

Methylprednisolone attenuates hypothermia- and rewarming-induced cytotoxicity and IL-6 release in isolated primary astrocytes, neurons and BV-2 microglia cells

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Abstract

Brain protection is crucial during neonatal and pediatric cardiac surgery. The major methods for brain protection are the administration of steroids and deep hypothermia. Therefore, we have investigated the impact of methylprednisolone (MP) administration and deep hypothermia on neonatal mouse astrocytes, neurons and BV-2 microglia cells. Brain cells were pretreated with MP (100 mM) and incubated according to a deep hypothermia protocol mimicking temperature changes during cardiac surgery in children: deep hypothermia (2 h at 17 °C, phase 1), slow rewarming (2 h up to 37 °C, phase 2), and normothermia (20 h at 37 °C, phase 3). In all brain-related cell types cytotoxicity was investigated as well as the release of the pro-inflammatory cytokine interleukin-6 (IL-6), which plays a major role in neuroprotection and neuroregeneration. Deep hypothermia induces substantial cytotoxicity and the secretion of IL-6 by astrocytes, BV-2 microglia cells and neurons. MP administration has no influence on the cell survival and IL-6 release of normothermic astrocytes, BV-2 microglia cells and neurons, while hypothermia-induced cytotoxicity and IL-6 secretion are significantly suppressed by MP. These data suggest that MP increases cell survival after deep hypothermia but also suppresses important neuroprotective and regenerative processes induced by IL-6. Hence, more specific immune modulation than that provided by MP may be needed to protect the brain during neonatal and pediatric cardiac surgery.

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Brain injury is still one of the important complications after corrective pediatric cardiac surgery using cardiopulmonary bypass (CPB) [6,11,39]. Global or focal cerebral ischemia involving all types of brain cells may occur during non-pulsatile low flow perfusion and periods of total circulatory arrest. One of the main protective procedures during cardiac surgery is systemic cooling, the main rationale being that hypothermia decreases cerebral blood flow and the metabolic rate of O₂ [7]. During surgical corrections of complex congenital cardiovascular mal-

formations in early childhood, neonates and infants are cooled down to a minimal rectal body temperature of below 20 °C [3,22]. Other factors which may contribute to brain injury are part of the inflammatory response to CPB; contact of the blood with the large non-physiological surfaces of the oxygenator and increased blood suctioning from the operative situs. This may trigger a cascade of inflammation in all organs including the brain [15].

However, the role of inflammation in neuroprotection and neuroregeneration is controversial [21,28,33]. In particular, the pro-inflammatory cytokine interleukin-6 (IL-6) protects the brain against neuronal degeneration [26,27,36] and is an important prerequisite for neuronal regeneration [8,25]. Nevertheless, pharmacological pretreatment with steroids before surgery

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has been suggested as an additive protective procedure to avoid cerebral inflammation although conflicting results on the potential neuroprotective effects of steroids have been reported [10,23,32,34,35].

Previously, we demonstrated the failure of pretreatment with MP to prevent ischemic neuronal cell damage in representative brain regions in a neonatal animal model of deep hypothermic circulatory arrest [32]. In addition, not only neurons but also other brain cells such as astrocytes and microglial cells are activated by hypothermic perfusion and this effect seems to precede the neuronal injury [1,2,4]. Therefore, we used a cell culture model to investigate the effect of hypothermia and rewarming with and without MP pretreatment not only on primary neurons but also on astrocytes and BV-2 microglia cells. In contrast to previous studies [17,20], which were performed using one cell type and only at one time interval immediately after hypothermia, our protocol was modified to assess the survival and IL-6 release of the cells at four different time points: at baseline, immediately after deep hypothermia, after rewarming, and after subsequent 20 h of normothermia, mimicking the clinical situation.

To improve neuroprotective strategies during CPB, it is important to evaluate the cellular response in isolated primary cell cultures after pretreatment with MP under the conditions of cooling and rewarming. In this study we analyzed the impact of dynamic cooling and rewarming with and without MP on the survival of isolated neonatal primary astrocytes, neurons and BV-2 microglia cells as well as the release of the neuroregenerative cytokine IL-6.

Tissue culture material was obtained from Becton Dickinson (Heidelberg, Germany). The cytotoxicity detection kit (LDH) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). DMEM high glucose medium, phosphate-buffered salt solution, and fetal calf serum were from GIBCO (Karlsruhe, Germany), all other reagents from Sigma-Aldrich (Munich, Germany).

All procedures involving animals were performed in accordance with the German guidelines on the use of laboratory animals. Primary neurons were prepared from fetal mice (E15) as described previously [18]. In brief, after removal of the meninges, cerebral cortices were dissociated with a glass pipette in HBSS containing 4 mg/ml trypsin and 0.5 mg/ml DNase (Worthington Biochemical Corporation, Lakewood, NJ, USA). After centrifugation ($165 \times g$, 10 min, 4°C) the cells from four cortices were pooled and plated into 75 cm tissue culture flasks containing DMEM with 4.5 g/l glucose supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. They were incubated in cell culture dishes for 10 days before starting the experiment and are therefore considered to have the characteristics of immature neonatal cells. Each experiment was performed three times and twelve 6-well plates of single cell cultures were prepared for each experiment. Primary astrocytes were prepared from newborn mice using a modification of a technique described previously [18]: brain tissue was dissociated with 0.25% trypsin, centrifuged at $470 \times g$ for 10 min, resuspended in Neurobasal medium, plated on poly-L-lysine-coated 24-well plates, and incubated at 37°C

in 5% CO_2 and 95% air. After the appearance of a confluent cell layer, adherent glial cells were mechanically dissolved the proliferation of the remaining glial cells was inhibited by $8 \mu\text{M}$ cytosine arabinoside for 3 days. The primary astrocyte cell cultures obtained were >95% pure (tested by double immunohistochemistry) [19].

BV-2 microglial cells are primary mouse microglial cells immortalized by stable transfection with the *c-myc* oncogene using a J7-retrovirus [5], leading to a phenotype functionally identical to native primary microglia [24].

We applied a dynamic time–temperature protocol used during pediatric cardiac surgery (Fig. 1A). Two hours before the study period, the culture medium was replaced with 2 ml serum-free DMEM supplemented with 5 mM glucose, 0.06 g/l penicillin, and 0.1 g/l streptomycin. Cells and brain slices were pretreated with MP (100 mM) before commencement of the time–temperature protocol (Fig. 1A): the protocol starts with 2 h of deep hypothermia at 17°C , followed by a rewarming phase for 2 h up to 37°C ($0,17^\circ\text{C}/\text{min}$), followed by 20 h normothermia (37°C). The temperature of the culture medium was continuously monitored using a thermocouple paratrend 7 probe (Diametrics Medical Inc., St. Paul, USA) inserted in one control petri dish. At every time point, two 6-well plates were removed and the supernatant collected. One culture dish was examined throughout the incubation protocol to monitor cell morphology microscopically. PBS or MP dissolved in PBS were applied before starting the experiment. The low MP concentration of 0.1 M was used, since extensive pilot experiments revealed strong effects even at such a low concentration.

Lactate dehydrogenase (LDH) release into cell culture supernatant was quantified using a Cytotoxicity Detection Kit (Roche) following the manufacturer's instructions. At the end of the experiments supernatants were collected and the LDH content was measured. LDH release is expressed as a percentage of the total content, determined by lysing an equal number of cells with 1% Triton X-100.

Conditioned medium from treated cells was tested for IL-6 using commercially available sandwich enzyme-linked immuno-sorbent assays (ELISA) according to the manufacturer's instructions (Pharmingen, Heidelberg, Germany). The total amount of IL-6 is correlated with the percentage of living cells, which was measured by LDH release in the supernatant.

Student's *t*-test was used to compare values of IL-6 and LDH between the different time intervals under normothermic and hypothermic conditions with and without MP. The Mann–Whitney *U*-test was used to compare the difference in IL-6 and LDH between the different groups. Differences were considered significant at $p < 0.05$.

Using a dynamic time–temperature protocol (Fig. 1A), we investigated the effects of deep hypothermia and rewarming on the cell survival of primary astrocytes, BV-2 microglial cells and primary neurons. Compared to controls, deep hypothermia (2 h at 17°C) and rewarming (2 h up to 37°C) significantly increase cytotoxicity of primary astrocytes (Fig. 1B), BV-2 microglial cells (Fig. 1C) and primary neurons (Fig. 1D). After 24 h of normothermia (37°C) the cytotoxicity in the cultures of primary

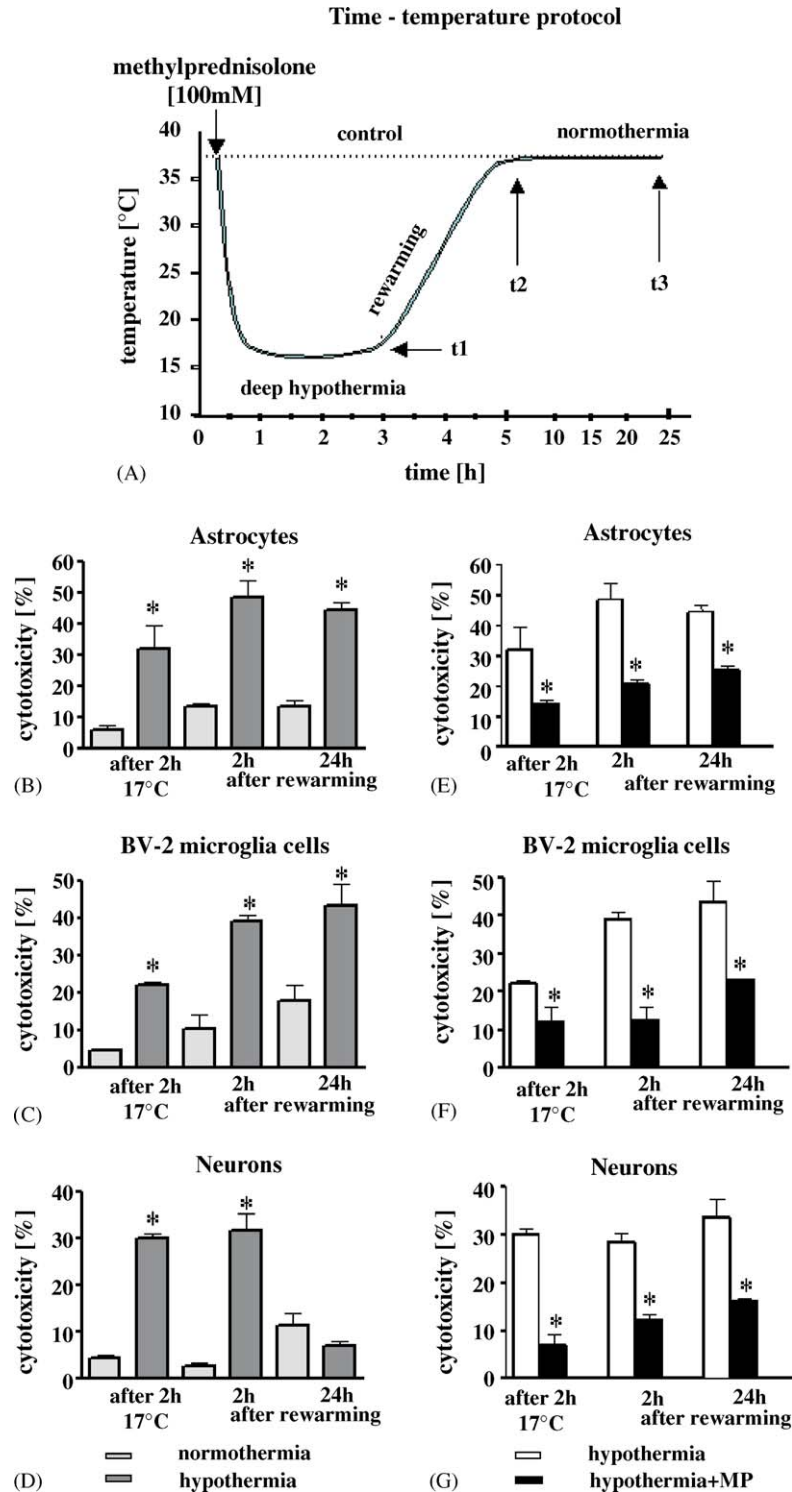


Fig. 1. (A) Time–temperature protocol. The time–temperature protocol simulates the *in vivo* temperature situation used for neuroprotection during cardiopulmonary bypass surgery. Primary cultures of neonatal cortical neurons, astrocytes, BV-2 microglial cells were used in a specially designed incubator allowing continuous changes of temperature. The protocol starts with 2 h of deep hypothermia at 17 °C, followed by a re-warming phase for 2 h up to 37 °C (0,17 °C/min), followed by 20 h normothermia (37 °C). Each experiment was performed three times and twelve 6-well plates of single cells cultures were prepared for each experiment. (B–D) Deep hypothermia promotes cell death of isolated brain cells. Deep hypothermia (2 h at 17 °C) and re-warming (2 h up to 37 °C) significantly increase cell death of primary astrocytes (B), BV-2 microglial cells (C) and primary neurons (D). After 24 h of normothermia (37 °C) the LDH release of primary astrocytes (B) and BV-2 microglial cells (C) is still significantly higher than in normothermic control cultures. (E–G) Methylprednisolone reduces deep hypothermia-induced cell death in isolated brain cells. After pretreatment with MP deep hypothermia-induced cytotoxicity is significantly suppressed and significantly more primary astrocytes (E), BV-2 microglial cells (F) and neurons (G) survive deep hypothermic conditions compared to controls without MP treatment. * $p < 0.05$.

astrocytes (B) and BV-2 microglial cells (C) is still significantly higher than in normothermic controls.

MP has no influence on cell survival of normothermic astrocytes, BV-2 microglial cells and primary neurons (not shown). In hypothermic primary astrocytes (Fig. 1E), BV-2 microglial cells (Fig. 1F) and primary neurons (Fig. 1G) treatment with MP significantly suppresses the cytotoxicity effect of deep hypothermia at all time points.

Under normothermic conditions there is minimal release of the pro-inflammatory cytokine IL-6 from primary astrocytes (Fig. 2A), BV-2 cells (Fig. 2B) and primary neurons (Fig. 2C) in single-cell cultures ($n = 30$). All measured levels are low concentrations and serve as controls in comparison to cells incubated according to the time–temperature protocol. Pretreatment with MP (100 mM) has no substantial effect on IL-6 release of normothermic cultures ($n = 30$) of primary astrocytes (Fig. 2A), BV-2 microglial cells (Fig. 2B) and primary neurons (Fig. 2C).

Compared to normothermic control cell cultures ($n = 30$) astrocytes (Fig. 2D), BV-2 microglia cells (Fig. 2E) and neurons (Fig. 2F) subjected to 2 h of deep hypothermia (17 °C) release significantly higher levels of IL-6 immediately after the hypothermic period. The highest IL-6 concentrations are found in astrocyte cultures (Fig. 2D). After rewarming up to 37 °C, IL-6 release by primary astrocytes (Fig. 2D) and BV-2 microglial cells (Fig. 2E) increases again in comparison to levels measured in baseline controls and immediately after 2 h of deep hypothermia. In contrast, neuronal cells release unchanged amounts of IL-6 (Fig. 2F).

Pretreatment with MP is associated with significant attenuation of IL-6 release from all brain cells after deep hypothermia and rewarming (Fig. 2D and F). The suppressive effect of MP on neuronal cells (Fig. 2F) is less pronounced than on astrocytes (Fig. 2D) and BV-2 microglial cells (Fig. 2E).

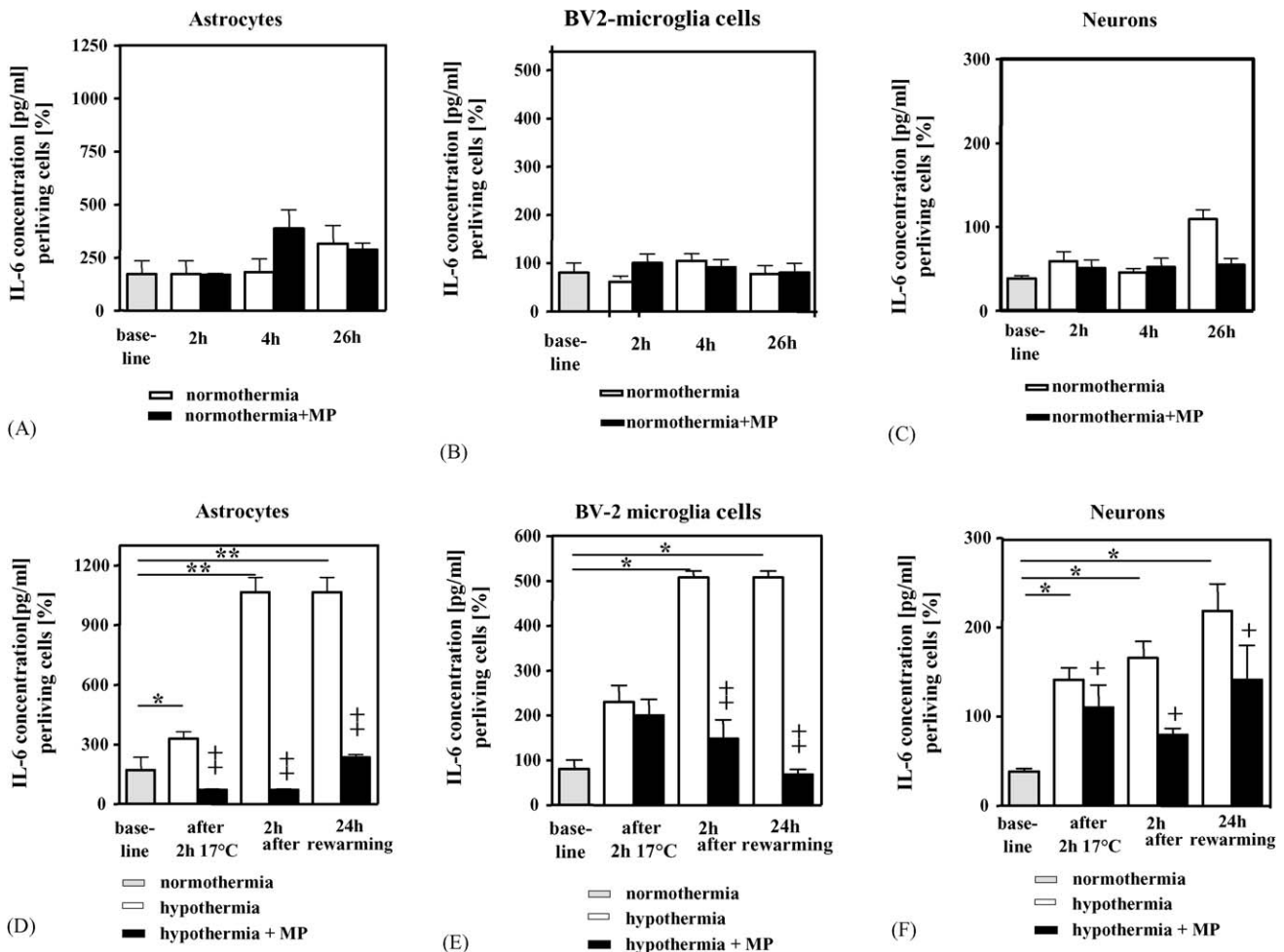


Fig. 2. Effects of methylprednisolone on hypothermic and normothermic primary astrocytes, primary neurons and BV-2 microglial cells. (A–C) Under normothermic conditions MP has no effect on the IL-6 release of primary astrocytes (A), BV-2 microglia cells (B) and neurons (C) over the study period of 24 h incubation. (D–F) Primary astrocytes secrete significantly higher levels of IL-6 after 2 h of hypothermia and again after rewarming compared to normothermic control cells (D). BV-2 microglia cells secrete significantly higher levels of IL-6 after 2 h of hypothermia, rewarming and 24 h of normothermia compared to control cells kept under normothermic conditions (E). Furthermore, after 2 h of rewarming there is a significant increase in IL-6 release compared to IL-6 release after deep hypothermia (E). Primary neurons secrete significantly higher levels of IL-6 at all time points in comparison to baseline (F). MP significantly reduces IL-6 secretion in all brain cells. Under deep hypothermic conditions MP significantly reduces IL-6 levels of hypothermic primary astrocytes at all time points (D). In BV-2 microglial cells, after rewarming the cells back to 37 °C and after 24 h normothermia, MP treatment led to significantly lower levels of IL-6 (E). In primary neurons, MP also significantly reduces IL-6 secretion in the hypothermia-treated cells at all time points (F). * $p < 0.05$, ** $p < 0.01$ compared to baseline levels during normothermia, + $p < 0.05$, ++ $p < 0.01$ compared to hypothermic cells without MP pretreatment at the corresponding time points.

Brain injury and altered psychomotor development are still important complications after cardiac surgery in pediatric and adult patients. The etiology of such brain cell injury is multifactorial and still needs extensive elucidation *in vivo* and *in vitro*. Here, we have investigated the effect of deep hypothermia and MP on neonatal brain cells. We could demonstrate that deep hypothermia leads to increased cytotoxicity, which is suppressed by MP.

Inflammatory response to hypothermic perfusion may be one of the contributing factors. In addition to improving the conventional hemodynamic parameters of cardiopulmonary bypass, such as hematocrit, perfusion patterns, acid and base management as well as cooling patterns [13,14,30,31], pharmacological pretreatment with steroids is still routinely used to attenuate the inflammatory response after CPB. However, inflammation in the brain is considered a “double-edged sword”, in that inflammatory cells and their mediators may exert protective as well as detrimental effects [21,28,33]. Interleukin-6 (IL-6) is a good example of a pro-inflammatory cytokine that may protect the CNS from degeneration [26,27,36] and supports the regeneration of the central nervous system after injury [8,25]. During CPB, there is an increase of IL-6 levels, which are substantially reduced by preoperative administration of MP [10]. Thus, pretreatment with steroids before surgery may suppress neuroprotective and pro-regenerative effects of IL-6 and understanding the effect of steroids in association with deep hypothermia on the morphology and function of different immature brain cells is fundamental.

Deep hypothermia has been suggested to reduce global cell metabolism. However, deep hypothermia and rewarming induced a significant increase of IL-6 release in a cell type-dependent manner. Compared to BV-2 microglia cells and neuronal cells, astrocytes released the highest amount of IL-6 under normothermic conditions and significantly more after hypothermia and rewarming. In the CNS, astrocytes are a major inducible source of interleukin-6 (IL-6). Since IL-6 exerts neuroprotective [26,27,36] and neuroregenerative effects [8,25] it is feasible that hypothermia-induced IL-6 upregulation may help to control and to compensate for hypothermia-induced cytotoxicity during and after rewarming.

Interestingly, deep hypothermia as an independent factor induced consistently cytotoxicity in all three types of brain cells. Similarly, in clinical settings deep hypothermic rather than normothermic CPB has been found to induce endothelial dysfunction and to trigger the inflammatory system during cardiac surgery [9,12,37,38]. Deep hypothermic circulatory arrest is associated with endothelial dysfunction in cerebral microvessels and apoptosis. Furthermore, vascular endothelial dysfunction and apoptosis may be involved in the pathophysiology of multi-organ failure after deep hypothermia [9]. It is important to note that hypothermia may exert cytotoxic effect on healthy neonatal cells, although there is ample evidence that hypothermia is neuroprotective under ischemic conditions [7]. In particular, in the treatment of perinatal hypoxia mild hypothermia is a well accepted neuroprotective strategy. In contrast, in the context of corrective pediatric cardiac surgery healthy neonatal brains are cooled down – normally in the absence of ischemic conditions.

Therefore, it is important to study the different effects of deep hypothermia on healthy and ischemic brain cells.

Methylprednisolone (MP) is one of the anti-inflammatory drugs commonly used in cardiac surgery [16]. Preoperative administration of MP diminishes the increase in levels of IL-6 and tumor necrosis factor- α (TNF α) after CPB [10] and has been discussed as a protective strategy to reduce CPB-induced systemic inflammation. However, the effects of MP in the brain are controversial [10,29,32,35].

In the present study, MP pretreatment led to a significant increase of cell survival after deep hypothermia and rewarming. This effect was found consistently in all three types of brain cells. On the other hand, MP suppressed the hypothermia-induced increase of the neuroprotective and neuroregenerative cytokine IL-6. These data indicate that MP may not only prevent the cytotoxicity associated with CPB and deep hypothermia but may also suppress important protective and regenerative effects of IL-6. Another interpretation may be that the downregulation of IL-6 levels is a symptom of successful neuroprotection by MP. Thus, the clinical value of such observations should be interpreted with caution because the significance of the IL-6 downregulation by MP is not fully clear. These results should be evaluated in further studies using organotypic brain slices and *in vivo* experiments to elucidate the interaction between astrocytes, microglial cells and neurons during and after deep hypothermia and rewarming. Here, we have focused on a key neuroprotective and neuroregenerative cytokine, which is produced by all the cells studied. However, further investigations are necessary to evaluate other protective and regenerative factors in the context of hypothermia, rewarming and pretreatment with MP.

In the present study, we were able to show that MP pretreatment improves cell survival after deep hypothermia but also suppresses the secretion of the neuroprotective and regenerative cytokine IL-6. These data suggest that more specific immune modulation than that achieved by MP administration is needed to protect the brain during neonatal and paediatric cardiac surgery.

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