

# Susceptibility of rat hippocampal neurons to hypothermia during development

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**Purpose:** This study evaluated the extent of damage due to hypothermia in the mature and immature brain.

**Methods:** Hippocampal tissue cultures at 7 and 14 days *in vitro* (DIV) were used to represent the immature and mature brain, respectively. The cultures were exposed at 25°C for 0, 10, 30, and 60 minutes (n=30 in each subgroup). Propidium iodide fluorescent images were captured 24 and 48 hours after hypothermic injury. Damaged areas of the cornu ammonis 1 (CA1), CA3, and dentate gyrus (DG) were measured using image analysis.

**Results:** At 7 DIV, the tissues exposed to cold injury for 60 minutes showed increased damage in CA1 ( $P<0.001$ ) and CA3 ( $P=0.005$ ) compared to the control group at 48 hours. Increased damage to DG was observed at 24 ( $P=0.008$ ) and 48 hours ( $P=0.011$ ). The 14 DIV tissues did not demonstrate any significant differences compared with the control group, except for the tissues exposed for 30 minutes in which DG showed less damage at 48 hours than the control group ( $P=0.048$ ). In tissues at 7 DIV, CA1 ( $P=0.040$ ) and DG ( $P=0.013$ ) showed differences in the duration of cold exposure.

**Conclusion:** The immature brain is more vulnerable to hypothermic injury than the mature brain.

**Key words:** Hippocampus, Hypothermia, Development

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## Introduction

Organotypic slice culture has several advantages, including preservation of the gross cytoarchitecture and the possibility of long-term observation and manipulation, and is frequently used to examine physiologic or pathologic processes<sup>1</sup>. The hippocampus is a major component of the brain. It belongs to the limbic system and plays important roles related to storing new memories. The hippocampus is the most susceptible brain region to various injuries. It has a simple anatomic structure and is easy to manipulate. As a result, organotypic hippocampal slice culture is commonly used to study neuronal cell death, neuroprotection, and synaptic plasticity<sup>2</sup>.

Newborn infants are prone to developing hypothermia<sup>3</sup>, which can result in metabolic acidosis, hypoxia, hypoglycemia, and multiorgan damage (e.g., pulmonary hemorrhage, renal failure, and disseminated intravascular coagulopathy) in neonates<sup>4,5</sup>. Hypothermia can be fatal, particularly in preterm infants. The mortality rate is approximately 10%, and approximately 10% of the survivors show evidence of brain damage<sup>3</sup>.

The immature brain is more vulnerable to unconjugated bilirubin, hypoxic-ischemic damage and acute hypoglycemia than the mature brain<sup>6-8</sup>. However, some studies have shown that the immature brain is more resistant to several types of injury, including N-methyl-D-aspartate (NMDA) toxicity and glutamate- and seizure-induced hippocampal damage, than the mature brain<sup>9-11</sup>. Although hypothermia is known to cause brain damage,

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few studies have investigated differences between the mature and immature brain as a result of hypothermic injury<sup>12,13</sup>. According to previous study, 7 days *in vitro* (DIV) hippocampal tissues are representative of the immature brain, and 14 DIV hippocampal tissues are representative of the mature brain<sup>1</sup>. Prominent damage to hippocampal tissues and loss of hippocampal neurons have been described in several experiments evaluating hypothermic injury<sup>13</sup>. The present study measured the damage to immature and mature hippocampal tissues following hypothermic exposure.

## Materials and methods

### 1. Organotypic slice culture

Organotypic slice cultures of the hippocampus were prepared from a total of 12 7-day-old Sprague Dawley rats (DooYeol Biotech, Seoul, Korea) using the Stoppini method<sup>14</sup>. The rats were stabilized for 24 hours prior to the experiment and decapitated using scissors. The heads were sterilized with 70% alcohol, and the hippocampi were quickly removed and placed in ice-cold Gey's Balanced Salt Solution (GBSS, Sigma Aldrich, St Louis, MO, USA). The hippocampi were sliced (450  $\mu$ m thick) with a tissue slicer (Stoelting Co., Wood Dale, IL, USA). Millicell culture inserts (Millipore, Ireland, 5 slices per insert) were placed into 6-well plates (SPL Life Sciences Co., Pocheon, Korea) with 1 mL of Gahwiler media<sup>15</sup> per well. The slice culture media consisted of 25% Hank's Balanced Salt Solution (HBSS, GibcoBRL/Life Technologies, USA), 25% heat inactivated horse serum (Hyclone, Logan, UT, USA), 50% Basal Medium Eagle (BME, GibcoBRL/Life Technologies, Grand Island, NY, USA), 6.5 mg/mL glucose, and 200 mM glutamax-I (GibcoBRL/Life Technologies). The slices were cultured at 37°C with 5% CO<sub>2</sub> in a humidified incubator (MCO175, Sanyo, Tokyo, Japan). The media was changed twice per week, and all procedures were performed aseptically in a horizontal flow hood.

### 2. Hypothermic injury

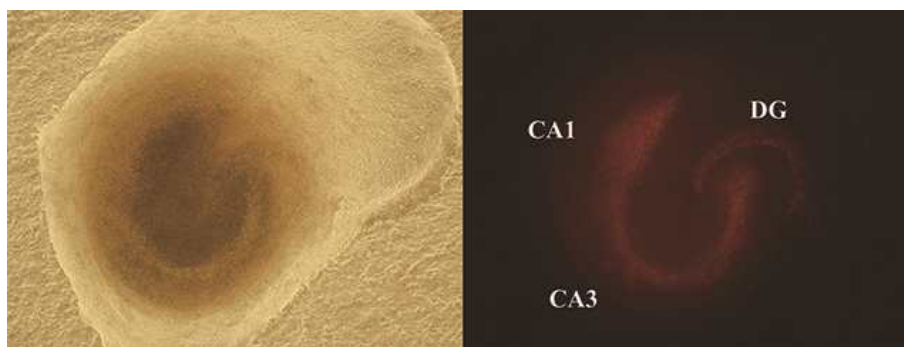
The slices were divided into two groups, 7 DIV and 14 DIV. The tissues in each group were maintained for 0, 10, 30, and 60 minutes at 25°C (0 minute, control; n=30 slices per subgroup) and rewarmed at 37°C with 5% CO<sub>2</sub> in a humidified incubator. Deteriorating tissues were excluded prior to induction of hypothermic injury.

### 3. Image analysis

Fluorescent images of the slices were captured under an inverted microscope (IX 71, Olympus, Japan) 24 and 48 hours after hypothermic injury. At 72 hours after hypothermic injury, the slices were exposed to 2 mM NMDA in serum free medium for 30 minutes at 37°C with 5% CO<sub>2</sub> in a humidified incubator. The slices were cultured with 1 mL of serum free media with 1  $\mu$ g propidium iodide (PI; Sigma Aldrich), and fluorescent images were obtained 96 hours after hypothermic injury. The images were analyzed with Image J (version 1.47c, National Institutes of Health, Bethesda, MD, USA). We defined threshold as amount of fluorescent area and a single researcher measured the individual areas of the cornu ammonis 1 (CA1), CA3, and dentate gyrus (DG) above the threshold (Fig. 1). After NMDA processing, the images were assumed to include the total area of the hippocampal tissue. We estimated the extent of damage 24 and 48 hours after hypothermic injury. The tissues in the 7 DIV and 14 DIV categories were compared, and the cold exposure groups (10, 30, and 60 minutes) in each category were compared with the control group. The ratio of damaged area at 24 and 48 hours to the total damaged area after NMDA treatment was calculated.

### 4. Statistical analysis

We evaluated brain injury by comparing tissues at 7 DIV with tissues at 14 DIV using the Student *t* test and Mann-Whitney *U* test. Repeated analysis of variance was used to assess the extent of injury based on the length of time after hypothermic injury and rewarming. Differences were considered significant when *P* < 0.05. All analyses were performed using IBM SPSS ver. 18.0



**Fig. 1.** Propidium iodide fluorescence staining with bright and dark fields. The areas of cornu ammonis 1 (CA1), CA3, and dentate gyrus (DG) were measured individually.

(IBM Co., Armonk, NY, USA).

## Results

### 1. Cultured hippocampal tissues at 7 DIV

#### 1) Cold exposed groups compared with the control group

Damage to CA1 in the hippocampal tissue at 7 DIV was  $0.00 \pm 0.00$  and  $0.01 \pm 0.02$  in the control group at 24 and 48 hours, respectively (mean  $\pm$  standard deviation,  $n=30$ , measured area/total area). In the tissues exposed to cold for 10 minutes, the damage was  $0.00 \pm 0.00$  and  $0.30 \pm 1.46$  at 24 and 48 hours. In the tissues exposed for 30 minutes, the damage was  $0.04 \pm 0.20$  and  $0.32 \pm 1.18$  at 24 and 48 hours. In the tissues exposed for 60 minutes, the damage was  $0.11 \pm 0.35$  and  $3.21 \pm 9.52$  at 24 and 48 hours. There were no significant differences between the tissues exposed to cold for 10 minutes and the control group ( $P=0.169$  and  $P=0.363$ ) and 30 minutes and the control group ( $P=0.361$  and  $P=0.307$ ) at 24 and 48 hours. The tissues exposed for 60 minutes showed increased damage compared to the control group at 48 hours ( $P<0.001$ ).

Damage to CA3 in the hippocampal tissue at 7 DIV was  $0.00 \pm 0.01$  and  $0.00 \pm 0.01$  in the control group at 24 and 48 hours, respectively. In the tissues exposed to cold for 10 minutes, the damage was  $0.00 \pm 0.00$  and  $0.00 \pm 0.00$  at 24 and 48 hours. In the tissues exposed for 30 minutes, the damage was  $0.00 \pm 0.0$  and  $0.03 \pm 0.10$  at 24 and 48 hours. In the tissues exposed for 60 minutes, the damage was  $0.01 \pm 0.02$  and  $1.20 \pm 3.97$  at 24 and 48 hours. There were no significant differences between the tissues

exposed to cold for 10 minutes and the control group ( $P=0.185$  and  $P=0.052$ ) at 24 and 48 hours and 30 minutes and the control group ( $P=0.056$  and  $P=0.852$ ) at 24 and 48 hours. The tissues exposed for 60 minutes showed a significant increase in damage compared to the control group at 48 hours ( $P=0.005$ ).

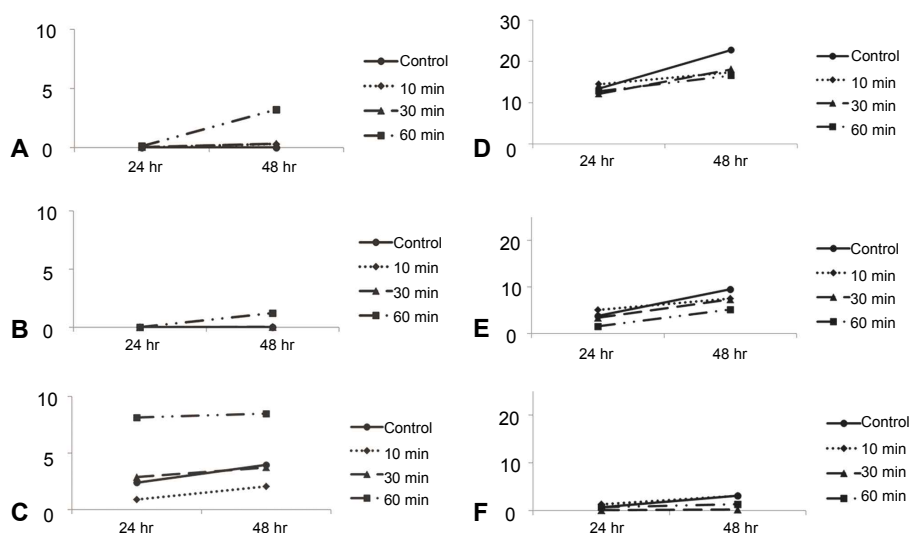
Damage to DG in the hippocampal tissue at 7 DIV was  $2.38 \pm 8.46$  and  $3.95 \pm 9.22$  in the control group at 24 and 48 hours, respectively. In the tissues exposed to cold for 10 minutes, the damage was  $0.88 \pm 2.26$  and  $2.05 \pm 3.66$  at 24 and 48 hours. In the tissues exposed for 30 minutes, the damage was  $2.87 \pm 8.05$  and  $3.72 \pm 9.28$  at 24 and 48 hours. In the tissues exposed for 60 minutes, the damage was  $8.14 \pm 11.84$  and  $8.48 \pm 10.80$  at 24 and 48 hours. The tissues exposed for 10 minutes ( $P=0.585$  and  $P=0.538$ ) and 30 minutes ( $P=0.077$  and  $P=0.153$ ) did not show significant differences compared with the control group at 24 and 48 hours. The tissues exposed for 60 minutes showed a significant increase in damage compared to the control group at 24 and 48 hours ( $P=0.008$  and  $P=0.011$ ). In summary, the tissues exposed for 60 minutes showed more damage to the CA1 and CA3 than the control group at 48 hours. Damage to DG was evident at 24 and 48 hours (Fig. 2).

#### 2) Changes in damage over time

Damage to CA1 in the control group and tissues exposed for 10, 30, and 60 minutes in the hippocampal tissues at 7 DIV showed increased damage between the 24 and 48 hours time points after hypothermic injury ( $P=0.049$ ). However, there was no significant increase in damage to CA3 and DG over time (CA3,  $P=0.107$ ; DG,  $P=0.710$ ) (Fig. 2)

#### 3) Damage and duration of exposure

On CA1 of 7 DIV, there were significant difference between



**Fig. 2.** Damage in the control and experimental groups over time following cold injury. Figs. A–C represents the tissues in the 7 days *in vitro* groups; CA1 (A), CA3 (B), DG (C). Figs. D–F represents the tissues in the 14 DIV groups; CA1 (D), CA3 (E), DG (F). CA1, cornu ammonis 1; DG, dentate gyrus.

control group, 10, 30, and 60 minutes exposed groups ( $P=0.040$ ). On CA3 of 7 DIV, there were no significant difference between control group, 10, 30, and 60 minutes exposed groups ( $P=0.066$ ). On DG of 7 DIV, also significant difference was observed between control group, 10, 30, and 60 minutes exposed groups ( $P=0.013$ ).

In the CA1 category, there were significant differences in damage between the control group, and the group of tissues exposed for 10, 30, and 60 min ( $P=0.040$ ). In the CA3 category, there were no significant differences in damage between the control group, and the group of tissues exposed for 10, 30, and 60 minutes ( $P=0.066$ ). Significant differences were observed between the control group, tissues exposed for 10, 30, and 60 minutes in the DG category ( $P=0.013$ ) (Fig. 2).

## 2. Cultured hippocampal tissues at 14 DIV

### 1) Cold exposure groups compared with the control group

Damage to CA1 in the hippocampal tissue at 14 DIV was  $13.40\pm 17.34$  and  $22.75\pm 22.46$  in the control group at 24 and 48 hours, respectively. In the tissues exposed to cold for 10 minutes, the damage was  $14.48\pm 32.13$  and  $17.41\pm 26.89$  at 24 and 48 hours. In the tissues exposed for 30 minutes, the damage was  $12.12\pm 19.03$  and  $18.09\pm 22.20$  at 24 and 48 hours. In the tissues exposed for 60 minutes, the damage was  $12.76\pm 20.03$  and  $16.54\pm 18.93$  at 24 and 48 hours. There were no significant differences between the control group and the tissues exposed for 10 minutes ( $P=0.849$  and  $P=0.367$ ), 30 minutes ( $P=0.767$  and  $P=0.387$ ), and 60 minutes ( $P=0.892$  and  $P=0.267$ ) at 24 and 48 hours.

Damage to CA3 in the hippocampal tissue at 14 DIV was  $3.75\pm 9.69$  and  $9.48\pm 15.95$  in the control group at 24 and 48 hours, respectively. In the tissues exposed to cold for 10 minutes, the damage was  $5.06\pm 15.89$  and  $7.47\pm 21.40$  at 24 and 48 hours. In the tissues exposed for 30 minutes, the damage was  $3.34\pm 11.40$  and  $7.31\pm 16.36$  at 24 and 48 hours. In the tissues exposed for 60 minutes, the damage was  $1.49\pm 3.36$  and  $5.13\pm 10.18$  at 24 and 48 hours. There were no significant differences between the control group and the tissues exposed for 10 minutes ( $P=0.656$  and  $P=0.648$ ), 30 minutes ( $P=0.868$  and  $P=0.575$ ), and 60 minutes ( $P=0.302$  and  $P=0.251$ ) at 24 and 48 hours.

Damage to DG was  $0.64\pm 3.25$  and  $3.08\pm 10.43$  in the control group at 24 and 48 hours, respectively. At 24 and 48 hours, the damage was  $1.31\pm 4.16$  and  $3.05\pm 7.50$  in the tissues exposed to cold for 10 minutes,  $0.05\pm 0.10$  and  $0.18\pm 0.37$  in the tissues exposed for 30 minutes, and  $0.69\pm 2.09$  and  $1.30\pm 2.88$  in the tissues exposed for 60 minutes. There was no significant difference between the control group and the tissues exposed for 10 min ( $P=0.443$  and  $P=0.988$ ) and 60 minutes ( $P=0.954$  and  $P=0.446$ ). The tissues exposed for 30 minutes showed significantly less damage at 48 hours than the control group ( $P=0.361$  and  $P=0.048$ ) (Fig. 2).

### 2) Changes in damage over time

Regarding CA1, CA3, and DG, all of the tissues at 14 DIV showed significant deterioration between the 24 and 48 hours time points (CA1,  $P<0.001$ ; CA3,  $P<0.001$ ; DG,  $P=0.025$ ). However, these results are not relevant because the tissues at 14 DIV were not significantly damaged compared with control group (Fig. 2).

### 3) Damage and duration of exposure

There were no significant differences in damage between the control group and the groups of tissues exposed to cold for 10, 30, and 60 minutes in all the categories of CA1, CA3, and DG at 14 DIV (CA1,  $P=0.182$ ; CA3,  $P=0.558$ ; DG,  $P=0.321$ ). All of these results were time compensated. In summary, there were no statistically significant differences in the duration of hypothermic exposure in the 7 DIV or 14 DIV groups (Fig. 2).

## Discussion

Cold injury in neonates and infants can cause death, multiple systemic dysfunction, and neurodevelopmental disturbances. Culic<sup>5)</sup> suggested that the infant brain is extremely vulnerable to deep hypothermia. The aim of this study was to determine the effects of hypothermic injury on the immature and mature brain. Neurons at 7 and 14 DIV represented the immature and mature brain, respectively. In hippocampal tissues at 7 DIV, the CA1 and CA3 showed an increase in damage after 60 minutes of exposure compared to the control group at 48 hours. DG showed increased damage at 24 and 48 hours. Hippocampal tissue at 14 DIV demonstrated no significant differences compared with the control group, except for the tissues exposed for 30 minutes in which decreased damage was shown compared to the control group at 48 hours. In general, the mature neurons showed less damage as a result of hypothermic injury than the immature neurons compared to the control groups, although direct comparisons are limited. According to Warren et al.<sup>13)</sup>, hippocampal neuronal injury from hypothermia and rewarming is primarily related to intracellular  $Ca^{2+}$  accumulation mediated by NMDA receptors. Neuronal expression of different glutamate receptor subtypes is highly dependent on developmental stage, and responses to glutamate differ between immature and mature neurons<sup>9,10,16)</sup>. These studies could explain the increased damage in immature neurons in response to hypothermic injury, as shown in this study. The effects of hypothermic injury on the immature brain compared to the mature brain are in contrast to the results of previous studies based on NMDA toxicity and glutamate- and seizure-induced hippocampal damage<sup>9-11)</sup>. Additional studies should be performed to evaluate the possible involvement of different glutamate receptor subtypes or unknown mechanisms.

In a clinical setting, induced mild hypothermia (brain temperature of  $32^{\circ}C-34^{\circ}C$ ) is applied for therapeutic purposes to treat

neonatal hypoxic ischemic brain damage<sup>17,18)</sup> or adult cardiac arrest<sup>19)</sup>. Further studies are needed to determine the most effective, safe target temperatures and duration of exposure on long-term neurodevelopmental outcomes, particularly in neonates because the immature brain is vulnerable to hypothermia, as indicated in the present study. Body temperature should be carefully monitored and hypothermia should be avoided at birth or during invasive procedures.

Although mature hippocampal tissues experienced gradual damage over time compared to the control group, immature hippocampal tissues only showed statistical significance in damage to CA1.

In this study, the cold exposure times were 0, 10, 30, and 60 minutes. There were statistical differences in the extent of damage to CA1 and DG in tissues at 7 DIV exposed for 0, 10, 30, and 60 minutes. Significant differences between the duration of cold exposure were not observed in tissues at 14 DIV. Although 25°C is considered profound hypothermia, any exposure to hypothermia can be harmful, and the duration of exposure affects the immature brain in particular.

In conclusion, hypothermia can damage systemic organs and the brain. Because the immature brain is more vulnerable to hypothermic injury than the mature brain, hypothermia should be carefully avoided, particularly in neonates. Studies on therapeutic hypothermia, effective temperatures, and duration of treatment should be performed.

Future studies could include evaluating the effects of hypothermia in a therapeutic range (32°C–34°C) and comparing the effects of repetitive hypothermic exposure with a single exposure on the brain.

## Conflict of interest

No potential conflict of interest relevant to this article was reported.

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