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## ORIGINAL ARTICLE

### Effects of Graded Hypothermia on Hypoxic-ischemic Brain Damage in the Neonatal Rat

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**Key words:** hypoxia-ischemia; brain; rat; hypothermia; heat shock protein 72

**Objective** To investigate the effect of graded hypothermia on neuropathologic alterations of neonatal rat brain after exposed to hypoxic-ischemic insult at 37°C, 33°C, 31°C, and 28°C, respectively, and to observe the effect of hypothermia on 72-kDa heat shock protein (HSP72) expression after hypoxic-ischemic insult.

**Methods** Seven days old Wistar rats were subjected to unilateral common carotid artery ligation followed by exposure to hypoxia in 8% oxygen for 2 hours at 37°C, 33°C, 31°C, and 28°C, respectively. The brain temperature was monitored indirectly by inserting a mini-thermocouple probe into the temporal muscle during hypoxia. After hypoxia-ischemia their mortality was assessed. Neuronal damage was assessed with HE staining 72 hours after hypoxia. HSP72 expression at 0.5, 24, and 72 hours of recovery was immunohistochemically assessed using a monoclonal antibody to HSP72.

**Results** Hypoxia-ischemia caused 10.5% (2/19) of mortality in rat of 37°C group, but no death occurred in 33°C, 31°C or 28°C groups. HE staining showed neuropathologic damage was extensive in rats exposed to hypoxia-ischemia at 37°C (more than 80.0%). The incidence of severe brain damage was significantly decreased in 33°C (53.3%) and 31°C groups (44.4%), and no histologic injury was seen in the 28°C group of rats. Expression of HSP72 was manifest and persistent in the rat brain of 37°C group, but minimum in the rat brain of 28°C group.

**Conclusion** Mild and moderate hypothermia might prevent cerebral visible neuropathologic damage associated with hypoxic-ischemic injury by decreasing stress response.

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**H**YPOXIC-ISCHEMIC encephalopathy (HIE) is an important cause of neonatal death and long-term disability.<sup>1</sup> It has been known that hypothermia provides neuroprotective effects on

hypoxic-ischemic brain injury in adult and neonatal animal models,<sup>2, 3</sup> and significantly reduces the risk of death or disability in infants with moderate or severe HIE.<sup>4</sup> However, its key mechanism still remains unclear.

Heat shock protein 72 (HSP72) is a member of a family of stress-inducible proteins.<sup>5</sup> The expression of HSP72 in the central nervous system is induced by hypoxia-ischemia.<sup>6</sup> Our previous study has been shown that the

induction of HSP72 is associated with protection of neuronal cell against the neonatal hypoxic-ischemic insult.<sup>7</sup> In this study, we explored the effect of intra-hypoxia hypothermia at 37°C, 33°C, 31°C, and 28°C, and chronologically assessed the effects of graded hypothermia on HSP72 expression by immunohistochemical technique using monoclonal antibodies to 72-kDa HSP.

## MATERIALS AND METHODS

### Experimental model and experimental design

This study was performed in accordance with the guidelines of the Experimental Animal Center of Miyazaki Medical College. Wistar rats with dated pregnancy, purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan), were individually housed in cages and fed on a standard laboratory diet. Offspring, delivered vaginally, were maintained with their dams until the day of experimentation. All experimental procedures were in accordance with guidelines of the institutional animal care and use committee.

Seven-day-old rats, weighing  $13.9 \pm 1.7$  g, were used in this study. The experimental procedures were performed as previously described.<sup>7</sup> Briefly, each rat was lightly anesthetized with ether. The left common carotid artery was doubly ligated with 5-0 surgical silk and transected. After the surgical procedure, the rats were allowed to recover for 1 to 2 hours without their dams, then placed in a chamber. The temperature in the chamber was maintained at 37°C, 33°C, 31°C or 28°C, respectively. Thirty minutes later, the rats were exposed to a humidified hypoxic gas mixture (8% oxygen with 92% nitrogen) for 2 hours. The oxygen content in the chamber was monitored by an oxygen monitor (OMD-1000, Aika Medical Co., Matsuda City, Japan) during hypoxia. The rectal temperature, taken as an indicator of core temperature, was measured with a liner-type probe inserted into rectum for a depth of 5 mm. The brain temperature was indirectly monitored by inserting a needle-type thermocouple probe into the left temporal muscle. The pups were gently swaddled to minimize movement and possible dislocation of the probes. They were allowed to recover for 30 minutes in room air after hypoxia, and then, returned to their dams and reared until sacrificed. After hypoxia-ischemia their mortality was examined.

### Neuropathologic examination

The rats were sacrificed at 72 hours of recovery (19 rats in 37°C group, 15 rats in 33°C group, 9 rats in 31°C group, and 6 rats in 28°C group). After decapitation, the brains were quickly removed to detect the gross brain abnormalities, and cut into 2 blocks (about 3 mm in thickness).

The blocks were immersion-fixed in a solution of ethanol and acetic acid (19:1) for 12 hours, then dehydrated in ethanol, and embedded in paraffin. Finally, 6- $\mu$ m-thick coronal sections containing dorsal hippocampus were prepared for hematoxylin and eosin staining. Neuronal histological damage was classified on a 3-point scale according to the percentage of neurons lost in the area, as described in our previous study: Grade 1 was mild neuronal damage, <25% neuronal loss; Grade 2 was moderate neuronal damage, 25%-50% neuronal loss; and Grade 3 was severe neuronal damage, >50% neuronal loss.<sup>7</sup> Cerebral structures scored were the cortex and the hippocampus (CA1, CA3 subfield and the dentate gyrus).

### Immunohistochemical staining for HSP72

Other 42 rats were used (11 rats in 37°C group, 12 rats in 33°C group, 10 rats in 31°C group, and 9 rats in 28°C group). These rats were sacrificed by perfusion-fixation at 0.5, 24, and 72 hours after hypoxia-ischemia. In 37°C group, 0.5-hour cohort had 3 rats, 24- and 72-hour cohorts included 4 rats, respectively. In 33°C group, there were 4 rats in each cohort. In 31°C group, 0.5-, 24- and 72-hour cohorts included 3, 4, and 3 rats, respectively. And in 28°C group, there were 3 rats in each cohort. Briefly, at each recovery period, rats were intraperitoneally anesthetized with pentobarbital and then perfused transcardially with normal saline solution followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). After this perfusion fixation, the brains were removed, immersed in the fixative for 6 hours, and sequentially transferred into an ascending order of sucrose concentrations (10%, 20%, and 30%) in 0.1 mol/L phosphate buffer during 2 days at 4°C. Frozen sections (6 to 10  $\mu$ m in thickness) were preserved at -20°C until HSP72 immunostaining. The immunohistochemical technique used to detect HSP72 was essentially described by Vass *et al.*<sup>5</sup> Sections were blocked with 10% normal horse serum for 2 hours followed by incubation with mouse anti-HSP72 monoclonal antibody (Amersham Pharmacia Biotech AB, Uppsala, Sweden) diluted 1:200 in buffer containing 10% normal horse serum and 0.3% Triton X-100 at 4°C overnight. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in 100% methanol for 20 minutes. Sections were then incubated with biotinylated secondary antibody at 1:200 dilution for 3 hours. The immunoreaction was visualized in the presence of 0.05% 3, 3'-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide for 5 to 7 minutes. The grading scale used for HSP72 immunoreactivity has been reported previously: weak, positive neurons seen in <25% of the surface area; medium, 25%-50%; and strong, >50%.<sup>7</sup>

### Statistical analysis

The collected data were processed with SPSS 10.0. The difference in temporal muscle temperature intra-hypoxia were evaluated by analysis of variance (ANOVA). The differences in mortality and incidence of neuronal damage in rat exposed to hypoxia at 33°C, 31°C, and 28°C were compared with those exposed to hypoxia at 37°C using Fisher's exact test. A  $P$  value  $<0.05$  was considered significant.

## RESULTS

### Mortality

The mortality was 10.5% (2/19) in rats exposed to hypoxia at 37°C. There were no deaths either in 33°C, 31°C or 28°C groups. The mortality had no significant difference between 37°C group and 33°C, 31°C as well as 28°C groups ( $P>0.05$ ).

### Profiles of intra-hypoxic temperature

When the rats suckling with their dams at Animal Center, the temperature of temporal muscle was  $34.5^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$ , the rectal temperature was  $33.8^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ . Thirty minutes after placement in the incubator, temporal muscle temperature of the rat in 37°C group was  $37.4^{\circ}\text{C} \pm 0.9^{\circ}\text{C}$ ; in 33°C group was  $36.4^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ ; in 31°C group was  $35.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ ; in 28°C group was  $33.3^{\circ}\text{C} \pm 1.2^{\circ}\text{C}$ , respectively. During hypoxia, the profiles of temporal muscle temperature are shown in Figure 1. During hypoxia, the temporal muscle temperature of rats was significantly higher in 37°C group than that in 33°C group ( $P<0.05$ ).

### Neuropathologic injury

Neuropathologic injury after hypoxia-ischemia underlying

different temperature are depicted in Figure 2. More than 80.0% of rats exposed to hypoxia at 37°C showed moderate to severe damage in the ipsilateral cerebral cortex and hippocampus. Neuropathologic injury declined to 53.3% when the rats exposed to hypoxia-ischemia at 33°C. The incidence of brain damage was dramatically reduced in 31°C group (44.4%) and no obvious damage occurred in brain of the rats at 28°C. Severe brain damage in cerebral cortex was significantly decreased in 33°C group as compared with 37°C ( $P<0.05$ ).

### HSP72 expression

HSP72 expression varied chronologically and topographically with the different temperature (Fig. 3).

In the 37°C group, HSP72 expression was induced in the cerebral cortex, hippocampal CA1, CA3, and dentate gyrus ipsilateral to ligation at 30 minutes of recovery. Expression of HSP72 became evident in those regions at 24 hours. After 72 hours, HSP72 was persistently expressed in those regions.

In the 33°C group, HSP72 was weakly induced in the cerebral cortex, dentate gyrus at 30 minutes of recovery. At 24 hours, 2 of 4 rats exhibited intense HSP72 induction in the cerebral cortex and hippocampus. After 72 hours, expression of HSP72 was still intense in the cerebral cortex and hippocampal CA3, but markedly decreased in the dentate gyrus.

In the 31°C group, the expression of HSP72 was too weak to be detected in the cerebral cortex and dentate gyrus at 30 minutes of recovery. At 24 hours, the expression of HSP72 was less intense in the cerebral cortex and hippocampus compared with that in the 33°C group. After 72 hours, HSP72 expression showed intensive induction in

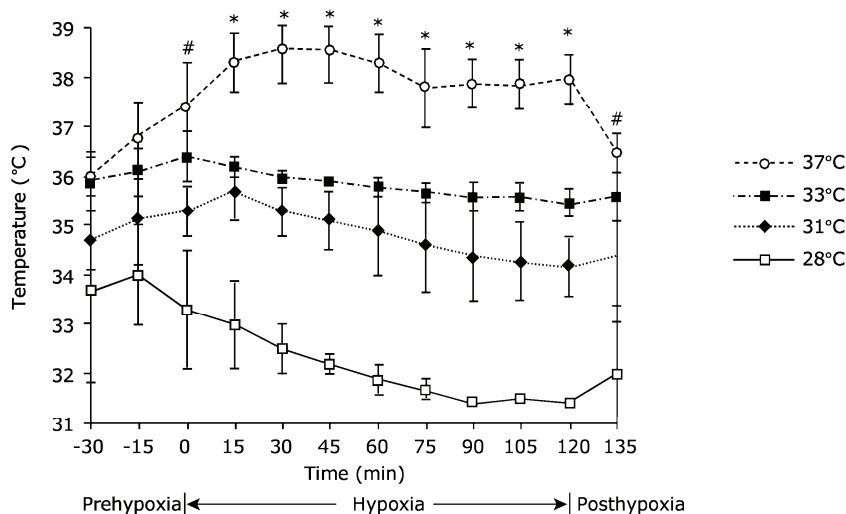


**Figure 1.** Profiles of intra-hypoxic temporal muscle temperature of 7-day-old rat at 37°C, 33°C, 31°C, and 28°C respectively.

$n=5$  in 37°C and 33°C groups;  $n=3$  in 31°C and 28°C groups.

\* $P<0.05$ , # $P<0.01$  compared with 33°C group.

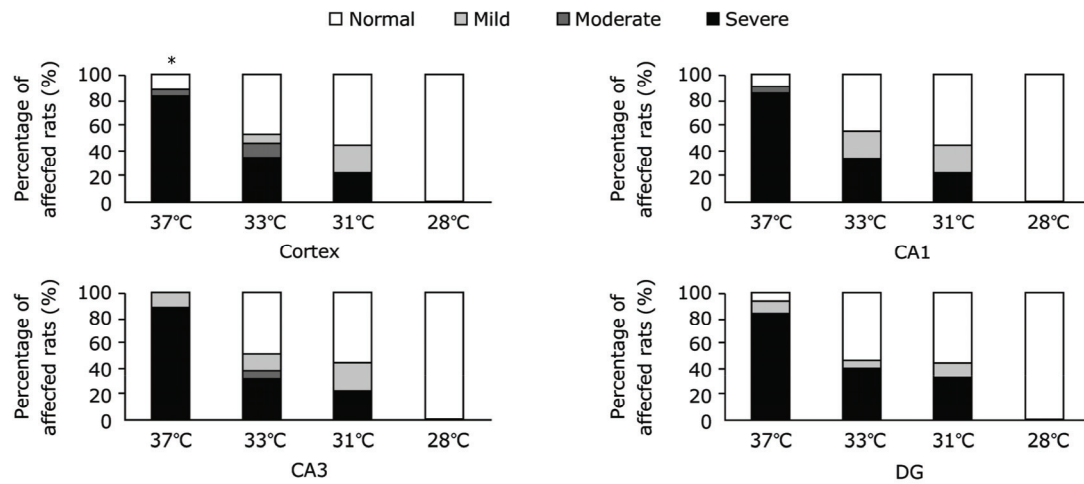


**Figure 2.** Comparison of neuronal damage examined with H&E staining after 72 hours of recovery in the cerebral cortex and the hippocampus [CA1, CA3, and dentate gyrus (DG)] at 37°C ( $n=17$ ), 33°C ( $n=15$ ), 31°C ( $n=9$ ), and 28°C ( $n=6$ ) hypoxia using 3-point scale as shown in the paper.

\* $P<0.05$  compared with severe brain damage of cerebral cortex in 33°C group.

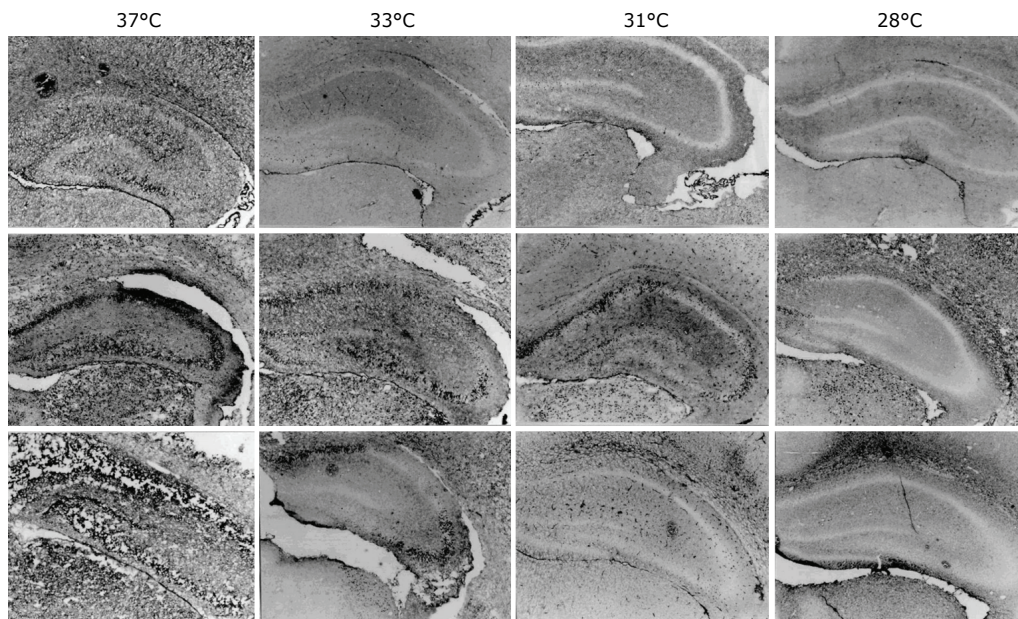


**Figure 3.** Typical expression of HSP72 in the brains of 7-day-old rats at 37°C, 33°C, 31°C, and 28°C of hypoxia in the cerebral cortex and hippocampus after 0.5 hour (the 1st line), 24 hours (the 2nd line), and 72 hours (the 3rd line) of recovery detected with HSP72 immunohistochemical staining. DAB×36

the cerebral cortex and hippocampus only in 1 rat.

In the 28°C group, the induction of HSP72 was only detected in the dentate gyrus in 2 of 3 rats at 30 minutes. At 24 hours, only 1 rat showed mild expression of HSP72 in the cerebral cortex, hippocampal CA1 and CA3, with weakly induction in the dentate gyrus. After 72 hours, no rats showed expression of HSP72 in the brain.

## DISCUSSION

In this study, measurement of neonatal rat brain

temperature is a critical issue. Direct measurement of the brain temperature causes small trauma to the brain.<sup>8</sup> To avoid direct trauma to the brain, temperature of the temporal muscle was recorded and external monitoring of head temperature can provide a reliable indirect estimate of brain temperature in the course of an ischemic insult.<sup>8</sup> We found that the mortality was decreased when the rats were exposed to hypoxia at 33°C, 31°C, and 28°C.

After hypoxia-ischemia insult, the mechanism of neuronal cell death in animals and humans includes neuronal necrosis and apoptosis.<sup>9</sup> The intensity of the initial insults

may determine the mode of death, with severe injury resulting in necrosis and milder insults resulting in apoptosis.<sup>9</sup> In our study, neuropathologic damage in the 37°C was manifest. The incidence of brain damage was much less either exposed to hypoxia at 33°C or 31°C. No obvious damage was observed at 28°C. This study demonstrates that moderate hypothermia may be effective in preventing the neuropathologic changes caused by hypoxic-ischemic damage in the neonatal rat.

Although moderate hypothermia is one of the most effective techniques available for reducing hypoxic-ischemic injury, its key mechanism still remains unclear. In this study, we found that intra-hypoxic hypothermia did decrease the expression of HSP72. This is consistent with a previous report that HSP expression is indeed inhibited by moderate hypothermia.<sup>10</sup> Olsson *et al*<sup>11</sup> found that intra-ischemic hypothermia prevented neuronal cell damage without noticeable expression of HSP70. Terao *et al*<sup>12</sup> reported that post-ischemic hypothermia did not increase even decrease the upregulation of HSP70 mRNA expression. We hypothesize that moderate intra-hypoxic hypothermia protects the neonatal neuronal necrosis by decreasing the stress response after hypoxia-ischemia insult.

In conclusion, mild and moderate hypothermia may prevent cerebral visible neuropathologic damage associated with hypoxic-ischemic injury by decreasing stress response.

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