J Anesth (2010) 24:726–732
DOI 10.1007/s00540-010-0999-x

ORIGINAL ARTICLE

Effect of mild and moderate hypothermia on hypoxic injury in nearly pure neuronal culture

Yu Hua · Kenjiro Hisano · Yuji Morimoto

Received: 1 December 2009 / Accepted: 5 July 2010 / Published online: 5 August 2010
© Japanese Society of Anesthesiologists 2010

Abstract
Purpose The effects of mild and moderate hypothermic therapy on cerebral injury are still controversial. Our hypothesis is that mild and moderate hypothermia should have some effects on neurons themselves if they really have protective effects. By using a nearly pure neuronal culture, we evaluated the effects and mechanism of hypothermia against hypoxic insult.
Methods A nearly pure neuronal culture from cortices of 18-day-old Wister rats was used. The neurons were exposed to below 1% oxygen at 3 different temperatures (30, 33 and 37°C). First, cell viability was measured by assessing viable neurons with trypan blue. Second, to evaluate the mechanism, the extracellular glutamate concentration was measured by high-performance liquid chromatography after hypoxia; cell viability after exposure to extrinsic glutamate was also evaluated. Next, mitochondrial membrane potential was estimated, by monitoring aggregation of MitoCapture™, and the percentage of apoptotic cells was evaluated by staining with Hoechst 33342 and propidium iodide.
Results After 24-h hypoxic insult, cell viability at 30 and 33°C was significantly higher than at 37°C. There was no significant difference between extracellular concentrations of glutamate after hypoxia or cell viability after glutamate exposure among the 3 temperature groups. In moderate hypothermia, the number of neurons with mitochondrial injury and the percentage of apoptotic cells were significantly reduced.
Conclusion Mild and moderate hypothermia inhibited hypoxic neuronal cell death. The mechanism of this effect may be related to protection of mitochondrial function, presumably followed by inhibition of apoptosis, at least in moderate hypothermia.

Keywords Hypothermia · Neurons · Hypoxia · Mitochondria · Glutamate

Y. Hua · K. Hisano · Y. Morimoto (✉)
Department of Anesthesiology and Critical Care Medicine, Hokkaido University Graduate School of Medicine, N15 W7, Sapporo 0608638, Japan
e-mail: morim2@med.hokudai.ac.jp

Introduction

Neuroprotective strategies against hypoxic, ischemic, and traumatic brain injury have not been sufficiently established [1, 2]. Among these, mild (33–36°C) and moderate (28–32°C) hypothermic therapy have furnished fascinating results for the central nervous system, including the hypoxic brain, in many laboratory studies [3, 4]. The mechanism of the protection by mild and moderate hypothermia is not explained by inhibition of cerebral metabolism. There have been many hypotheses, including inhibition of the glutamate surge, intracellular signaling pathways, disruption of the blood–brain barrier, proliferation or activation of microglia, and the production of free radicals; however, the mechanism remains unclarified [3].

In the clinical setting, 2 randomized controlled studies from Europe and Australia demonstrated the efficacy of mild hypothermic therapy for survivors of out-of-hospital cardiac arrest [5, 6]. In addition, in 2 other randomized controlled studies neurologic outcomes and mortality were improved by induction of mild hypothermic therapy for neonates with hypoxic–ischemic encephalopathy [7, 8]. On the other hand, treatment with mild hypothermia was not effective in improving outcome for patients with severe brain injury [9]. Nor did mild intraoperative hypothermia

improve outcome after craniotomy among good-grade patients with aneurismal subarachnoid hemorrhage [10]. Thus, the effectiveness of this therapy in the clinical setting is still controversial.

These laboratory and clinical findings prompted us to reappraise the effects of mild and moderate hypothermia. We hypothesized that mild and moderate hypothermia should have some effects on neurons themselves if they really have a protective effect against hypoxic or ischemic neuronal injury. In this study, we utilized a nearly pure cortical neuronal culture and evaluated the effect of concomitant mild and moderate hypothermia during hypoxic insult.

As a mechanism of ischemic/hypoxic cerebral injury, the glutamate–calcium theory, called excitotoxic neuronal cell death, is well known [11]. However, all clinical pharmacological approaches targeting this cascade have resulted in failure [11]. Meanwhile, the cell death-signaling pathway related to mitochondrial injury leading to apoptosis has recently been demonstrated in the ischemic/hypoxic brain [12]. Overloading of mitochondrial Ca^{2+} induces mitochondrial permeability transition, which leads to cytochrome *c* release and caspase activation [11, 13]. Accordingly, we also examined the mechanism of the effect of mild and moderate hypothermia with reference to these two aspects. As far as we know, there have been no studies evaluating the involvement of these 2 cascades simultaneously using the same experimental model.

Materials and methods

Nearly pure neuronal culture

With institutional approval of animal care and use, a nearly pure neuronal culture was prepared from the cortices of 18-day-old Wister rat fetuses. The cortices were digested with trypsin (Invitrogen, Carlsbad, CA, USA) and DNase I (Sigma–Aldrich, St Louis, MO, USA), followed by mechanical dissociation. Poly-L-lysine-coated plastic plates (BD Bioscience, Franklin Lakes, NJ, USA) were seeded with cortical cells at a density of 3000 cells/mm^2. The cells were grown in neurobasal medium (Invitrogen) with B27 minus AO (Invitrogen) and N_2 supplements (Invitrogen) and L-glutamine (Sigma–Aldrich). Serum-free neurobasal medium was optimized for cell survival and neurite outgrowth of neurons and the almost complete absence of glial cells without a reagent to inhibit them [14]. Cultures were kept at 37°C in 5% CO_2 in a humidified incubator. The medium was changed every third day and the cells were grown for 14 days. A pilot study showed that the proportion of neurons among the cells was over 90% in our culture system [15].

Hypoxic insult under mild and moderate hypothermia

After 14 days of culture, three dishes of the same sister culture were randomly allocated to chambers in which the temperature was maintained at 37, 33 (mild hypothermia), or 30°C (moderate hypothermia) in a 100% humidified atmosphere containing below 1% oxygen with the remainder nitrogen. The temperature of each chamber was accurately maintained using a thermostat (Digimulti D611; Techno Seven Yokohama, Japan) and the concentration of oxygen was continuously monitored with an oximeter (JKO-25 II; Jiko, Tokyo, Japan). After 24 h of hypoxic insult at each temperature, cell viability was measured.

In the preliminary study, we evaluated the changes in the cell viability over time. Until 6 h after hypoxia, almost no cells died. Cell viability finally decreased to below 50% at 24 h after the start of hypoxia. Accordingly, we set the exposure time at 24 h.

Cell viability

To evaluate cell viability, we used Shibuta's method as described previously [15]. Briefly, photomicrographs of 3 or 4 areas within the dish were taken shortly before exposure to hypoxia. After 24 h, the cells were exposed to 0.4% trypan blue with phosphate-buffered saline (Invitrogen) to stain nonviable cells, and photomicrographs were taken again in the same areas as before the exposure. Approximately 1000 viable neurons per culture dish were subjected to manual counting. A second observer unaware of the arrangement of the photographs, the study design, and the treatment procedure replicated all manual counts to ensure count accuracy and minimal inter-observer variability. Survival was calculated by use of the formula: 100 × (detected non-stained cells at the end of the experiment/detected whole cells shortly before exposure).

Each dish of the same sister culture was assigned to one of the three different temperatures. Cell viability in each dish was measured after hypoxic exposure for 24 h. Measurement was performed 16 times.

Glutamate–calcium cascade

Measurement of extracellular glutamate concentration

The extracelluar glutamate concentration was measured by use of high-performance liquid chromatography (HPLC) with an electrochemical detector (FLD-370; Eicom, Tokyo, Japan) [16]. After 24 h hypoxia, the culture supernatants were collected after the cells were sedimented by centrifugation and then stored at −80°C until assay within 2 weeks. The solution used to form fluorescent derivatives consisted of *o*-phthalaldehyde containing mercaptoethanol.

10 μl of this solution was added to 30 μl of dialysate sample diluted with 50% methanol and this mixture was left to react for 150 s before HPLC analysis with fluorimetric detection.

Each dish of the same sister culture was assigned to one of the three different temperatures. The extracellular glutamate concentration in each dish was measured after 24 h of hypoxic exposure. Measurement was performed 11 times.

Cell viability after exposure to extrinsic glutamate

After 14 days of culture, fresh medium containing 250 μM glutamate warmed at 37, 33, or 30°C was added to the 3 dishes from the same sister culture and each dish was incubated for 30 min in a chamber set at the corresponding temperature with 100% humidified room air. The medium was then changed to the normal one and the dishes were incubated for 23 h 30 min in the same atmosphere followed by evaluation of the cell viability by the method described above. The degree of glutamate exposure was established as the cell viability was compatible with that after 24 h of hypoxia at 37°C.

Each dish of the same sister culture was assigned to one of the three different temperatures. Cell viability in each dish was measured 24 h after glutamate exposure for 30 min. Measurement was performed 16 times.

Mitochondrial injury–apoptosis cascade

Quantitative evaluation of mitochondrial injury

Disruption of the mitochondrial transmembrane potential was evaluated by using a MitoCapture™ mitochondrial apoptosis detection kit (Biovision, Mountainview, CA, USA). In healthy cells, MitoCapture accumulates and aggregates in the mitochondria, emitting bright red fluorescence. When mitochondrial transmembrane potential disappears (i.e., the mitochondria are injured), it remains in the cytoplasm in its monomer form, fluorescing green. After 6 h of hypoxia at 37, 33, or 30°C, cells from the same sister culture were incubated with MitoCapture for 20 min and analyzed under a nonconfocal fluorescence microscope (Axiophoto; Zeiss, Jena, Germany). Because cell viability did not decrease after 6 h of hypoxia in the preliminary study, we estimated mitochondrial injury at that time. Quantitative analysis counting approximately 1000 cells was performed by a second observer unaware of the arrangement of photographs, study design, and treatment procedure. The percentage of the cells with mitochondrial injury was calculated.

Each dish of the same sister culture was assigned to one of the three different temperatures. The percentage of cells with mitochondrial injury was evaluated after 6 h of hypoxia. Measurement was performed 8 times.

Percentages of viable, apoptotic, and necrotic cells

The relative frequencies of apoptotic cells were examined after 24 h of hypoxia at 37, 33, and 30°C by the methods of Shimizu et al. as described before [15]. The cells were stained for 30 min at 37°C with Hoechest 33342 (Sigma–Aldrich) and propidium iodide (Sigma–Aldrich). They were then analyzed under a nonconfocal fluorescence microscope (Axiophoto; Zeiss). Quantitative analysis counting about 1000 cells was performed by a second observer unaware of the arrangement of photographs, study design, and treatment procedure. Necrotic cells were regarded as those with round red nuclei whereas apoptotic cells had fragmented nuclei regardless of their color. The percentages of viable, apoptotic, and necrotic cells were calculated by use of the formula: 100 × (viable, apoptotic, or necrotic cells/whole cells).

Each dish of the same sister culture was assigned to one of the three different temperatures. The percentages of viable, apoptotic, and necrotic cells in each dish were measured after 24 h of hypoxia. Measurement was performed 14 times.

Statistics

Values are expressed as mean ± standard deviation. One-factor ANOVA was performed among the temperature groups in each experiment. When significant differences were observed, the Tukey–Kramer test was performed as a post-hoc test. Statistical significance was assumed when $p < 0.05$.

Results

Cell viability (Fig. 1)

Cell viability was 34.6 ± 4.7, 48.1 ± 11.4, and 47.5 ± 6.9% at 37, 33, and 30°C, respectively ($p < 0.01$ in one-factor ANOVA). Cell viability at 33 and 30°C was significantly higher than that at 37°C.

Measurement of extracellular glutamate concentration (Fig. 2)

Extracellular glutamate concentrations were 1.32 ± 0.72, 1.34 ± 0.87, and 1.22 ± 0.85 pmol/μl at 37, 33, and 30°C respectively ($p = 0.94$ in one-factor ANOVA).

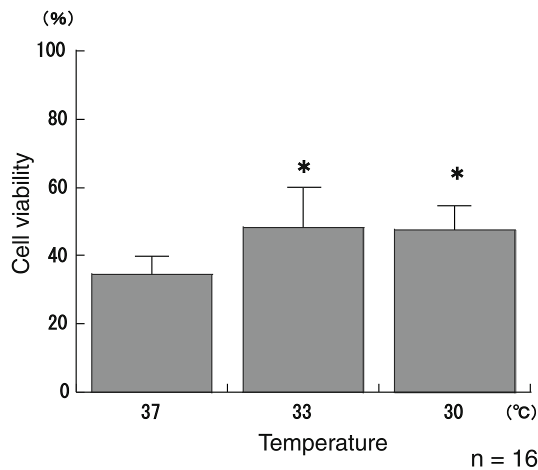

Fig. 1 Cell viability 24 h after hypoxic exposure. There was a significant difference in one-factor ANOVA ($p < 0.01$), and cell viability at 33 and 30°C was significantly higher than that at 37°C (indicated by *$p < 0.05$)

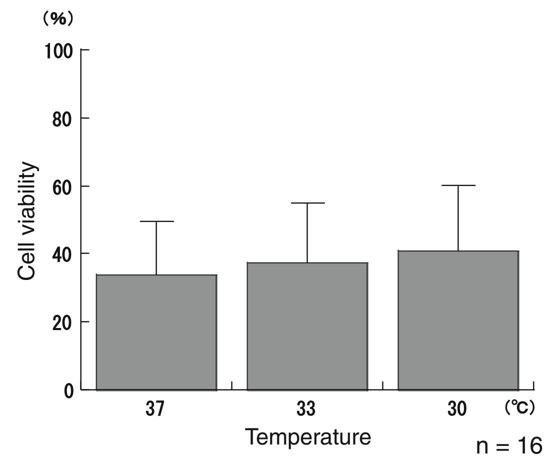

Fig. 3 Cell viability 24 h after the exposure to 250 μM extrinsic glutamate for 30 min. There was no significant difference in one-factor ANOVA ($p = 0.51$)

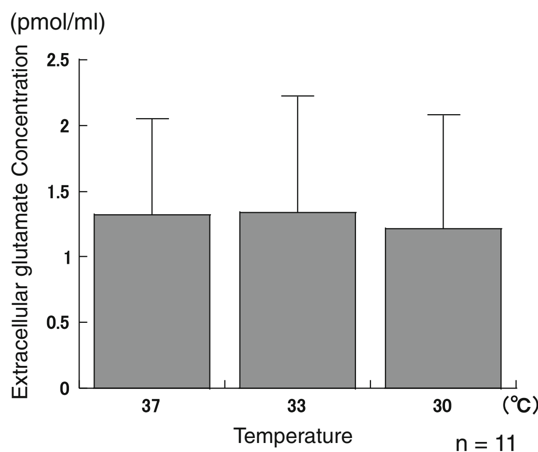

Fig. 2 Extracellular glutamate concentrations 24 h after hypoxic exposure. There was no significant difference in one-factor ANOVA ($p = 0.94$)

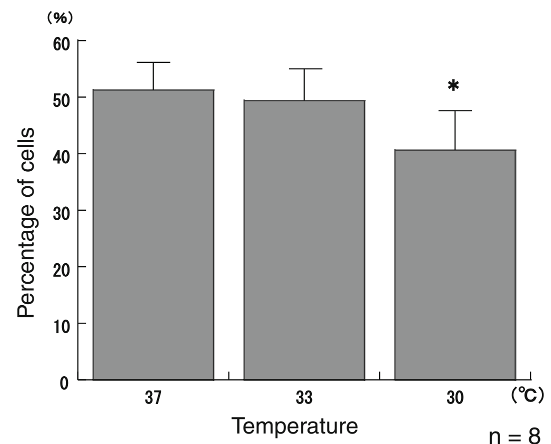

Fig. 4 Percentages of cells with mitochondrial injury 6 h after hypoxic exposure. There was a significant difference in one-factor ANOVA ($p < 0.01$) and the percentage at 30°C was significantly lower than that at 37°C (indicated by *$p < 0.05$)

Cell viability after exposure to extrinsic glutamate (Fig. 3)

Cell viability was 34.0 ± 15.1, 37.5 ± 17.0, and 41.0 ± 18.4% at 37, 33, and 30°C respectively ($p = 0.51$ in one-factor ANOVA).

Quantitative evaluation of mitochondrial injury (Fig. 4)

Percentages of the cells with mitochondrial injury were 51.2 ± 4.6, 49.4 ± 5.5, and 40.6 ± 6.7 at 37, 33, and 30°C, respectively ($p < 0.01$ in one-factor ANOVA). The percentage at 30°C was significantly lower than that at 37°C.

Percentages of viable, apoptotic, and necrotic cells (Fig. 5)

Percentages of apoptotic cells were 25.9 ± 8.2, 25.3 ± 7.2, and 17.8 ± 6.4 at 37, 33, and 30°C, respectively ($p = 0.01$ in one-factor ANOVA) and the percentage at 30°C was significantly lower than that at 37°C. Percentages of viable cells were 44.4 ± 13.3, 49.6 ± 11.9, and 58.2 ± 10.8 at 37, 33, and 30°C, respectively ($p = 0.01$ in one-factor ANOVA) and the percentage at 30°C was significantly higher than that at 37°C. For the necrotic cells, there was no significant difference among the temperature groups ($p = 0.09$ in one-factor ANOVA).

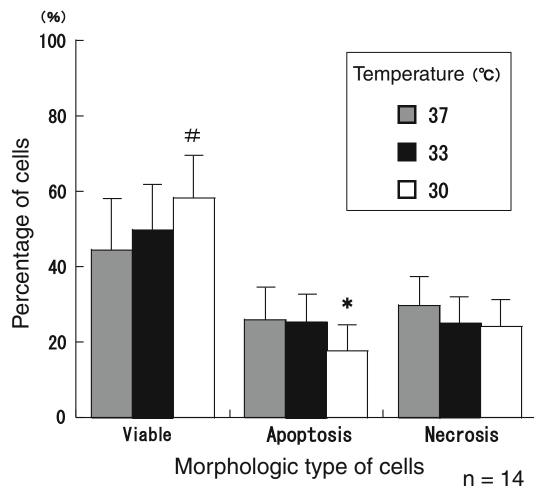

Fig. 5 Percentages of viable, apoptotic, and necrotic cells 24 h after hypoxic exposure. For apoptotic cells, there was a significant difference in one-factor ANOVA ($p = 0.01$) and the percentage at 30°C was significantly lower than that at 37°C (indicated by $*p < 0.05$). For viable cells, there was a significant difference in one-factor ANOVA ($p = 0.01$) and the percentage of viable cells at 30°C was significantly higher than that at 37°C (indicated by $^{\#}p < 0.05$)

Discussion

This study demonstrated that neuronal survival was significantly increased by mild and moderate hypothermia concomitant with 24 h hypoxia in nearly pure cortical neuronal culture. To the best of our knowledge, there has been only one previous report that examined the effect of mild or moderate hypothermia on hypoxic neuronal insult by using a nearly pure neuronal culture. That report by Bossenmeyer-Pourié et al. evaluated the effect of concomitant hypothermia at 32°C on hypoxic insult by using a nearly pure neuronal culture obtained from the rat embryo forebrain [17]. In their study, cultures were returned to normothermic and normoxic atmospheres for the next 96 h after 6 h of hypoxia. Cell viability of the neurons under normothermic hypoxia significantly decreased from 72 h after the insult and hypothermia inhibited this decrease. They also showed that both apoptosis and necrosis were inhibited by hypothermia; however, the mechanism could not be explored although they evaluated the time course of DNA and protein synthesis by measuring the incorporation of radiolabeled thymidine and L-leucine and also measured the expression of Bcl-2 and HSP 70 [17]. In their study, cell viability did not decrease after 6 h of hypoxia. Therefore, the focus of that study was the effect of hypothermia on reoxygenation injury. In our study, accordingly, we tried to observe the effect of concomitant hypothermia on ongoing hypoxic neuronal injury.

Our study demonstrated that mild and moderate hypothermia did not reduce the extracellular concentration of glutamate after hypoxia. Previous studies of global [18] and focal [19] ischemia demonstrated that mild hypothermia inhibited the increase in glutamate release. In-vitro studies using hippocampal slices [20] and mixed cortical cell culture [21] also indicated that mild hypothermia reduced the glutamate release after oxygen–glucose deprivation. On the other hand, Asai et al. demonstrated, by real-time measurement of glutamate, that intraischemic elevation of glutamate did not differ between normothermia and mild hypothermia in severe global ischemia [22]. However, the glutamate level after ischemia markedly decreased in hypothermia, suggesting the promotion of post-ischemic reuptake of glutamate. In our study, the change in the glutamate level after hypoxia was not evaluated, therefore, a significant difference might not be noted.

Cell viability after exposure to extrinsic glutamate did not significantly differ among the groups. Indeed, 15 min of exposure to either 100 μM or 1 mM glutamate uniformly induced a marked increase in intracellular calcium, with delayed recovery and massive neuronal death under the conditions of both normothermia and moderate hypothermia at 30°C in cultured hippocampal neurons [23]. Moreover, hypothermia at 34 and 28°C did not affect changes in the cytosolic free calcium concentration induced by NMDA in rat cortical brain slices [24]. These studies indicate that mild and moderate hypothermia cannot save neurons once glutamate is released during ischemia/hypoxia. Thus, inhibition of the glutamate–calcium cascade might not have contributed much to the decrease in hypoxic neuronal cell death due to mild and moderate hypothermia in our experimental model.

On the other hand, in moderate hypothermia, the percentage of cells with mitochondrial injury was significantly reduced and apoptosis due to hypoxic insult was significantly inhibited. Accordingly, moderate hypothermia might inhibit neuronal cell death induced by hypoxia by suppression of the mitochondrial injury–apoptosis cascade in our experimental model. However, the target of hypothermia remains controversial. Yenari et al. [25] showed that mild hypothermia significantly reduced the amount of cytochrome *c* release 5 h after the onset of focal ischemia, which indicated that the target of hypothermia was the mitochondrion itself. In contrast, Zhao et al. [13] showed biphasic cytochrome *c* release after global ischemia (5 and 48 h after ischemia). Caspase activity significantly increased after the first phase of cytochrome *c* release. Mild hypothermia did not block the first phase of cytochrome *c* release, but significantly blocked caspase activity and the second phase of cytochrome *c* release. This finding suggested that the target of hypothermia was caspase activity rather than mitochondria. In a study using gastric cancer cells, mitochondrial injury detected by MitoCapture was

seen 2 h after the induction of apoptosis by ceramide, and release of cytochrome *c* and activation of caspase-3 and caspase-9 were observed 3 and 24 h after that [26]. This suggested that mitochondrial changes detected by MitoCapture preceded the release of cytochrome *c* and the activation of caspase. Our study showed that moderate hypothermia inhibited the mitochondrial injury detected by MitoCapture 6 h after the onset of hypoxia, at which time almost no cells had died. Accordingly, in our experimental model, moderate hypothermia might inhibit the mitochondrial injury–apoptosis cascade by acting on the mitochondrion itself.

With regard to the relationship between neuronal apoptosis and hypothermia, Zhu et al. [27] evaluated the effect of hypothermia at 30°C on brain injury and apoptotic neuronal cell death in 7-day-old rats subjected to left common carotid artery ligation and hypoxia for 1 h. Brain infarct volumes and neuronal loss were significantly reduced 72 h after ischemia/hypoxia. Cytochrome *c* release and activation of caspase-3 and caspase-2 were significantly diminished by hypothermia. The numbers of cytochrome *c*-positive and TUNEL-positive cells (i.e., apoptotic cells) were also significantly reduced in the hypothermia group. Xu et al. examined the effect of hypothermia at 33°C on apoptosis induced by serum deprivation in a nearly pure mouse cortical neuronal culture [28]. Hypothermia significantly reduced the number of morphologically apoptotic neurons to less than half the number seen in normothermic culture after 48 h. Shibano et al. used serum-deprived PC 12 cells as the neuronal apoptotic model and examined the direct effects of mild and moderate hypothermia (29–35°C) [29]. After 96 h, the number of apoptotic cells was over 90% and this proportion decreased in a temperature-dependent fashion falling to below 50% at 29°C.

To the best of our knowledge, this is the first preliminary evaluation of the effects of mild and moderate hypothermia on hypoxic neuronal injury from the aspect of the glutamate–calcium and mitochondrial injury–apoptosis cascades, simultaneously, using the same experimental model. However, there are some limitations in our study. First, the objective of this study was to evaluate the effect of concomitant hypothermia on ongoing hypoxic neuronal injury. However, our results might indicate that hypothermia merely delayed cell death and the intracellular cascade. Accordingly, we may not be able to use the word "protection" in this study. It is unknown whether cell viability decreased further after reoxygenation in our experimental model, because cell injury was advanced compared with that in Bossenmeyer-Pourié's study. However, if intrahypoxic hypothermia inhibits the newly developed cell injury after reoxygenation, we may be able to use the word "protection" for the first time. Second, it is not uncommon for patients in critical conditions or during surgery to be exposed to hypoxic conditions. In this study, accordingly, we chose only hypoxia as a model of ischemic/hypoxic insult. However, other types of insult, for example oxygen–glucose deprivation, must be evaluated in the future. Third, we could not clarify the mechanism of mild hypothermia in this study. It was, however, demonstrated that concomitant mild and moderate hypothermia could directly affect ongoing hypoxic neuronal changes leading to cell death and that, at least in moderate hypothermia, the mechanism was related to suppression of the mitochondrial injury–apoptosis cascade.

In conclusion, by using a nearly pure neuronal culture, we showed that mild and moderate hypothermia could inhibit ongoing neuronal injury by hypoxia, suggesting that neurons themselves are a target for mild and moderate hypothermic therapy. It is speculated that suppression of the intracellular mitochondrial injury–apoptosis cascade is the mechanism of the effect of, at least, moderate hypothermia, whereas inhibition of the glutamate–calcium cascade may not contribute to this inhibition in either mild or moderate hypothermia.

Acknowledgments The authors thank Professor M. Yoshioka and former Assistant Professor M. Matshumoto, Department of Neuropharmacology in our university, for their teaching of HPLC measurement. The authors also thank Ms Naoko Kimura for her technical assistance.

References

1. Kawaguchi M, Furuya H, Patel PM. Neuroprotective effects of anesthetic agents. J Anesth. 2005;19:150–6.
2. Grocott HP, Yoshitani K. Neuroprotection during cardiac surgery. J Anesth. 2007;21:367–77.
3. Zhao H, Steinberg GK, Sapolsky RM. General versus specific actions of mild-moderate hypothermia in attenuating cerebral ischemic damage. J Cereb Blood Flow Metab. 2007;27:1879–94.
4. Miyazawa T, Tamura A, Fukui S, Hossmann KA. Effect of mild hypothermia on focal cerebral ischemia. Review of experimental studies. Neurol Res. 2003;25:457–64.
5. Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. N Engl J Med. 2002;346:557–63.
6. Hypothermia after Cardiac Arrest Study G. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N Engl J Med. 2002;346:549–56.
7. Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, Polin RA, Robertson CM, Thoresen M, Whitelaw A, Gunn AJ. Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. Lancet. 2005;365:663–70.
8. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, Fanaroff AA, Poole WK, Wright LL, Higgins RD, Finer NN, Carlo WA, Duara S, Oh W, Cotten CM, Stevenson DK, Stoll BJ, Lemons JA, Guillet R, Jobe AH, National Institute of Child Health, Human Development Neonatal

Research. Whole-body hypothermia for neonates with hypoxic–ischemic encephalopathy. N Engl J Med. 2005;353:1574–84.

9. Clifton GL, Miller ER, Choi SC, Levin HS, McCauley S, Smith KR Jr, Muizelaar JP, Wagner FC Jr, Marion DW, Luerssen TG, Chesnut RM, Schwartz M. Lack of effect of induction of hypothermia after acute brain injury. N Engl J Med. 2001;344:556–63.
10. Todd MM, Hindman BJ, Clarke WR, Torner JC. Intraoperative Hypothermia for Aneurysm Surgery Trial. Mild intraoperative hypothermia during surgery for intracranial aneurysm. N Engl J Med. 2005;352:135–45.
11. Uchino H, Kuroda Y, Morota S, Hirabayashi G, Ishii N, Shibasaki F, Ikeda Y, Hansson MJ, Elmer E. Probing the molecular mechanisms of neuronal degeneration: importance of mitochondrial dysfunction and calcineurin activation. J Anesth. 2008;22:253–62.
12. Chan PH. Mitochondria and neuronal death/survival signaling pathways in cerebral ischemia. Neurochem Res. 2004;29:1943–9.
13. Zhao H, Yenari MA, Cheng D, Sapolsky RM, Steinberg GK. Biphasic cytochrome *c* release after transient global ischemia and its inhibition by hypothermia. J Cereb Blood Flow Metab. 2005;25:1119–29.
14. Xie C, Markesbery WR, Lovell MA. Survival of hippocampal and cortical neurons in a mixture of MEM+ and B27-supplemented neurobasal medium. Free Radic Biol Med. 2000;28:665–72.
15. Hisano K, Watanabe M, Morimoto Y. Protective effects of free radical scavenger edaravone against glutamate neurotoxicity in nearly pure neuronal culture. J Anesth. 2009;23:363–9.
16. Matsumoto M, Togashi H, Kaku A, Kanno M, Tahara K, Yoshioka M. Cortical GABAergic regulation of dopaminergic responses to psychological stress in the rat in the rat dorsolateral striatum. Synapse. 2005;56:117–21.
17. Bossenmeyer-pourie C, Koziel V, Daval J-L. Effects of hypothermia on hypoxia-induced apoptosis in cultured neurons from developing rat forebrain: comparison with preconditioning. Pediatr Res. 2000;47:385–91.
18. Busto R, Globus MY-T, Dietrich WD, Martinez D, Valdes I, Ginsberg MD. Effect of mild hypothermia on ischemia-induced release of neurotransmitters and free fatty acids in rat brain. Stroke. 1989;20:904–10.
19. Winfee CJ, Connolly ES, Fiore AJ, Solomon RA. Mild hypothermia reduces penumbral glutamate levels in the rat focal cerebral ischemia model. Neurosurgery. 1996;38:1216–22.
20. Berger R, Jensen A, Hossmann KA, Paschen W. Effect of mild hypothermia during and after transient in vitro ischemia on metabolic disturbances in hippocampal slices at different stages of development. Dev Brain Res. 1998;105:67–77.
21. Bruno VM, Goldberg MP, Dugan LL, Giffard RG, Choi DW. Neuroprotective effect of hypothermia in cortical cultures exposed to oxygen–glucose deprivation or excitatory amino acids. J Neurochem. 1994;63:1398–406.
22. Asai S, Zhao H, Kohno T, Takahashi Y, Nagata T, Ishikawa K. Quantitative evaluation of extracellular glutamate concentration in postischemic glutamate re-uptake, dependent on brain temperature, in the rat following severe global brain ischemia. Brain Res. 2000;864:60–8.
23. Arai H, Uto A, Ogawa Y, Sato K. Effect of low temperature on glutamate-induced intracellular calcium accumulation and cell death in cultured hippocampal neurons. Neurosci Lett. 1993;163:132–4.
24. Bickler PE, Buck LT, Hansen BM. Effects of isoflurane and hypothermia on glutamate receptor-mediated calcium influx in brain slices. Anesthesiology. 1994;81:1461–9.
25. Yenari MA, Iwayama S, Cheng D, Sun GH, Fujimura M, Morita-Fujimura Y, Chan PH, Steinberg GK. Mild hypothermia attenuates cytochrome *c* release but does not alter Bcl-2 expression or caspase activation after experimental stroke. J Cereb Blood Flow Metab. 2002;22:29–38.
26. Belkhiri A, Dar AA, Zaika A, Kelley M, El-Rifai W. t-Darpp promotes cancer cell survival by up-regulation of Bcl2 through Akt-dependent mechanism. Cancer Res. 2008;68:395–403.
27. Zhu C, Wang X, Cheng X, Qiu L, Xu F, Simbruner G, Blomgren K. Post-ischemic hypothermia-induced tissue protection and diminished apoptosis after neonatal cerebral hypoxia–ischemia. Brain Res. 2004;996:67–75.
28. Xu L, Yenari MA, Steinberg GK, Giffard RG. Mild hypothermia reduces apoptosis of mouse neurons in vitro early in the cascade. J Cereb Blood Flow Metab. 2002;22:21–8.
29. Shibano T, Morimoto Y, Kemmotsu O, Shikama H, Hisano K, Hua Y. Effects of mild and moderate hypothermia on apoptosis in neuronal PC12 cells. Br J Anaesth. 2002;89:301–5.