

# Edaravone Inhibits DNA Peroxidation and Neuronal Cell Death in Neonatal Hypoxic-Ischemic Encephalopathy Model Rat

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**ABSTRACT:** Neonatal hypoxic-ischemic encephalopathy (HIE) is the most frequent neurologic disease in the perinatal period. Its major cause is oxidative stress, which induces DNA peroxidation and apoptotic neuronal death. We examined 8-hydroxy-2'-deoxyguanosine (8-OHdG) expression to evaluate brain damage in neonatal HIE and the therapeutic effect of edaravone, a free radical scavenger. Using HPLC and immunohistochemistry, the 8-OHdG levels of neonatal HIE model Sprague-Dawley rats that were subjected to left common carotid artery ligation and 2-h hypoxia significantly increased after 24–48 h of hypoxic-ischemic (HI) insult, but decreased after 72 h. Moreover, the number of apoptotic cells with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and karyorrhexis significantly increased after 24–72 h of HI insult. In a therapeutic experiment, edaravone was administered i.p. (9 mg/kg) after HI insult every 24 h. Edaravone reduced both the apoptotic neuronal cell number and 8-OHdG expression after 24–48 h of HI. From a double immunofluorescent study, DNA peroxidation occurred in apoptotic neuronal cells with 8-OHdG expression. Edaravone may inhibit the number of apoptotic neuronal cells and 8-OHdG expression within 48 h after HI insult. (*Pediatr Res* 65: 636–641, 2009)

Neonatal asphyxia, which is defined as impairment of gas exchange with metabolic acidosis, often develops into hypoxic-ischemic encephalopathy (HIE) (1). Neonatal HIE is sometimes accompanied with the neurologic sequelae, such as cerebral palsy, mental retardation, and intractable epilepsy, although most cases have no such complication. However, the morbidity of neonatal asphyxia is estimated to be 0.22% and 13.8% of the babies afflicted reportedly have neurologic sequelae in Japan (2). Another report estimates the incidence of neonatal asphyxia at 0.1–0.8% in live births (3). The number of victims is not small. Hence, it is necessary to rescue the immature brain from asphyxia and to assure development without neurologic sequelae.

The pathophysiology of neonatal HIE is mainly glutamate neurotoxicity due to the high concentration of free glutamate in intersynaptic spaces and reduction of energy supply from blood (4). As a result of the latter, mitochondrial release of cytochrome *c* is induced, which activates apoptosis through the caspase-pathway (5). Actually, asphyxiated brains often demonstrate apoptotic neuronal death (6). Additionally, it was

recently found that in the early stage after hypoxic-ischemic (HI) insult, reactive oxygen species (ROS), such as nitric oxide and hydroxyl radicals, contribute to apoptotic neuronal death (7–11). ROS, which are induced by oxidative stress, are an important factor in initiating DNA peroxidation, which can directly trigger DNA fragmentation and thereby initiate apoptosis (12). 8-Hydroxy-2'-deoxyguanosine (8-OHdG), which is caused by pathologic oxygen radicals, is a hydroxylated form of guanosine of DNA structure (13,14). Therefore, 8-OHdG measurement may be useful to evaluate DNA damage and neuronal apoptosis in the early stage of HIE (15).

Animal models of neonatal HIE have demonstrated apoptotic neuronal death, and its pathologic mechanism has been discussed. Furthermore, several studies have shown a therapeutic approach. Recently, antioxidative stress agents have been successful in cases of brain circulation insufficiency (16,17). Among them, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is thought to be the most beneficial agent for patients with acute ischemic stroke (18). Edaravone can interact with both peroxy and hydroxyl radicals, followed by the formation of a stable oxidation product through a radical intermediate (19). This scavenging effect cannot only inhibit neuronal apoptosis (20) and lipid peroxidation (21) but may also protect neurons against DNA peroxidation by excess production of ROS.

In this study, we reveal that the 8-OHdG amount is useful to evaluate DNA peroxidation and neuronal cell death in the acute stage of neonatal HIE model rat and demonstrate that edaravone is useful to reduce brain damage.

## MATERIALS AND METHODS

**Neonatal hypoxic-ischemic encephalopathy model rat.** All experiments were performed with the approval of the Animal Experiment Ethical Committee of the National Center of Neurology and Psychiatry. We used unsexed Sprague-Dawley rat pups (15–21 g; CLEA Japan Inc., Tokyo, Japan) on postnatal-day 7. A model of neonatal HIE was developed at postnatal-day 7 (22). Under anesthesia using isoflurane (Forane; Dainippon Pharma, Osaka, Japan) inhalation, the left common carotid artery was isolated, double ligated, and cut. After recovery for 2–4 h with their mothers, the pups were exposed to 8% oxygen with an ambient temperature 36°C for 2 h and then returned to their mothers. The pups were kept in a room (12 h/12 h light/dark cycle) until they were killed. The same mother littermates as age-matched controls

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**Abbreviations:** 8-OHdG, 8-Hydroxy-2'-deoxyguanosine; EdG, edaravone group; HI, hypoxic-ischemic; HIE, hypoxic-ischemic encephalopathy; LIC, left ipsilateral cortices; RCC, right contralateral cortices; VehG, vehicle group

underwent neither surgical procedure nor hypoxic exposure and were killed at postnatal-day 9.

**Tissue preparation.** Rat pups were killed at 6, 24, 48, and 72 h after the hypoxic ischemic insult for histopathological and HPLC analyses. They were anesthetized with diethyl ether and perfused intracardially with normal saline. Decapitated brains (frontal and parietal cortices) were dissected coronally at the level of 1 mm forward of the pituitary stalk.

For HPLC analysis, we separated the posterior region from the cortex, and froze the damaged cortex (left side) and healthy cortex (right side) separately in liquid nitrogen. Frozen samples were preserved at  $-80^{\circ}\text{C}$ .

For histopathological analysis, the anterior sections of remaining brains were fixed for 24 h with 4% paraformaldehyde in PBS at  $4^{\circ}\text{C}$  and embedded in paraffin. We made  $4\text{-}\mu\text{m}$ -thick coronal serial sections and performed staining with Nissl, hematoxylin and eosin (H & E), then TUNEL detection for apoptosis and immunohistochemistry, as well as immunofluorescent double staining.

**HPLC analysis of 8-hydroxy-2'-deoxyguanosine.** The 8-OHdG levels of cerebral cortex were measured with a method previously described (23,24). Briefly, nuclear DNA was isolated with a DNA extractor WB kit (Wako Pure Chemical Industries, Osaka, Japan) and digested into deoxynucleotides by treatment with nuclease P1 enzyme (Yamasa Corp., Chiba, Japan) and alkaline phosphatase (Sigma-Aldrich Chemical Co., St. Louis, MO). The concentration of 8-OHdG is defined as an 8-OHdG volume/(deoxyguanosine volume  $\times 10^5$ ) with HPLC (Ultrasphere-ODS,  $5\ \mu\text{m}$ ,  $4.6 \times 250\ \text{mm}^2$ ; Beckman Coulter, Fullerton, CA) and an electrochemical detection system (CoulArray Detector 5600A; ESA, Bedford, MA).

**TUNEL assay and immunohistochemistry.** TUNEL was performed using Klenow FragEL DNA Fragmentation Detection Kit (Calbiochem, Cambridge, MA) for apoptotic cell counting and In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany) for immunofluorescent double staining.

For immunohistochemistry, the mouse MAb against 8-OHdG (dilution of 1:50; JaiCA Inc., Fukuroi, Japan) was incubated for two overnights at  $4^{\circ}\text{C}$  (25). We confirmed specificity of the anti-8-OHdG antibody to react positively on a mouse hepatocellular carcinoma tissue. We used aminoethyl carbazole (Nichirei, Tokyo, Japan) as a chromogen to detect the immunoreactivity. For immunofluorescence, the primary antibodies against glial fibrillary acidic protein (GFAP, 1:1000; DAKO, Glostrup, Denmark) and neuron specific enolase (NSE, 1:400; Biomol, Plymouth, MA) were incubated overnight at  $4^{\circ}\text{C}$ . The stained sections were observed with fluorescence microscope (BX51; Olympus, Tokyo, Japan).

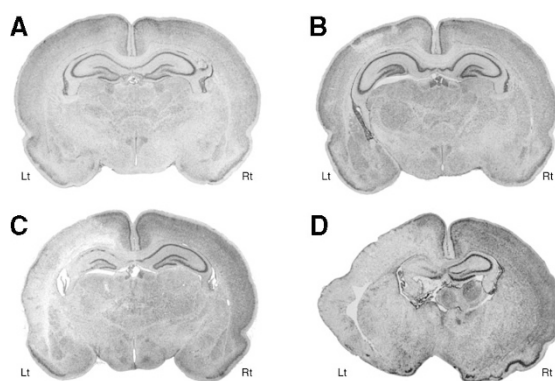
**Counting of karyorrhectic cells, TUNEL-positive cells, and 8-OHdG-positive cells.** Karyorrhectic cells observed on H & E, TUNEL-positive cells, and 8-OHdG-positive cells were counted in both somatosensory cortices in each of three fields at a magnification of 400 times. We found apoptotic cells to have karyorrhectic features and TUNEL-positivity. The numbers of these marked cells were converted into percent number in each section. The denominator of the ratio was decided to be the number of all nuclei in each field observed. Then we calculated the average and SD as the concentration in each individual.

**Drug preparation and administration.** Pups were divided into three groups, the vehicle group (VehG), edaravone group (EdG), and control (no treatment) group, consisting of 19, 20, and 8 pups, respectively. After the HI insult, pups of the EdG were immediately administered edaravone (gift from Mitsubishi Tanabe Pharma Inc., Tokyo, Japan) i.p. at a dose of 9 mg/kg and pups of the VehG with normal saline. The same dose of edaravone or normal saline was then administered every 24 h until being killed. The dose and time course of edaravone were based on the previous data (26). Rat pups were killed 6, 24, 48, and 72 h after HI insult. Pups of the control group were killed on postnatal-day 8 and 9.

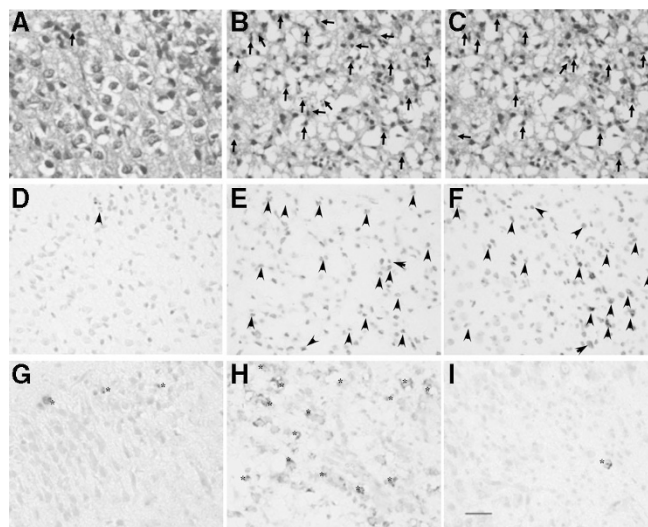
**Statistical analysis.** All data were expressed as average  $\pm$  SD. Data were analyzed with ANOVA, followed by Bonferroni's post hoc test.  $p < 0.05$  was considered as a significant difference.

## RESULTS

**Histopathological analysis of apoptotic cell death in cortex.** For histologic analysis, we used 4–6 samples at each time point, 6 controls at postnatal-day 9, and 26 samples were used in total. In the Nissl staining, the left ipsilateral cortices (LIC) demonstrated normal architecture 6 h after HI insult (Fig. 1A). However, after 24 h, the cortical damaged area gradually



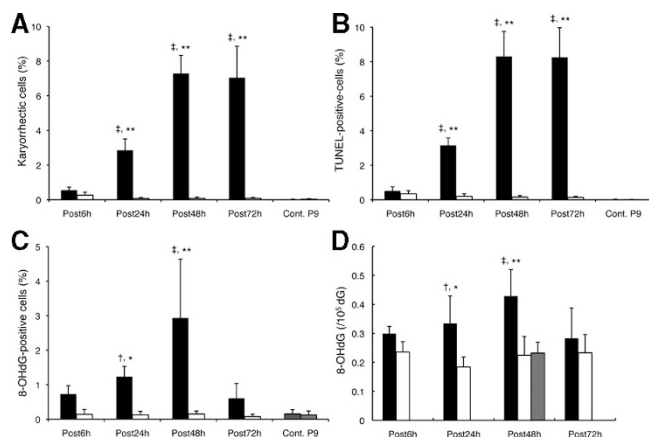
**Figure 1.** Evolution of brain injury in neonatal HIE with time. Damage to the left ipsilateral brain (Lt) worsens gradually over time, whereas the right contralateral brains (Rt) show no pathologic changes. Nissl staining of 6 h (A), 24 h (B), 48 h (C), and 72 h (D) after HI insult. Scale bar: 2 mm.



**Figure 2.** Histologic change of cortical damage with time. Karyorrhectic cells (arrows), TUNEL-positive cells (arrowheads) and cleaved caspase 3-positive cells appear 6 h after HI insult, and the numbers increase 48 and 72 h after HI insult. 8-OHdG-positive cells (asterisks) also appear 6 h after HI insult, increase at 48 h, but then decrease at 72 h. All figures are in the ipsilateral cortices. H & E-stained cells 6 h (A), 48 h (B), and 72 h (C) after HI insult. TUNEL-positive cells 6 h (D), 48 h (E), and 72 h (F) after HI. 8-OHdG-positive cells 6 h (G), 48 h (H), and 72 h (I) after HI. Scale Bar: 25  $\mu\text{m}$ .

became more extensive and severe, while spreading to the hippocampus, white matter, and striatum (Fig. 1B–D). The right contralateral cerebrum was normal at all times.

Karyorrhectic cells and TUNEL-positive cells were counted under high magnification ( $\times 400$ ). These cells gradually increased from 6 h after HI insult, and we noticed drastic increases at 48 and 72 h (Fig. 2A–F). From the cell counting (Fig. 3A and B), the percent concentrations of karyorrhectic cells in the LIC and right contralateral cortices (RCC) 24 h after HI insult were  $2.86 \pm 0.65\%$  of the LIC and  $0.07 \pm 0.05\%$  of the RCC ( $p < 0.001$ ), whereas those of TUNEL-positive cells at the same time were  $3.15 \pm 0.43\%$  of the LIC and  $0.43 \pm 0.15\%$  of the RCC ( $p < 0.001$ ), respectively. Significant increases were already seen 24 h after HI insult. Moreover, the concentrations of karyorrhectic cells and

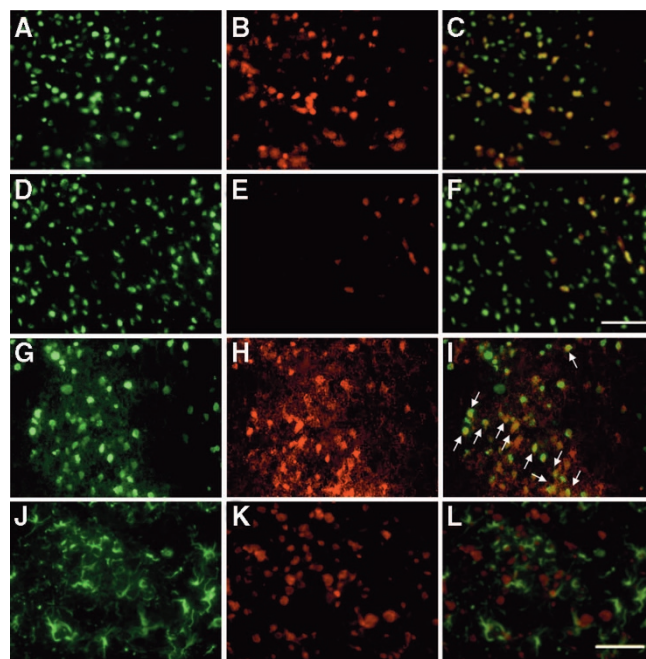


**Figure 3.** Quantitative analyses of apoptotic cells and 8-OHdG expression in the cortices. In the somatosensory cortices, significant increases of apoptotic cells, which are karyorrhetic (A) and TUNEL-positive (B), are recognized 24 h after HI insult and strengthen at 48 and 72 h. There are significant increases of 8-OHdG-positive cell number (C) and concentration (D, frontal and parietal cortices) 24 and 48 h after HI insult. Six hours after HI insult, significant increases of 8-OHdG-positive cells and concentration are shown. Post6 h, post24 h, post48 h, and post72 h mean 6, 24, 48, and 72 h after HI, respectively ( $n = 4-6$ ). ■, the left (HI ipsilateral) cortices; □, the right (only hypoxic contralateral) cortices. Controls were killed at postnatal-day 9 (■, Cont.P9 in A–C) or 48 h after HI insult or (■, Post48h in D) ( $n = 6$ ). Data are expressed as average  $\pm$  SD † $p < 0.05$ , ‡ $p < 0.001$  vs. contralateral cortices at the same time. \* $p < 0.05$ , \*\* $p < 0.001$  vs. ipsilateral cortices (A–C) or left cortices (D) of controls.

TUNEL-positive cells were drastically increased 48 h after HI insult. The percent concentration of karyorrhetic cells were  $7.27 \pm 1.05\%$  of the LIC and  $0.08 \pm 0.07\%$  of the RCC ( $p < 0.001$ ), and the percent concentration of TUNEL-positive cells were  $8.30 \pm 1.45\%$  and  $0.15 \pm 0.09\%$  ( $p < 0.001$ ), respectively. Seventy-two hours after HI insult, the percent concentration of karyorrhetic cells were  $7.03 \pm 1.82\%$  of the LIC and  $0.08 \pm 0.06\%$  of the RCC ( $p < 0.001$ ), and the percent concentration of TUNEL-positive cells were  $8.26 \pm 1.71\%$  and  $0.14 \pm 0.06\%$  ( $p < 0.001$ ), respectively.

**8-hydroxy-2'-deoxyguanosine immunohistochemistry and HPLC for evaluation of DNA peroxidation.** The numbers of 8-OHdG-positive cells were increased 24 and 48 h after HI insult, but decreased at 72 h (Fig. 2G–I). The percent concentrations of 8-OHdG-positive cells were  $1.23 \pm 0.30\%$  of the LIC and  $0.12 \pm 0.10\%$  of the RCC at 24 h after HI insult ( $p < 0.05$ ), and  $2.94 \pm 1.71\%$  of the LIC and  $0.15 \pm 0.08\%$  of the RCC at 48 h after HI insult ( $p < 0.001$ ) (Fig. 3C). A significant difference was recognized at 24 and 48 h, but not at 72 h. Interestingly, 6 h after HI insult, the number of 8-OHdG-positive cells obviously tended to increase.

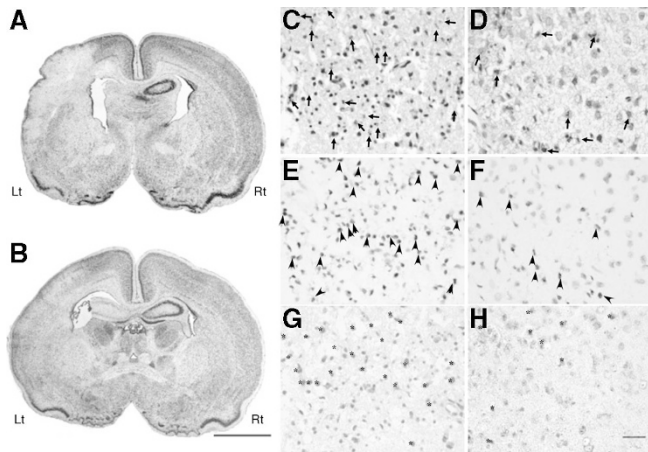
From 8-OHdG measurement with HPLC, the percent concentrations of 8-OHdG significantly increased 24 and 48 h after HI insult, at  $0.33 \pm 0.10$  of the LIC and  $0.18 \pm 0.03$  of the RCC at 24 h after HI insult ( $p < 0.05$ ), and  $0.43 \pm 0.09$  of the LIC and  $0.22 \pm 0.06$  of the RCC at 48 h after HI insult ( $p < 0.001$ ), respectively, per  $1 \times 10^5$  deoxyguanosine, but decreased at 72 h (Fig. 3D). This result was consistent with the immunohistochemical data. Moreover, the 8-OHdG level at 6 h tended to be higher than that of control.



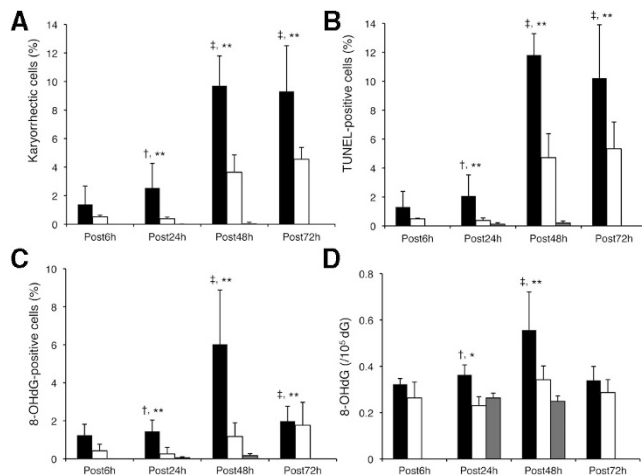
**Figure 4.** Immunofluorescence of neonatal HIE somatosensory cortices. TUNEL-positive cells are merged with 8-OHdG-positive cells 48 h after HI (A–C), but merged cells decrease at 72 h (D–F). 8-OHdG-positive cells have NSE signals (G–I), but no GFAP signals (J–L). Arrows show cells merged with 8-OHdG and NSE (I). TUNEL-stained ipsilateral cortex 48 h (A), and 72 h (D) after HI insult. 8-OHdG-stained ipsilateral cortex 48 h (B) and 72 h (E) after HI insult. Merged image 48 h (C) and 72 h (F) after HI insult. At the cortex 48 h after HI insult, NSE-stained ipsilateral cortex (G) and GFAP-stained ipsilateral cortex (J). 8-OHdG-stained ipsilateral cortex (H, K). Merged image of G and H (I) and that of J and K (L). Scale bar: 50  $\mu$ m.

**Immunofluorescent double staining for 8-hydroxy-2'-deoxyguanosine localization.** We confirmed that 8-OHdG localized in TUNEL-positive cells (Fig. 4A–F). Forty-eight hours after HI insult, many cells colocalized with TUNEL and 8-OHdG were observed (Fig. 4A–C). However, double-labeled cells were clearly fewer in number at 72 h than at 48 h (Fig. 4D–F). This phenomenon may be related to the change in the 8-OHdG-positive cell concentration between 48 and 72 h after HI insult. Moreover, we could detect 8-OHdG located in NSE-positive cells (Fig. 4G–I), but not in GFAP-positive cells (Fig. 4J–L). Therefore, it was confirmed that 8-OHdG was located in neurons.

**Evaluation of therapeutic effect by edaravone.** On the basis of the this result, we conducted edaravone therapy for our neonatal HIE rat pups, using 4–6 samples for each group at each time point and four controls of postnatal-day 8 or 9. We used 47 samples in total. Macroscopically, compared with the VehG brain, the EdG brain damage was reduced 48 h after HI insult (Fig. 5A and B). Microscopically, karyorrhetic cells, TUNEL-positive cells, and 8-OHdG-positive cells were remarkably decreased in the EdG (Fig. 5C–H). We also confirmed that the numbers of karyorrhetic cells and TUNEL-positive cells were significantly decreased 24, 48, and 72 h after HI insult in the EdG (Fig. 6A and B). The percent concentrations of karyorrhetic cells 24, 48, and 72 h after HI



**Figure 5.** Histopathology of edaravone therapy 48 h after HI insult. The lesion of edaravone-treated brain (B) is obviously smaller than that of vehicle-treated brain (A). Numbers of karyorrhectic cells (arrows), TUNEL-positive cells (arrowheads), and 8-OHdG-positive cells (asterisks) are decreased in edaravone-treated cortices. Nissl staining of vehicle-treated brain slice (A) and edaravone-treated brain (B). H & E staining of vehicle-treated (C) and edaravone-treated (D) cortices. TUNEL staining of vehicle-treated (E) and edaravone-treated (F) cortices. 8-OHdG staining of vehicle-treated (G) and edaravone-treated (H) cortices. Lt, the left ipsilateral brain; Rt, the right contralateral brain. Scale bar: 2 mm (A, B); 25  $\mu$ m (C–H).



**Figure 6.** Quantitative analyses of the edaravone-treated cortices. The numbers of karyorrhectic cells (A) and TUNEL-positive cells (B) in the edaravone-treated somatosensory cortices are significantly decreased 24, 48, and 72 h after HI insults. The significant differences increase at 48 and 72 h. The number of 8-OHdG-positive cells (C) and concentration (D: frontal and parietal cortices) in the edaravone-treated cortices are significantly decreased 24 and 48 h after HI insult, but the differences disappear at 72 h. Post6 h, post24 h, post48 h, and post72 h mean 6, 24, 48, and 72 h after HI insult, respectively ( $n = 4-6$ ). Controls in post24h means postnatal-day 8 rats, and control in post48h means postnatal-day 9 ( $n = 4$ , respectively). Data are expressed as average  $\pm$  SD  $\dagger p < 0.05$ ,  $\ddagger p < 0.001$  vs. edaravone-treated group at the same time. \* $p < 0.05$ , \*\* $p < 0.001$  vs. control groups. ■, VehG; □, EdG; ▨, control group.

insult were  $2.54 \pm 1.72\%$  of the VehG and  $0.38 \pm 0.12\%$  of the EdG at 24 h after HI insult ( $p < 0.05$ ),  $9.71 \pm 2.08\%$  of the VehG and  $3.64 \pm 1.22\%$  of the EdG at 48 h after HI insult ( $p < 0.001$ ), and  $9.31 \pm 3.19\%$  of the VehG and  $4.55 \pm 0.83\%$  of the EdG at 72 h after HI insult ( $p < 0.001$ ), respectively. Those of TUNEL-positive cells were  $2.07 \pm$

$1.45\%$  of the VehG and  $0.38 \pm 0.17\%$  of the EdG at 24 h after HI insult ( $p < 0.05$ ),  $11.80 \pm 1.47\%$  of the VehG and  $4.72 \pm 1.66\%$  of the EdG at 48 h after HI insult ( $p < 0.001$ ), and  $5.33 \pm 3.68\%$  of the VehG and  $0.07 \pm 1.85\%$  of the EdG at 72 h after HI insult ( $p < 0.001$ ), respectively.

In addition, the concentration of 8-OHdG-positive cells in the EdG was significantly inhibited 24 and 48 h after HI insult (Fig. 6C). The percent concentrations of 8-OHdG-positive cells were  $1.44 \pm 0.60\%$  of the VehG and  $0.26 \pm 0.24\%$  of the EdG at 24 h after HI insult ( $p < 0.05$ ), and  $6.02 \pm 2.85\%$  of the VehG and  $1.17 \pm 0.73\%$  of the EdG at 48 h after HI insult ( $p < 0.001$ ), respectively. No significant difference was recognized 72 h after HI insult.

For the HPLC analysis, we used 4–6 samples for each group. As with the quantitative result, a significant difference was recognized 24 and 48 h after HI insult and not demonstrated at 72 h (Fig. 6D). The concentrations of 8-OHdG were  $0.36 \pm 0.04$  of the VehG and  $0.23 \pm 0.04$  of the EdG at 24 h after HI insult ( $p < 0.05$ ), and  $0.56 \pm 0.16$  of the VehG and  $0.34 \pm 0.06$  of the EdG at 48 h after HI insult ( $p < 0.001$ ) per  $1 \times 10^5$  deoxyguanosine, respectively.

## DISCUSSION

We demonstrated that in neonatal HIE model rats, the 8-OHdG levels significantly increased after 24–48 h of HI insult but decreased after 72 h, and the number of apoptotic cells significantly increased after 24–72 h of HI insult. 8-OHdG measurement may be a useful biochemical tool to evaluate DNA peroxidation and consider neuronal apoptosis within 48 h of the neonatal HIE event. Moreover, edaravone inhibited both apoptotic neuronal cell number and 8-OHdG expression after 24–48 h of HI.

Neuronal excitotoxicity due to high-dose glutamate in intersynaptic space is one of the major factors inducing neonatal brain injury (27). Another factor inducing neuronal death, mitochondrial dysfunction, has been known to lead to the apoptotic process (27). This phenomenon is likely to be observed in the immature brain. Oxidative stress is also thought to be one of the major factors to induce neuronal cell death of immature brain.

Among various oxidative stress pathways, DNA peroxidation correlates with apoptotic cell death expansion within 48 h after HI insult. In adult brain infarction, the 8-OHdG amount reportedly increases in the damaged cortex 24–48 h after ischemic insult and decreases at 72 h (28,29). These findings are compatible with the present results. The early stage change of poly(ADP-ribose) polymerase, a DNA repair-associated enzyme, is similar to that of 8-OHdG (30). The overexpression of poly(ADP-ribose) polymerase with cleaved caspase-3 appearance is recognized 24 h after HI, ends at 72 h, and the expression pattern is steep. Although in dividing cells the damaged DNA is enzymatically repaired with or without 8-OHdG production, post-mitotic cells such as neurons show low enzyme activity for DNA repair and are quite vulnerable to a HI insult (31). Neurons are especially sensitive to ROS, which cause DNA strand breaks (32). It is presumed that when excessive DNA

peroxidation occurs in neurons, the affected neurons easily fall into DNA repair dysfunction, DNA fragmentation and apoptosis in burst. Our data indicate that DNA peroxidation is the key factor in the drastic increase of neuronal apoptosis at the acute stage of neonatal HIE.

Interestingly, both the number of 8-OHdG-positive cells and the 8-OHdG concentration decreased 72 h after HI insult, whereas the number of apoptotic cell deaths did not show an obvious decrease. The apoptotic cells after 48 h of HI insult can be involved in other factors, such as inflammatory cytokines, in relation to microglial activation. IL-1 $\beta$  (IL-1 $\beta$ ) expression gradually increases during 1–14 d after HI insult, and erythropoietin inhibits microglial activation and reduces brain damage (33). On the contrary, under the condition of hypoglycemia and hypoxemia by HI insult, IL-1 $\beta$  directly or indirectly attacks neurons and leads to neuronal cell death (34). Moreover, IL-1 $\beta$  induces microglial activation in cortex 3–4 d after HI in the neonatal rat (35,36). This cycle causes many neuronal cell deaths and expansion of lesions. Although we could not detect IL-1 $\beta$  expression, we can speculate that the different pathophysiological changes may take place between events within and after 48 h of HI insult. DNA peroxidation plays an important role in forming brain lesions in the early stage of the neuronal death cycle.

Our data demonstrated that edaravone therapy for neonatal HIE inhibits 8-OHdG overexpression at the acute stage, an effect which appears within 48 h after HI insult. In this connection, it is very interesting to consider DNA peroxidation from the standpoint of oxidation stress pathology. It is already reported that 2-d edaravone treatment is more effective than 5-d or 10-d treatment in this model (26). We also showed that 8-OHdG overexpression correlated to apoptotic neuronal cell death within 48 h after HI insult.

The edaravone pharmacological mechanism serves to inhibit not only hydroxyl radicals, but also nitrogen oxide (NO) *via* production of peroxy-nitrate radicals (37,38). Although NO contributes to the brain damage, it also has neuroprotective roles, promoting vasodilation, and inhibiting microvascular aggregation (39). On the other hand, edaravone may cause some adverse effect with chronic administration. Therefore, in clinical application, the dosing period should be clinically within 2 wk. It is known that edaravone inhibits lipid peroxidation in neonatal HIE rat model (21). Here, we revealed that edaravone also inhibits DNA peroxidation. Edaravone may be a useful agent as a scavenger inhibiting both lipid and DNA peroxidation. Moreover, this study indicates that edaravone treatment should be started immediately within 48 h after HI insult.

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