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Effect of Hypothermia on Brain Cell Membrane Function and Energy Metabolism after Transient Global Hypoxia-Ischemia in the Newborn Piglet

This study was done to determine the effects of hypothermia on brain cell membrane function and energy metabolism after transient hypoxia-ischemia (HI) in the newborn piglet. Cerebral HI was induced by temporarily complete occlusion of bilateral common carotid arteries with surgical clips and simultaneous breathing with 8% oxygen for 30 min, followed by release of carotid occlusion and normoxic ventilation for 4 hr. Rectal temperature was maintained between 38.0 and 39.0℃ in normothermic groups, and between 34.0 and 35.0℃ in hypothermic groups for 4 hr after HI. During HI, heart rate, glucose and lactate level in the blood and cerebrospinal fluid increased, and base excess, pH and blood pressure decreased significantly in both normothermic and hypothermic groups. After HI, these abnormalities returned to normal in normothermic group, but lactic acidosis persisted in hypothermic group. Decreased cerebral Na^+,K^+-ATPase activity and increased lipid peroxidation products, indicative of HI-induced brain injury, were more profound in hypothermic group than in normothermic group. Brain ATP and phosphocreatine levels were not different between normothermic and hypothermic groups. In summary, hypothermia applied immediately after HI for 4 hr did not improve the recovery of brain cell membrane function and energy metabolism in the newborn piglet.

Key Words : *Hypoxia-Ischemia, Brain; Hypothermia; Reperfusion Injury; Metabolism; Animals, Newborn*

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INTRODUCTION

Perinatal asphyxia remains a primary cause of hypoxic-ischemic brain injury during the neonatal period, ultimately leading to mortality or permanent neurodevelopmental sequelae in survivors (1). Hypoxic-ischemic brain damage is an evolving process, which begins during the primary insult and extends into the recovery period after oxygenation and circulation have been restored (2, 3). Since it is practically impossible to institute some neuroprotective treatment during the primary asphyxial insult, it would be clinically more relevant to develop an effective therapeutic strategy that can improve the prognosis even when applied after the hypoxic-ischemic insult.

Although it is well known that hypothermia during hypoxia-ischemia is neuroprotective (4-6), the results from experimental studies on potentially beneficial effects applied after asphyxia are still conflicting. Some studies have confirmed a beneficial effect of hypothermia after hypoxia-ischemia (7-10), but others have not been able to confirm this (4, 5, 11, 12). Furthermore, hypothermia was associated with increased mortality in premature infants (13) and newborn animals (11). Therefore, evidence for or against beneficial effect of post-hypoxic-ischemic hypothermia in the neonatal period is still controversial, and further studies will be necessary to clarify this.

This study was done to determine whether post-hypoxic-ischemic hypothermia could reduce cerebral injury in the developing brain. We tested the hypothesis that moderate hypothermia applied after transient global hypoxia-ischemia improves recovery in brain cell membrane function and energy metabolism in the newborn piglet. Changes in brain cell membrane component, function and energy metabolism were determined by measuring lipid peroxidation products (conjugated dienes), Na^+,K^+-ATPase activity and concentrations of cerebral high-energy phosphate compounds in the cerebral cortex.

MATERIALS AND METHODS

Animal preparation

The experimental protocols described herein were reviewed and approved by the Institutional Animal Care and Use Committee of the Samsung Biomedical Research Center, Seoul, Korea.

Newborn piglets less than 3 days old and of mixed strain (Yorkshire, conventional breed, purchased from Paju farm, Paju, Korea) were used in this study. Animals inhaled ether for sedation, and anesthesia was induced with thiopental sodium (5 mg/kg, i.v.). Supplemental doses were given as required to maintain anesthesia. After local injection with lidocaine (1%), a tracheostomy was performed and the piglet was paralyzed with pancuronium (0.1 mg/kg, i.v.) and ventilated with neonatal pressure-limited time-cycled mechanical ventilator (Sechrist Infant Ventilator, IV-100V, Sechrist Industries, Anaheim, CA, U.S.A.). Ventilator settings were adjusted to keep the arterial partial oxygen pressure at 80-100 mmHg and the arterial partial carbon dioxide pressure at 35-45 mmHg. Femoral artery and vein were cannulated for monitoring blood pressure, blood sampling, and for medication and fluid infusion respectively. Heart rate, oxygen saturation and blood pressure were monitored continuously using the Hewlett-Packard neonatal monitoring system (Hewlett-Packard Model M1276A, MA, U.S.A.). Bilateral common carotid arteries were isolated at the level of the fourth cervical vertebral level and encircled with silk surgical thread (size 4).

Experimental protocol

Thirty-nine newborn piglets were divided randomly into the following four experimental groups: 9 in the normothermia control group (NC); 10 in the hypothermia control group (HC); 10 in the normothermia hypoxia-ischemia group (NH); 10 in the hypothermia hypoxia-ischemia group (HH).

The experimental protocols for each group are described in Fig. 1. The total duration of each experimental treatment was 4.5 hr after obtaining stabilization. After surgery and stabilization, all animals were ventilated in order to maintain normal blood gas levels. In control groups (NC, HC), normoxic ventilation was maintained for 4.5 hr. In hypoxic-ischemic groups (NH, HH), acute cerebral hypoxia-ischemia was induced by temporarily complete occlusion of bilateral common carotid arteries with surgical clips and simultaneous breathing with 8% oxygen for 30 min. After then, carotid artery occluders were released, inspired oxygen concentration was increased to keep oxygen saturation at 90-95%, and normoxic ventilation was continued for 4 hr.

In normothermic groups (NC, NH), rectal temperature was monitored and maintained between 38.0 and 39.0℃ using heated operating table and the servo-controlled warmer throughout the experiment. In hypo-

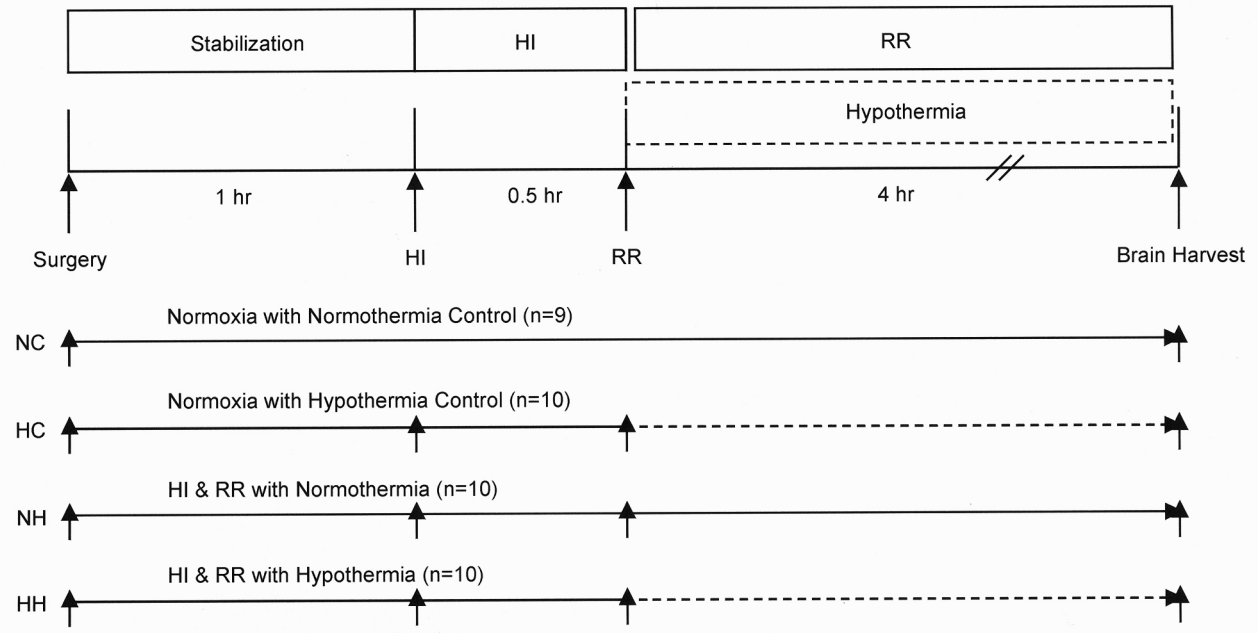

Fig. 1. Diagram of experimental protocol. The total duration of each experimental treatment was 4.5 hr after stabilization period. HI means hypoxia-ischemia. RR means reoxygenation-reperfusion. NC, normothermia control group; HC, hypothermia control group; NH, normothermia hypoxia-ischemia group; HH, hypothermia hypoxia-ischemia group.

thermic groups (HC, HH), animals were cooled rapidly by applying ice water packs, and rectal temperature was kept between 34.0 and 35.0℃ for 4 hr during the immediate reoxygenation-reperfusion period after acute transient hypoxia-ischemia.

Arterial blood gases, concentrations of glucose and lactate of blood and cerebrospinal fluid (CSF) were measured at baseline, immediately after hypoxia-ischemia, and after then, every 1 hr during the experiment in all groups. Arterial blood gases were measured on a blood gas analyzer (Ciba-Corning) and concentrations of glucose and lactate were measured using a YSI model 2300 dual analyzer (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). At the end of each experiment brain cortex was harvested within a second using guillotine, frozen rapidly in liquid nitrogen, and stored at -80℃ for further biochemical analyses.

Biochemical analyses of brain cortex

Methods of brain cell membrane preparation and determination of cerebral cortical cell membrane Na^+,K^+-ATPase activity, levels of conjugated dienes, tissue glucose and lactate concentrations, ATP and phosphocreatine were described in detail previously (14). Briefly, brain cell membranes were prepared according to the method described by Harik et al. (15). The activity of Na^+,K^+-ATPase was determined by subtracting the enzyme activity in the presence of ouabain from the total activity in the absence of ouabain. The level of conjugated dienes was determined using the method of Recknagel and Glende (16). The concentrations of glucose and lactate in the cerebral cortex were determined spectrophotometrically using a commercial kit (Sigma Chemical Co., St. Louis, MO, U.S.A.). Brain ATP and phosphocreatine levels were determined with a coupled enzyme assay using the method of Lamprecht et al. (17).

Statistical analysis

The principal statistical tests used were one-way analysis of variance and Schaffe's correction. To detect significant changes over time within each group, data were compared using repeated measures analysis of variance with Bonferroni correction. Statistical analysis described above was done using SAS software program version 6.12. A p-value of <0.05 was considered significant. Data were given as mean±standard deviation.

RESULTS

Physiologic variables

The mean physiologic variables from the four experimental groups are summarized in Table 1. In NC group, there were no significant changes in the values of physiologic variables including heart rate, arterial blood gases, and mean arterial blood pressure during the experiment. In HC group, heart rate, and arterial base excess decreased progressively, and became significantly lower than corresponding values in NC group at 4 hr of hypothermia. In NH and HH groups, heart rate increased, and mean arterial pressure, base excess and pH decreased significantly compared to the corresponding values in NC and HC groups at the end of hypoxic-ischemic episodes.

Table 1. Physiological data in each experimental group of newborn piglets

		NC (n=9)	HC (n=10)	NH (n=10)	HH (n=10)
Heart rate (/min)	Baseline	204±36	198±28	186±37	185±16
	HI	195±35	185±14	227±13*,†	217±50*,†
	4 hr	204±36	123±20*	173±25*,†	155±4*
Mean arterial pressure (mmHg)	Baseline	67±12	61±19	59±17	65±10
	HI	69±14	69±14	41±7*,†	48±19*,†
	4 hr	70±18	64±14	85±7	69±12
Arterial base excess (mEq/L)	Baseline	0.77±4.67	0.12±4.08	0.16±2.96	-1.59±3.18
	HI	1.94±5.36	-0.46±2.92	-16.90±2.85*,†	-16.37±7.99*,†
	4 hr	2.31±3.13	-3.44±3.06*	-2.10±2.98*	-11.40±7.16*,†,‡
Arterial pH	Baseline	7.44±0.15	7.42±0.09	7.41±0.04	7.40±0.09
	HI	7.44±0.17	7.42±0.06	7.12±0.06*,†	7.10±0.21*,†
	4 hr	7.47±0.14	7.32±0.04	7.42±0.05	7.18±0.15*,†,‡

NC, normothermia control group; HC, hypothermia control group; NH, normothermia hypoxia-ischemia group; HH, hypothermia hypoxia-ischemia group

'HI' means hypoxia-ischemia, '4 hr' means 4 hr after hypoxia-ischemia

Values given represent mean±standard deviation

*$p<0.05$ compared to NC; †$p<0.05$ compared to HC; ‡$p<0.05$ compared to NH

After hypoxia-ischemia, these abnormalities gradually improved in NH group. However, the decrease in arterial base excess and pH persisted in HH group at 4 hr after hypoxia-ischemia.

Glucose and lactate concentration in the blood, cerebrospinal fluid, and brain

Glucose and lactate concentrations in the blood and CSF from the four experimental groups are summarized in Table 2. In NC group, the glucose and lactate levels in the blood and cerebrospinal fluid did not change significantly during the experiment. In HC group, the glucose level in the blood and CSF increased progressively, and became significantly higher than the corresponding values in the NC group. At the end of hypoxic-ischemic episodes, the lactate concentration in the blood and CSF increased significantly in NH and HH groups compared to the corresponding values in NC and HC groups. After hypoxia-ischemia, the increased lactate level in the blood and CSF observed during hypoxia-ischemia became normalized in NH group. However, the abnormalities persisted in HH group at 4 hr after hypoxia-ischemia. In NH and HH groups, the CSF glucose levels at 4 hr after hypoxia-ischemia became significantly higher than the corresponding values in NC and HC groups, and these changes were more profound in HC group than in NH group.

The brain glucose and lactate levels were not significantly different among the four experimental groups (Table 3).

Biochemical data in cerebral cortex

In HC group, cerebral cortical cell membrane Na^+,K^+-ATPase activity slightly but significantly decreased and

Table 2. Glucose and lactate concentrations in the blood and cerebrospinal fluid in each experimental group of newborn piglets

		NC (n=9)	HC (n=10)	NH (n=10)	HH (n=10)
Blood glucose (mg/dL)	Baseline	85±21	86±10	91±6	84±28
	HI	97±41	96±12	108±22	115±5
	4 hr	79±18	104±19*	97±16	125±33*,†
CSF glucose (mg/dL)	Baseline	63±18	78±14	77±3	76±11
	HI	73±19	86±14	92±11	103±20*
	4 hr	64±17	100±16*	109±23*	142±44*,†,†
Blood lactate (mmol/L)	Baseline	2.6±0.7	1.4±0.5	1.6±0.5	1.5±0.6
	HI	2.4±0.6	1.4±0.4	8.5±2.3*,†	7.7±2.2*,†
	4 hr	1.3±0.6	1.8±0.6	1.9±1.0	3.9±2.9*,†,†
CSF lactate (mmol/L)	Baseline	2.8±0.6	2.3±0.5	2.3±0.3	2.3±0.5
	HI	2.6±0.3	2.2±0.5	7.7±1.8*,†	5.3±1.4*,†,†
	4 hr	2.5±0.9	2.1±0.3	3.2±0.8	4.2±2.4†

NC, normothermia control group; HC, hypothermia control group; NH, normothermia hypoxia-ischemia group; HH, hypothermia hypoxia-ischemia group
'HI' means hypoxia-ischemia, '4 hr' means 4 hr after hypoxia-ischemia
Values given represent mean±standard deviation
*$p<0.05$ compared to NC; †$p<0.05$ compared to HC; †$p<0.05$ compared to NH

Table 3. Biochemical data in the cerebral cortex in each experimental group of newborn piglets

Group	NC (n=9)	HC (n=10)	NH (n=10)	HH (n=10)
Glucose (mM/kg)	3.84±0.95	4.82±0.96	4.91±0.87	5.0±0.77
Lactate (mM/kg)	3.36±1.11	2.18±0.46	3.20±0.51	4.60±4.01
Na^+,K^+-ATPase activity (μM Pi/mg protein/hr)	53.6±3.0	50.4±1.5*	48.7±0.9*,	47.0±1.2*,†
Conjugated dienes (μM/g protein)	0.82±0.07	0.89±0.05*	0.90±0.07*	0.99±0.06*,†,†
ATP (mM/kg)	3.53±0.79	4.11±0.19	3.83±0.20	3.71±0.13
Phosphocreatine (mM/kg)	3.83±0.82	4.06±0.20	3.77±0.20	3.65±0.24

NC, normothermia control group; HC, hypothermia control group; NH, normothermia hypoxia-ischemia group; HH, hypothermia hypoxia-ischemia group
Values given represent mean±standard deviation
*$p<0.05$ compared to NC; †$p<0.05$ compared to HC; †$p<0.05$ compared to NH

levels of lipid peroxidation products (conjugated dienes) were increased compared to the corresponding values in NC group (Table 3). In NH and HH groups, Na^+,K^+-ATPase activity decreased and conjugated dienes increased significantly compared to the corresponding values in NC and HC groups, and these abnormalities were more profound in HH group than in NH group.

The brain ATP and phosphocreatine levels measured at the end of the experiment were not significantly different among the four experimental groups (Table 3).

DISCUSSION

The findings in this study do not support the hypothesis that post-hypoxic-ischemic hypothermia is neuroprotective in the developing brain. In the present study, moderate hypothermia applied during the immediate reoxygenation-reperfusion period after acute transient global hypoxia-ischemia did not improve the recovery in brain cell membrane function and energy metabolism in the newborn piglet. Furthermore, persistent lactic acidosis in the blood and CSF, and more profound increase in brain lipid peroxidation products (conjugated dienes) with hypothermia treatment suggest that post-hypoxic-ischemic hypothermia is not beneficial, and might even be harmful in the newborn piglet.

The recent evidence indicates that oxygen free radicals play a key role in the reoxygenation-reperfusion brain injury after primary hypoxic-ischemic insult (2, 3). Oxygen free radicals attack double bonds of polyunsaturated fatty acids in cell membranes in a process called membrane lipid peroxidation (18). Therefore, increased lipid peroxidation products (conjugated dienes) in NH group indicates an oxygen free radical-induced brain cell membrane injury after hypoxic-ischemic insult. As hypothermia has been known to suppress free radical induced brain injury (7, 19), our data of increased lipid peroxidation products even in the HC group were rather unexpected. These results could be explained by a study demonstrating that hypothermia may hinder the brain's intrinsic free radical scavenging systems by suppressing the enzymes involved in antioxidant metabolism (20), and aggravate hypoxia-ischemia induced brain injury.

The precise neuroprotective mechanism of post-hypoxic-ischemic hypothermia has not been established. As hypothermia produces a graded reduction in cerebral metabolism (21), the resulting reduction in the rate of depletion of high-energy metabolites seems to make a substantial contribution to its neuroprotective effects. Twelve hr of cooling by 4℃ after hypoxia-ischemia produced a major reduction in the delayed impairment in cerebral energy metabolism, seen 8-12 hr after hypoxia-ischemia in the newborn piglet (10). However, it is not clear if preservation of cerebral energy metabolism in the delayed injury is the primary neuroprotective mechanism by which hypothermia operates, or whether it merely represents an indicator of cellular protection mediated by other pathways. In the present study, hypothermia during the immediate reoxygenation-reperfusion period after hypoxia-ischemia did not have any significant effects on cerebral ATP and phosphocreatine levels, which already returned to normal levels at 4 hr after hypoxia-ischemia.

As a large portion of basal energy production is required for maintenance of normal Na^+,K^+-ATPase activity (22), the bulk of energy saving may be derived from active suppression of this enzyme activity. Our data of decreased Na^+,K^+-ATPase activity observed in the hypothermia groups support this assumption. In the present study, decreased Na^+,K^+-ATPase activity observed in NH group suggests post-hypoxic-ischemic neuronal dysfunction and brain injury. Since this enzyme maintains transmembrane gradients of sodium and potassium ions, decreased Na^+,K^+-ATPase activity would result in brain cell membrane dysfunction that may lead to osmotic cell swelling and neuronal death (23). However, hypothermia prevented intracellular ion and water entry and consequent cell swelling by slowing the rate of ion leakage (24, 25), in vitro, even if the ATP-dependent Na^+,K^+-ATPase activity is inhibited by ouabain (25). These findings suggest that the neuroprotective effects of hypothermia might be independent of metabolic rate, brain ATP levels or Na^+,K^+-ATPase activity. These findings are also of interest in view of the action of hypothermia to preserve the membrane potential for a longer time, thus delaying the onset of secondary cytotoxic edema after hypoxia-ischemia (7, 26).

There is a complex trade-off between optimizing neuroprotection and minimizing side effects, which will involve decisions about the methods, duration and depth of hypothermia (7, 27). As severe or long lasting hypothermia has well known adverse effects (7, 11, 28), it is important to establish the shortest and mildest degree of hypothermia offering neuroprotection. Recent studies have shown that brain cooling by 3-4℃ after hypoxia-ischemia was most effective in reducing the severity of brain injury with least systemic toxicity (7, 10, 26). For duration, at least 3 hr of hypothermia seemed to be necessary for some neuroprotection in the newborn piglet (10). For methods of hypothermia, whole body reduction of temperature instead of selective brain cooling (27) were chosen in this study because this method is easier and simpler to apply, no adverse effects have been reported in other studies (6-8, 10), and if successful, it could be a useful guide for clinical application. However, relatively short (4 hr) and moderate (4℃ reduction) sys-

temic hypothermic intervention applied immediately after hypoxia-ischemia in the present study failed to show any beneficial effects, and was associated with adverse effects including increased levels of cerebral lipid peroxidation products, metabolic acidosis, and persistently increased lactate levels in the blood and CSF. Therefore, further studies will be necessary to determine the optimal duration, depth, and methods of brain cooling with maximum benefits and minimum adverse effects in the developing brain.

Like other studies (9, 26, 28), we have observed a moderate increase in the blood and CSF glucose levels in response to hypothermia. At the end of hypoxia-ischemia, heart rate, base deficit, glucose and lactate levels in the blood and CSF increased, and arterial pH and blood pressure decreased significantly both in NH and HH groups. After hypoxia-ischemia, these abnormalities returned to normal values in NH group, but metabolic acidosis and increased levels of glucose and lactate in the blood and CSF persisted in HH group. Metabolic acidosis was also observed in HC group after 4 hr of hypothermia. These adverse systemic effects of hypothermia might be attributable to reduced tissue perfusion (7) or catecholamine-mediated effects (26, 28).

In summary, moderate systemic hypothermia after transient global hypoxia-ischemia failed to improve the recovery in brain cell membrane function and energy metabolism in the newborn piglet. Furthermore, hypothermia was associated with adverse effects such as increased cerebral lipid peroxidation products and systemic lactic acidosis after the hypoxic-ischemic insult. These findings suggest that post-hypoxic-ischemic hypothermia is not beneficial and might even be harmful in neonates.

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