Fișa suspiciunii de plagiat / Sheet of plagiarism’s suspicion

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<td><strong>ARDELEAN, A., PRIBAC, G.C., BODO, E., CZAPAR, M., MOS, L., COTORACI, C.,</strong></td>
<td><strong>SHIBANO, T., MORIMOTO, Y., KEMMOTSU, O., SHIKAMA, H., HISANO, K., HUA, Y.,</strong></td>
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**Incidența minimă a suspiciunii / Minimum incidence of suspicion**

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Effects of mild and moderate hypothermia on apoptosis in neuronal PC12 cells

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Background. There is still a possibility that mild hypothermic therapy may be useful as a neuroprotective tool during the intraoperative period, although the mechanism of cerebral protection by mild hypothermia is not well understood. We hypothesized that mild hypothermia may be protective against cerebral ischaemia by inhibiting post-ischaemia apoptosis. In this study, we used serum-deprived PC12 cells as the neuronal apoptotic model and examined the direct effects of mild and moderate hypothermia.

Methods. Apoptosis was induced by depriving the cell culture medium of serum, which is one of the most representative methods to induce apoptosis, but not necrosis, in PC12 cells. Effects of mild (35 and 33°C) and moderate (31 and 29°C) hypothermia on apoptosis were evaluated. Cytotoxicity (lactate dehydrogenase leakage) and the percentage of apoptotic cells (calculated by flow cytometry with propidium iodide) were evaluated 4 days after induction of apoptosis. As a control, cells without induction of apoptosis were incubated under the same conditions as the apoptosis group.

Results. Without induction at 37°C, cytotoxicity and the percentage of apoptotic cells were over 60 and 90%, respectively. At each temperature examined below 35°C, significant decreases in cytotoxicity and the percentage of apoptotic cells were observed. Mean cytotoxicity at 31 and 29°C was 50.2 (SD 4.2)% and 47.9 (4.4)% respectively. The percentage of apoptotic cells at 31 and 29°C was 42.5 (7.4)% and 36.5 (7.3)% respectively. In the control group, cytotoxicity and the percentage of apoptotic cells were significantly higher at 29°C than at 37°C.

Conclusions. Mild and moderate hypothermia (29–35°C) inhibited apoptosis, although hypothermia below 30°C may induce apoptosis in intact cells.

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Hypothermia for cerebral protection was first introduced in the 1950s.1 2 Prior to this, hypothermic treatment was not widespread due to its harmful effects, including cardiovascular and respiratory depression and severe infection. However, Busto and colleagues reported that a small reduction of intracerebral rat cerebral temperature (from 36°C to 34°C) markedly attenuated ischaemic cell damage over 20 min in the four-vessel occlusion model.3 Although a recent multicentre randomized controlled trial questioned the effectiveness of mild hypothermic therapy in patients with severe brain injury,4 there is still a possibility that mild hypothermic therapy may be useful as a neuroprotective tool during the intraoperative period.5 6

The mechanism of cerebral protection by mild hypothermia is still unclear. However, 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%.7 In fact, Busto and colleagues 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.3
Recently, it was reported that apoptosis was detected following focal cerebral ischaemia, especially in the penumbral region.\textsuperscript{8,9} It has also been reported that delayed neuronal death following global ischaemia may be partly attributable to apoptosis.\textsuperscript{10,11} Accordingly, it is speculated that ischaemic 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. In contrast, apoptosis occurs due to activation of intracellular cascades.\textsuperscript{12} Therefore, mild hypothermia may protect against cerebral ischaemia by inhibiting the intracellular apoptotic cascades activated by ischaemia. In this study, therefore, we used serum-deprived PC12 cells as the neuronal apoptotic model and examined the direct effects of mild and moderate hypothermia on apoptosis.

**Methods**

**Cell culture and induction of apoptosis**

PC12 cells, which were originally derived from rat pheochromocytoma, were provided by Riken Gene Bank (Tsukuba Science City, Japan). Cells having undergone up to 10 passages from the original cell line were used in the experiments. PC12 cells were maintained on collagen-coated dishes (Biocoat Cellware, Bedford, MA, USA) in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA), supplemented with 5% heat-inactivated fetal bovine serum (MoreGate, Melbourne, Australia) and 5% heat-inactivated horse serum (Gibco), at 37°C in a 100% humidiﬁed atmosphere containing 5% carbon dioxide/95% air. Apoptosis was induced by depriving the medium of serum, which is one of the most representative methods of inducing only apoptosis in PC12 cells.\textsuperscript{13} We have previously demonstrated that apoptosis, but not necrosis, is induced in our cells by serum deprivation through DNA electrophoresis and fluorescence microscopic analyses.\textsuperscript{14}

**Effects of hypothermia: cytotoxicity assay**

Cells (1×10⁵) were subcultured to 35-mm collagen-coated dishes. Two days after normal culture at 37°C, apoptosis was induced in phenol-red-free DMEM (Gibco) without serum. The cells were randomly incubated in chambers, in which the temperature was maintained at 37, 35 or 33°C (mild hypothermia series), or 37, 31 or 29°C (moderate hypothermia series), in a 100% humidiﬁed atmosphere containing 5% carbon dioxide/95% air. The temperature of each chamber was accurately maintained by a thermostat (Digimulti D611; Techno Seven, Yokohama, Japan). As a control, cells in which apoptosis was not induced were incubated under the same conditions.

Cytotoxicity was evaluated 4 days after serum deprivation using the lactate dehydrogenase (LDH) leakage assay as reported previously.\textsuperscript{14} Briefly, the culture supernatants were collected after the cells were sedimented by centrifugation. Next, they were incubated with a LDH reaction mixture using a cytotoxicity detection kit (Boehringer Mannheim, GmbH, Mannheim, Germany). Changes in absorbance at 490 nm were measured with a spectrophotometric microplate reader (model 3350; Bio-Rad, Hercules, CA, USA). LDH activity of each sample was obtained from the change in absorbance of the LDH standard. LDH activity of the cells was also measured after cells were lysed in 1% Triton X-100. Cytotoxicity (%) was defined as follows: (LDH activity that leaked from the cells)/(LDH activity that leaked from the cells + LDH activity within the cells).

**Effects of hypothermia: percentage of apoptotic cells**

Cells (1×10⁵) were subcultured to 35-mm collagen-coated dishes. Two days after normal culture at 37°C, the medium was deprived of serum. The cells were randomly allocated into a mild or moderate hypothermia series as in the cytotoxicity assay. As a control, non-induced cells were incubated under the same conditions.

The method for flow cytometric analysis was as described previously.\textsuperscript{14} Briefly, cells were ﬁxed in 70% ethanol following harvest. Fixed cells were incubated with 0.4 mg ml⁻¹ RNase A (Sigma, St Louis, MO, USA) and stained with propidium iodide (Sigma). Cellular DNA content was measured by ﬂow cytometry (FACS Calibur™; Becton Dickinson, San Jose, CA, USA). Calculation of the percentage of apoptotic cells was based on the cumulative frequency curves of the appropriate DNA histograms. Apoptotic cells were regarded as the population that contained less DNA than at the G₁ peak (Fig. 1).

**Statistics**

Values are expressed as mean (SD). To compare cytotoxicity and the percentage of apoptotic cells, one-factor analysis of variance (ANOVA) was used. When a signiﬁcant difference was observed, post hoc analysis was performed with Sheffe’s test. Statistical signiﬁcance was assumed when \( P<0.05 \).

**Results**

**Cytotoxicity: apoptosis group (Fig. 2)**

Cytotoxicity at 37°C was >60% 4 days after induction. At each temperature below 35°C, cytotoxicity decreased signiﬁcantly (\( P<0.01 \)) compared with 37°C.

**Cytotoxicity: control group**

We performed 10 and nine experiments in the mild and moderate hypothermia groups, respectively. Cytotoxicity at
37, 35, 33 and 31°C was approximately 10–11%. At 29°C, cytotoxicity was 17.0 (5.8)% and was significantly higher than that at 37°C ($P=0.03$).

**Percentage of apoptotic cells: apoptosis group**
(Fig. 3)
The percentage of apoptotic cells at 37°C was >90%. At each temperature below 35°C, this was significantly decreased compared with 37°C ($P<0.01$) (Fig. 3). At 29°C, this value decreased to 36.5 (7.3)%.

**Percentage of apoptotic cells: control group**
We performed 20 and 17 experiments in the mild and moderate hypothermia groups, respectively. At 37, 35, 33 and 31°C, the percentage of apoptotic cells was <10%. This value was 12.3 (4.2)% at 29°C and was significantly higher than that at 37°C ($P<0.01$).

**Discussion**
Several hypotheses have been suggested with regard to the neuroprotective effect of mild hypothermia. For example, depression of the glutamate surge,15 delayed onset of intracellular calcium mobilization,16 inhibition of reactive oxygen species (ROS) production17 or early recovery of protein synthesis18 have all been reported. However, the exact mechanism has not been elucidated. Recently it has been reported that cerebral apoptosis is decreased under hypothermia at 33°C after forebrain and focal ischaemia in rats.19 20 Bossenmeyer-Pourie and colleagues reported that hypothermia at 32°C reduced apoptosis following 6 h of hypoxia in neuronal cultures from the forebrain of 14-day-old rat embryos.21 However, necrosis in addition to apoptosis was observed in their model so that the effect of hypothermia on apoptosis alone was not clarified. In addition, the effects of several temperatures were not examined. To our knowledge, the direct effects of mild and moderate hypothermia on apoptosis, but not necrosis, are not well understood. Cell survival by hypothermia itself has not been studied extensively.
Studies of cytotoxicity and percentage of apoptotic cells revealed that hypothermia directly inhibited neuronal apoptosis in the mild and moderate range (29–35°C). As we reported previously, several mechanisms such as production of ROS and induction of caspase activity are suggested as intracellular cascades leading to apoptosis induced by serum deprivation in PC12 cells. They are also observed after cerebral ischaemia. Kil and colleagues examined the levels of the salicylate hydroxylation product as an index of ROS production in the rat forebrain ischaemia model at brain temperatures of 30, 36 and 39°C. Levels of the salicylate hydroxylation product significantly decreased in a temperature-dependent fashion. It was reported that expression of caspase-3 proteins was decreased during hypothermia at 33°C after rat forebrain ischaemia. Thus, the inhibition of apoptosis under hypothermia observed in this study might also be related to depression of such intracellular cascades.

Lucas and colleagues reported that there were no morphological or electrophysiological changes in spinal cord neurone culture, even when the temperature was decreased to 17°C for 2 h. In our study, however, cytotoxicity and the percentage of apoptotic cells significantly increased at 29°C in the control group. This result suggests that hypothermia may damage intact cells through induction of apoptosis if the period of hypothermia is lengthy, even if the range of hypothermia is moderate. The result may be noteworthy because as of late, hypothermic therapy is usually performed for several days.

In conclusion, we evaluated the relationship between hypothermia and neuronal apoptotic processes using PC12 cells, in which apoptosis but not necrosis was induced by serum deprivation. Mild and moderate hypothermia (29–35°C) inhibited apoptosis, although hypothermia below 30°C may induce apoptosis.

References