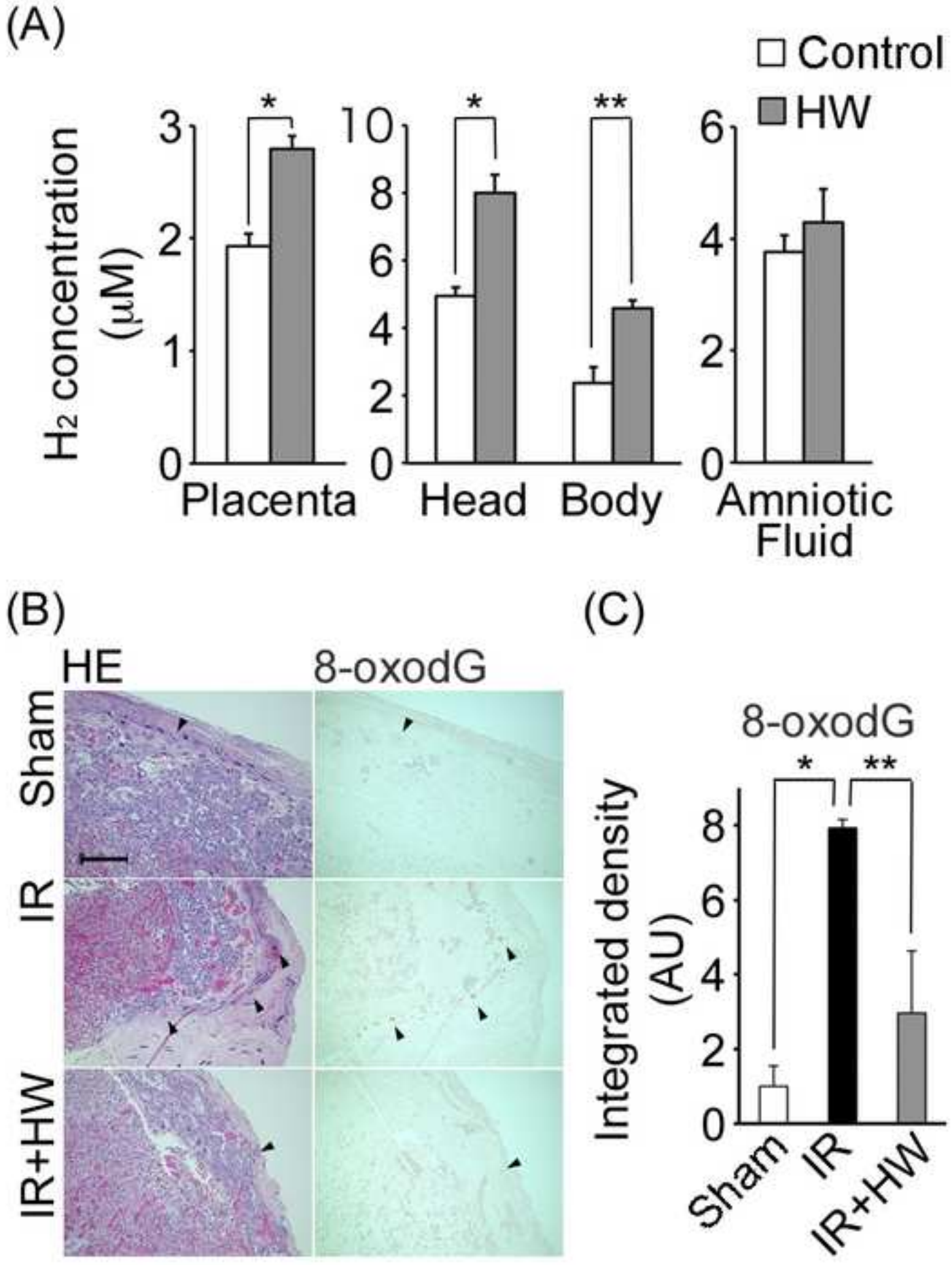


Figure 4

Figure 5



Graphical Abstract (for review)