PROTECTIVE EFFECTS OF MILD AND MODERATE HYPOTHERMIA ON CULTURED NEUROBLASTOMA-GLIOMA HYBRID NG108-15 CELLS

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Summary

We have evaluated if mild hypothermia could have a significant protection against cerebral ischemia by its inhibition of post-ischemic apoptosis. Recent studies on cultured neuroblastoma-glioma hybrid NG108-15 cells used as a neuronal apoptotic model were examined on direct effects of mild hypothermia. Nevertheless, the mechanism of cerebral protection isn’t completely known. We hypothesized that mild hypothermia may be protective against cerebral ischemia by inhibiting post-ischemia apoptosis. In this study, we used serum-deprived NG108-15 cells as the neuronal apoptotic model and examined the direct effects of mild and moderate hypothermia. Apoptosis was induced by depriving the cell culture medium of serum, which is one of the most representative methods to induce apoptosis. Cytotoxicity and the percentage of apoptotic cells were evaluated 3 days after induction of apoptosis. With induction at 37°C, cytotoxicity and the percentage of apoptotic cells were over 50 and 80%, respectively. At each temperature examined below 35 °C, significant decreases in cytotoxicity and the percentage of apoptotic cells were observed. In the control group, cytotoxicity and the percentage of apoptotic cells were significantly higher at 29°C than at 37°C. We observed that mild and moderate hypothermia inhibited apoptosis.

Key words: hypothermia, apoptosis, NG108-15 cells, cytotoxicity

Introduction

Studies after 1950s shown that a small reduction of intra-ischemic rat cerebral temperature (from 36°C to 34°C) markedly attenuated ischemic cell damage over 20 min in the four-vessel occlusion model. Even so, the mechanism of cerebral protection by mild hypothermia is still unclear. It is not wholly attributable to metabolic inhibition, because a temperature reduction of 1-2°C decreases the cerebral metabolic rate of oxygen consumption by only 7-14% (Wass et al., 1995). In fact, a group of researchers also reported that cerebral energy metabolites such as ATP were depleted to a similar degree at 33, 34 and 37°C at the end of 20 min of four vessel occlusion (Busto et al., 1987).

It was reported that apoptosis was detected following focal cerebral ischemia, especially in the penumbral region. It has also been reported that delayed neuronal death following global ischemia may be partly attributable to apoptosis (Ardelean A. et al. 2007; MacManus et al. 1999). It is speculated that ischemic cell death is partly due to apoptosis in addition to necrosis. Necrosis occurs due to intracellular energy depletion, after which metabolic depression may inhibit necrosis. Apoptosis occurs due to activation of intracellular cascades.
Therefore, mild hypothermia may protect against cerebral ischemia by inhibiting the intracellular apoptotic cascades activated by ischemia. In this study, we used serum-deprived cultured neuroblastoma-glioma hybrid NG108-15 cells as the neuronal apoptotic model and examined the direct effects of mild and moderate hypothermia on apoptosis.

Other studies were used to treat transitory cerebral hypoxia-ischemia (HI) in newborn animals that appears as a result of difficulties during labor or during heart attack. Perinatal HI in its most severe form could cause defects of permanent neuronal development that includes spastic tetraparesis, severe cognitive deficiencies and epilepsy (Nelson and Ellenberg, 1981). Advanced methods of obstetrical and neonatal care haven’t significantly modified the results of HI encephalopathy (Nelson and Ellenberg, 1988). The treatment of newborn with HI remains symptomatic in spite of the efforts of several researchers to evaluate the neuroprotective interventions (Vannucci and Perlman, 1997). In revived adults after heart attack, mild hypothermia for 12-24 hours is one of the few interventions with neurologic benefits (Bernard et al. 2002). In a study of newborn with cerebral disorders, there are recent encouraging results (Battin et al. 2001).

**Material and methods**

Cell culture and induction of apoptosis NG108-15 cells were originally derived from a hybrid of neuroblastoma and glioma cells. NG108-15 cells stop dividing and terminally differentiate when treated with nerve growth factor. This makes NG108-15 cells useful as a model system for neuronal differentiation. Cells having undergone up to 11 passages from the original cell line were used in the experiments. NG108-15 cells were maintained on collagen coated dishes in DMEM medium, supplemented with 5% heat-inactivated fetal bovine serum, at 37°C in a 100% humidified atmosphere.

Preliminary data of this study reports the safety of mild hypothermia use on affected newborns (Gunn et al. 1998).

In some studies, asphyxic cardiac arrest was chosen as a simulation model of neonatal HI. This model of systemic hypoxia followed by asphyxic cardiac arrest is well physiologically and pathologically characterized (Martin et al. 1997; Barkovich et al. 1995), and the model of cerebral lesion is very similar to newborn HI encephalopathy (Barkovich et al. 1995; Low et al. 1989). The mentioned study was focused on the striatum, this being the most vulnerable region and because cell death is the most rapid in these regions, thus requiring a fast and early intervention. The experiment was designed to determine whether whole body hypothermia after HI ameliorates neuronal cell death before rewarming in the putamen and caudate nucleus at 24 h, at which time striatal injury is near maximal (Marint et al. 2000; Brambrink et al. 1999). The hypotheses tested were that sedated piglets subjected to 24 h of whole body hypothermia (34°C) after asphyxic cardiac arrest demonstrate decreased acute neuronal necrosis in striatum at 24 h in the absence of rewarming, sustained neuroprotection at 7 days of recovery, and improved functional recovery.

**Cytotoxicity evaluation**

After two days of normal culture at 37°C, apoptosis was induced in phenol-red-free DMEM without serum. The cells were randomly incubated in chambers, in which the temperature was maintained at 37, 35 or
33°C (mild hypothermia), or 37, 31 or 29°C (moderate hypothermia), in an atmosphere containing carbon dioxide and air 5/95. The temperature of each chamber was accurately controlled. As a control, cells in which apoptosis was not induced were incubated under the same conditions.

Cell death can be assayed by quantifying plasma membrane damage or rupture. The LDH Cytotoxicity Detection Kit offers a simple way to measure plasma membrane damage, based on the release of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme present in most cells. Culture supernatants were collected after the cells were sedimented by centrifugation (cooling centrifuge Sigma 2-16K) and were incubated with a LDH reaction mixture using a cytotoxicity detection kit (Rocher Diagnostics Corporation). At 490 nm were measured changes in absorbance with a Microplate Reader Benchmark (Bio-Rad). Change in absorbance of the LDH standard determines the LDH activity of each sample. LDH activity of the cells was also measured after cells were lysed in 1% Triton X-100. Cytotoxicity (%) can be defined using the following formula:

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\text{Cytotoxicity(%) = \frac{LDH leakage activity}{LDH leakage activity + LDH activity within cells}}
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Apoptotic cells under hypothermia

After two days of normal culture at 37°C, apoptosis was induced in phenol-red-free DMEM without serum. Cells were randomly placed into mild or moderate hypothermia as described previously. The control, non-induced cells were incubated under the same conditions.

Cells were mixed in 70% ethanol following harvest. Fixed cells were incubated with 0.4 mg/ml RNase A (Sigma) and stained with propidium iodide (Sigma). Cellular DNA content was measured by flow cytometry (FACS Calibur; Becton Dickinson). Calculation of the percentage of apoptotic cells was based on the frequency curves of DNA histograms. Apoptotic cells were regarded as the population that contained less DNA than at the G1 peak (Fig. 1).

The number of apoptotic cells in apoptotic group is almost identical to that reported in Shibano-2002, despite the cell types and harvesting times are different.

Statistics

To compare cytotoxicity and the percentage of apoptotic cells, was used the ANOVA one-way analyses. Statistical significance was used when P<0.05 and values were expressed as mean.
Results and discussion

Cytotoxicity in apoptosis group (Fig. 2), at 37°C was >55%, 3 days after induction. At each temperature below 35°C, cytotoxicity decreased significantly (P < 0.01) compared with 37°C. In control group, we performed some experiments in the mild and moderate hypothermia groups. Cytotoxicity at 37, 35, 33, and 31°C was approximately 9-10%. At 29°C, cytotoxicity was 16.5% and was significantly higher than that at 37°C (P=0.02). The percentage of apoptotic cells, in apoptosis group (Fig. 3), at 37°C was >80%. At each temperature below 35°C, this was significantly decreased compared with 37°C (P<0.01). At 29°C, this value decreased to 36.0 (7.1%).

Fig. 2 Hypothermia effect on cytotoxicity (apoptosis group). Mean values indicate cytotoxicity (LDH leakage) in the mild (A) and moderate (B) hypothermia groups. There is a significant difference between mild and moderate hypothermia (P<0.01).

Fig. 3 Hypothermia effect on apoptotic cells (apoptosis group). Mean values indicate percentage of apoptotic cells in mild (A) and moderate (B) hypothermia. There is a significant difference between mild and moderate hypothermia (P<0.01).

In control group, in order to assay the percentage of apoptotic cells we performed a number of experiments in mild and moderate hypothermia. At 37, 35, 33 and 31°C, the percentage of apoptotic cells was less than 10%. This value was 11.2 (3.9%) at 29°C and was significantly higher than that at 37°C (P<0.01).

The authors do not specify the number of experiments in the mild and, respectively, moderate hypothermia groups, as is mentioned in Shibano-2002.
Conclusions

There are some theories on the neuroprotective effect of mild hypothermia: inhibition of reactive oxygen species (ROS) production or early recovery of protein synthesis (Lawrence et al. 2005), depression of the glutamate surge, delayed onset of intracellular calcium mobilization (Mitani et al. 1991). Nevertheless, the precise mechanism is still unclear. It has been reported that cerebral apoptosis is decreased under hypothermia at 33°C after forebrain and focal ischemia in rats and piglets (Phanithi et al. 2000). Others reported that hypothermia at 32°C reduced apoptosis following 6 h of hypoxia in neuronal cultures (Bossenmeyer-Pourie et al. 2000). We observed necrosis in addition to apoptosis, so the effect of hypothermia on apoptosis alone is not clearly expressed. Moreover, not all temperatures’ effects were studied. As we know it, to this day, the effects of mild and moderate hypothermia directly on apoptosis need more clarifications. The same thing can be affirmed about cell survival by hypothermia.

Hypothermia directly inhibited neuronal apoptosis in the mild and moderate range (29-35°C) in the studies on cytotoxicity and percentage of apoptotic cells. Previous studies reported (Kaasik et al. 2001) that mechanisms such as production of ROS and induction of caspase activity are suggested as intracellular cascades leading to induced apoptosis in NG108-15 cells. They are also observed after cerebral ischemia (Ozkul et al. 2007). Others have examined the levels of salicylate hydroxylation product as an index of ROS production in models temperatures of 30, 36 and 39°C (Hashimoto et al. 2003). Levels of the salicylate hydroxylation product significantly decreased depending on temperature. The expression of caspase-3 proteins was decreased during hypothermia at 33°C after rat forebrain ischaemia according to some studies (Phanithi et al. 2000). In this respect, the apoptosis inhibition under hypothermia observed in this study might be related to depression of intracellular cascades.

Others have reported that there were no morphological or electrophysiological modifications in spinal cord neuron culture, even on 17°C temperature for 2 h (Craenen et al. 1996). Nonetheless, cytotoxicity and the percentage of apoptotic cells increased at 29°C in our control group. This suggests that hypothermia may damage intact cells if the period of hypothermia is prolonged by inducing apoptosis, even on moderate hypothermia.

Concluding, apoptosis but not necrosis was induced by serum deprivation in the relationship between hypothermia and neuronal apoptotic processes using neuroblastoma-glioma cells. Even though hypothermia below 30°C may induce apoptosis, mild and moderate hypothermia (29-35°C) inhibited apoptosis.

References


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