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Fast rewarming after deep hypothermic circulatory arrest in rats impairs histologic outcome and increases NFκB expression in the brain

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Abstract

Objective: Deep hypothermia is used as a neuroprotectant during cardiac surgery utilizing deep hypothermic circulatory arrest (DHCA), although the ideal rewarming strategy is not known yet. Some of the neuroprotective properties of hypothermia seem to be mediated by Nuclear Factor Kappa B (NFκB) as an important transcription factor. The current study was designed to investigate the effect of the rewarming rate on histologic outcome and cerebral NFκB expression one day following DHCA in rats.

Methods: With IRB approval, 20 rats were cannulated for cardiopulmonary bypass (CPB), cooled to a rectal temperature of 15-18°C, subjected to 45min of DHCA and randomly assigned to either a slow (40 min) or a fast (20 min) rewarming protocol. At 24 hours post DHCA, the number of eosinophilic neurons was analyzed with hematoxylin and eosin (HE) staining, and NFκB expression immunohistochemically. The two experimental groups were compared with untreated control rats.

Results: HE staining showed more eosinophilic neurons in the motor cortex following fast rewarming (60 [15-388]) compared to slow rewarming (15 [10–21]) ($p<0.05$). Neuronal expression of NFκB was increased in the fast rewarming group in both brain areas, the motor cortex (fast: 258 [135-393]; slow: 165 [80–212]; control: 73 [44-111]) as well as the hippocampus (fast: 243 [209-314]; slow: 202 [187-239]; control: 86 [68-108]) ($p<0.05$). Hyperthermic episodes were strictly avoided.

Conclusions: Fast rewarming with strict avoidance of hyperthermia after DHCA in rats was accompanied by pronounced histologic damage and accentuated cerebral NFκB expression.

Introduction

Cardiac surgery requiring circulatory arrest employs deep hypothermia as the neuroprotectant in order to reduce the risk of global cerebral ischemia, although an ideal temperature management especially for rewarming has not been clearly defined yet. In this context, the rewarming period has been shown to be of particular importance as the neuroprotective effect of deep hypothermia might be counteracted by hyperthermic episodes during this phase, presenting a risk factor for neurologic injury. However, whether the rewarming rate per se is harmful for the brain in the absence of hyperthermia after DHCA is not known yet.

The rat model of CPB with DHCA allows investigation of cerebral inflammation and cerebral histologic outcome. A previous study has shown significant histologic damage and a maximum expression of nuclear Factor Kappa B (NFκB) on postoperative day one[1,2]. In this

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context, NFκB expression is of interest, as Yenari and colleagues showed an inhibitory influence of hypothermia on NFκB activity and consequent inflammatory response, suggesting that the protective effect of hypothermia is, in part, related to an inhibition of NFκB[3]. Considering the influence temperature has on NFκB expression and the role this important transcription factor plays within the inflammatory cascade, its investigation within differing rewarming strategies seems to be of particular interest.

The current study was performed to investigate whether, in the absence of hyperthermia, the rewarming strategy has an impact on (1) cerebral histologic outcome and (2) on neuronal NFκB expression one day following DHCA in rats.

Methods

Study design

After institutional animal care committee approval, male Sprague Dawley rats (330 – 400g, Charles River, Sulzfeld, Germany) were housed under standard laboratory conditions: 12 h light / 12 h dark, 22°C, 60% humidity and free access to water and standard rat chow three weeks prior to the experiments for acclimatization.

Animals were randomly assigned to one of two groups: a slow or a fast rewarming group (n=10 each). These two experimental groups were compared to untreated control animals which were neither anesthetized nor cannulated, but sacrificed in order to harvest brain tissue for further analyses (n=10, "control" group). One day following DHCA, the rats were sacrificed. In the harvested brains, histologic damage was analyzed using hematoxylin and eosin (HE) staining and neuronal NFκB expression was determined using immunohistochemistry.

Cardiopulmonary bypass and deep hypothermic circulatory arrest

Rats were intubated and mechanically ventilated with oxygen in air FiO_2 = 40% ($PaCO_2$ at 32-40 mmHg). All instruments were disinfected, catheters gas sterilized and surgery was carried out in an aseptic manner. Surgical sites were infiltrated with 2% xylocaine. During surgical preparation, anesthesia was maintained with 2-2.5% isoflurane and 5μg fentanyl boluses. One hundred and fifty international units of heparin were administered. The tail artery (aortic inflow), the right superficial epigastric artery (blood pressure monitoring) and the right external jugular vein (venous drainage) were cannulated as previously described[2]. Pericranial and rectal temperatures were monitored.

CPB was set up as previously described[2]. It consisted of a venous reservoir, a peristaltic pump, a membrane oxygenator with integrated water quench and an arterial inflow cannula, all connected via 1.6 mm internal diameter plastic tubing. An in-line flow probe (2N806 flow probe and T208 volume flowmeter; Transonics Systems, Inc., Ithaca, NY) was used to continuously measure CPB flow. CPB was instituted at a flow rate of 160-180 ml/min/kg and was consecutively decreased by half during the cooling period. With the rectal temperature reaching 15-18°C after 30 min, the roller pump was turned off and venous blood was drained to the reservoir. Circulatory arrest, as confirmed by asystole and no mean arterial pressure (MAP), was maintained for 45 min at 15-18°C. With the reinstitution of CPB, animals were rewarmed according to group assignment as described below. After a rectal temperature of at least 35.5°C was reached, CPB was terminated.

During CPB, anesthesia consisted of 0.8-1% isoflurane, cisatracurium (1.6 mg/h) and fentanyl boluses (5μg). A continuous positive airway pressure mode (5 cmH_2O, FiO_2 = 0.21) was applied to avoid atelectasis. During DHCA, anesthesia was discontinued. During rewarming, MAP was kept above 50 mmHg by norepinephrin, as soon as a rectal temperature of 30 °C and a blood flow of 150 ml/min/kg were achieved. Arterial blood gas values were controlled using the pH-stat strategy ($PaCO_2$ of 31-40 mmHg).

Following decannulation, rats remained anesthetized with 1.0-1.5% isoflurane, intubated and ventilated for one hour (rectal temperature of 36.5°C). Bicarbonate was administered to treat acidosis and calcium was injected to prevent a drop of ionized calcium below 1 mmol/l. The heparin-induced anticoagulation was allowed to dissipate spontaneously. When animals resumed spontaneous breathing, the tracheas were extubated and the rats put for continuous observation into a transparent, oxygen-enriched box for 12 hours with free access to water and food. Animals were returned to their cages on the first postoperative day and housed in their familiar groups. Twenty-four hours following DHCA, rats were anesthetized, serum samples harvested and brains removed, snap frozen on dry ice and stored at minus 80°C for further analyses.

Rewarming protocols

Before reconnecting to CPB, 100 IU heparin were added to the circuit. CPB restarted at a rectal temperature of 15-18°C and a flow rate of 105 ml/min/kg. The in-flow rate was gradually increased so that it reached 120 ml/kg/min at 20°C, 150 ml/kg/min at 30°C and the full rate of 160-180 ml/kg/min at the end of the rewarming phase. After reconnection of CPB, rats were rewarmed using a water bath integrated in the oxygenator, a heating blanket and a convective forced-air heating system.

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- *slow rewarming protocol (40 min):* Rectal temperature increased to 27-28°C over 15 min at a rate of 0.6-0.9°C/min, to 32.5°C over 10 minutes at a rate of 0.5°C/min and to 35.5°C over 15 min at a rate of 0.1-0.2°C/min. The maximum temperature of in-flow blood was 35.5°C, the gradient between oxygenated blood temperature and rectal temperature was, at a maximum, 4°C. After a rectal temperature of 35.5°C was reached, CPB was terminated. During the whole rewarming period, hyperthermic temperatures (pericranial and rectal) were avoided.
- *fast rewarming protocol (20min):* Rectal temperature increased gradually with 0.7-1°C/min over 20 min. The maximum in-flow blood temperature was 38.5°C, the gradient between oxygenated blood temperature and rectal temperature was, at a maximum, 5°C. After a rectal temperature of 35.5°C was reached, CPB was terminated. During the whole rewarming period, hyperthermic temperatures (pericranial and rectal) were avoided.

Assessment of histological outcome

Cell damage in the motor cortex and hippocampus was assessed in brain slices (10 µm, bregma –3.3) stained with hematoxylin and eosin (HE) by the number of eosinophilic neurons throughout the motor cortex and the hippocampus (x 400) of the rats sacrificed 24 hours postoperatively.

Assessment of biochemical outcome

To detect NFκB (polyclonal rabbit anti- NFκB p65, Abcam, Cambridge, UK) -positive neurons in both the motor cortex and the hippocampus, an immunohistochemical double staining was performed (10µm, bregma –3.3). Neurons were labeled, using an anti-neuronal nuclei antibody (Mouse Anti-Neuronal Nuclei Monoclonal Antibody, Chemicon International, Temecula, CA, USA). NFκB-positive neurons were counted throughout both regions within five high magnification fields (x400) per region.

Statistics and data management

Physiologic values were analyzed, using general linear models with the between-groups factor rewarming (slow versus fast), the within-groups factor time and their interaction term ($time^2 \times$ rewarming) followed by one factorial ANOVA. Effects of time levels were analyzed quadratically ($time^2$), focusing on biphasic changes of physiologic variables during the observation period. The number of eosinophilic neurons and the number of NFκB positive neurons were analyzed using non-parametric analyses of variance with the Kruskal-Wallis test followed by the Mann-Whitney U-test. Figures display box and whisker plots while, in the result section, the values are given as the median [10%-90% percentile]. Statistical analyses were performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Three animals were excluded from further data analysis due to cannulation problems or insufficient venous return. Those animals were replaced to keep the sample size equal. Physiological values of rats are summarized in Table 1. Rectal temperature was controlled, according to the experimental protocol, with strict avoidance of hyperthermia. Pericranial temperatures were comparable between the two groups, again without any hyperthermic episodes. Priming the circuit with 6% hetastarch led to a drop in hemoglobin concentration, which was restored by retransfusion of centrifuged whole blood following CPB. The higher PaO_2 during CPB is due to a high concentration of O_2 in the gas mixture. The amount of norepinephrine administered was comparable between groups, as was the amount of bicarbonate (see Table 1).

Eosinophilic neurons were increased in both investigated brain regions of animals subjected to DHCA compared to untreated controls. The fast rewarming group showed more eosinophilic neurons in the motor cortex compared to the slow rewarming group (fast: 60 [15-388]; slow: 15 [10–21]; Figure 1A and B, $p < 0.05$). Immunohistochemistry showed more NFκB-positive neurons in the motor cortex and the hippocampus of animals subjected to DHCA compared to untreated controls. Rats subjected to the fast rewarming protocol showed significantly more NFκB-positive neurons than rats of the slow rewarming group in the motor cortex (fast motor cortex: 258 [135-393]; slow motor cortex: 165 [80–212]; control motor cortex: 73 [44-111]) and in the hippocampus (fast hippocampus: 243 [209-314]; slow hippocampus: 202 [187-239]; control hippocampus: 86 [68-108]; Figure 2A and B, $p < 0.05$).

Discussion

Fast rewarming after DHCA impaired histologic outcome on postoperative day one compared to slow rewarming. This impaired histologic outcome was accompanied by an increased expression of neuronal NFκB after fast rewarming.

The recovery model of DHCA in rats induces neuronal damage and cerebral inflammation dependent on the duration of DHCA[2]. In the current study, 45 min of circulatory arrest were chosen in order to induce a

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Table 1. Physiologic data during the operative procedure in two groups of 45 min of deep hypothermic circulatory arrest (DHCA) followed by slow or fast rewarming. Variables were obtained prior to cardiopulmonary bypass (pre-CPB), prior to DHCA (pre-DHCA), 5 min following restart of CPB (post-DHCA), 5 min before cessation of CPB (CPB end) and 1h after CPB (post-CPB (1h)). Data are presented as means ± SEM. Some values were controlled within defined ranges at certain times and, therefore, they are not presented and not statistically analyzed: (1) Mean arterial pressure was controlled above 50 mmHg by repeated 1-2 µg norepinephrine boluses with a total of 10 ± 2 µg (slow rewarming) and 8 ± 6 µg (fast rewarming) (t test n.s.) (2) base excess (BE) was controlled between –3 and +3 mmol/l by total doses $NaHCO_3$ of 73.9 ± 5.3 mmol (slow rewarming) and 72.2 ± 7.5 mmol (fast rewarming) (t test n.s.). Respective p-values (n.s.: not significant) indicate statistically significant changes during the observation period within each group ($time^2$) and between groups related to the time course ($time^2$ x rewarming)

	Group	pre-CPB	pre-DHCA	post-DHCA	CPB-end	post-CPB (1h)	$time^2$ x rewarming	$time^2$
mean arterial pressure [mmHg]	slow rewarming fast rewarming	89 ± 1 86 ± 4	39 ± 1 42 ± 1	46 ± 3 48 ± 5	1)	89 ± 3 87 ± 3	n.s.	p<0.01
hemoglobin [mg/dl]	slow rewarming fast rewarming	14.0 ± 0.2 14.1 ± 0.2	6.7 ± 0.2 6.8 ± 0.3	6.3 ± 0.2 6.3 ± 0.3	7.0 ± 0.2 6.3 ± 0.3	10.5 ± 0.2 10.8 ± 0.2	n.s.	p<0.01
PaO_2 [mmHg]	slow rewarming fast rewarming	163 ± 10 171 ± 9	514 ± 23 523 ± 29	365 ± 15 366 ± 13	313 ± 35 330 ± 24	210 ± 17 192 ± 13	n.s.	p<0.01
$PaCO_2$ [mmHg]	slow rewarming fast rewarming	35 ± 1 36 ± 2	35 ± 1 36 ± 1	33 ± 1 33 ± 1	40 ± 1 38 ± 1	35 ± 1 37 ± 2	n.s.	n.s.
BE [mmol/l]	slow rewarming fast rewarming	1.9 ± 0.8 2.0 ± 0.8	–2.4 ± 0.7 –1.6 ± 0.8	–6.3 ± 0.7 –5.6 ± 1.0		2)	n.s.	p<0.01
glucose concentration [mg/dl]	slow rewarming fast rewarming	114 ± 6 132 ± 8	200 ± 21 198 ± 13	282 ± 23 306 ± 22	304 ± 37 320 ± 22	193 ± 20 204 ± 18	n.s.	p<0.01
pericranial temperature [°C]	slow rewarming fast rewarming	36.1 ± 0.2 35.6 ± 0.2	16.7 ± 0.4 18.1 ± 0.2	19.9 ± 0.2 20.9 ± 0.4	35.5 ± 0.1 35.6 ± 0.1	36.3 ± 0.1 36.5 ± 0.1	n.s.	p<0.01
rectal temperature [°C]	slow rewarming fast rewarming	36.2 ± 0.2 35.6 ± 0.2	16.9 ± 0.1 17.0 ± 0.2	20.0 ± 0.0 20.2 ± 0.2	35.3 ± 0.1 35.3 ± 0.1	36.5 ± 0.0 36.5 ± 0.0	n.s.	p<0.01

minor cerebral insult with higher survival rate, but sufficient impact on histologic damage, as well as cerebral inflammatory reaction, to detect any influence the rewarming protocol might have on those parameters. As intended, this model of DHCA induced both a prominent histologic damage and activation of the inflammatory cascade, as depicted by the up-regulation of NFκB. We based the decision to investigate histologic outcome and NFκB-expression 24 hours following DHCA on own data: while histologic damage persisted from postoperative day 1 until day 28, NFκB-expression in hippocampal neurons peaked at the first postoperative day and was within normal ranges from postoperative day 3 to 28[1]. These results highlight the first postoperative day as the most vulnerable period to study the impact of potentially neurodestructive or neuroprotective rewarming strategies.

As hypothesized, the fast rewarming protocol led to higher numbers of damaged neurons one day following DHCA compared to the slow rewarming protocol. This is in accordance with a clinical trial where fast rewarming after mild hypothermic CPB has been shown to have an adverse effect on neurocognitive outcome[4]. However, this effect was attributed to higher peak temperatures in the fast rewarming group, with well-known deleterious effects of hyperthermia on brain function. In the current study, hyperthermia (pericranial and rectal) was strictly avoided, questioning hyperthermia as the sole culprit for adverse cerebral outcome. As a consequence, factors such as the perfusate temperature, the gradient between perfusate and body temperature, or abnormalities in flow-metabolism coupling need to be considered as well. Perfusate temperature is discussed to closely reflect brain temperature shortly after the onset of rewarming[5]. However, in our study, pericranial temperature (closely reflecting brain temperature[6]) was comparable in both groups at any time within rewarming, although perfusate temperature differed between groups. These results question the assumed relationship between perfusate and brain temperature. Taking a look at the role of perfusate to body temperature gradient in our study, it just marginally differed between groups and was, as recommended, kept well below 10°C in both groups[5]. Whether fast rewarming leads to a mismatch between brain metabolism and cerebral blood flow, as speculated by Enomoto and colleagues[7], cannot be answered with this study and remains speculative. Of particular interest, though, is the fact that the prolonged CPB duration of 20 minutes in the slow rewarming group did not show any detrimental effect, even though some clinical studies

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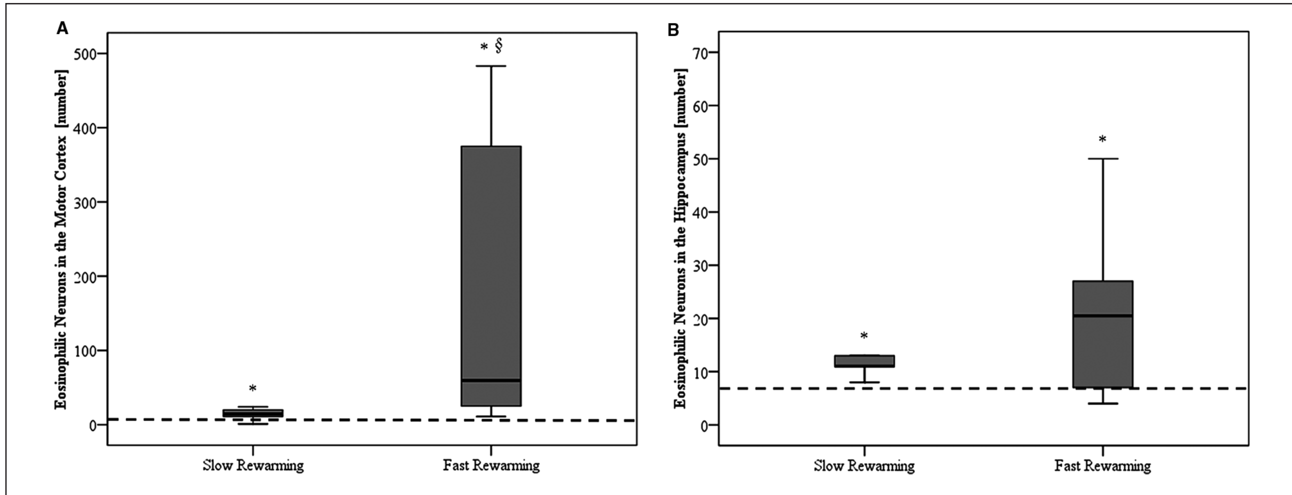

Figure 1. Number of eosinophilic neurons in the motor cortex and the hippocampus counted in five high-magnification fields (x400) on the first postoperative day after 45min of DHCA followed by slow or fast rewarming

In the motor cortex (1A) as well as in the hippocampus (1B), the number of eosinophilic neurons was elevated compared to untreated controls. Additionally, there was a significant increase in eosinophilic neurons in the motor cortex of the fast rewarming group compared to the slow rewarming group. Data are presented as median ± percentile, dashed line marks 95% percentile of untreated control animals, * = $p<0.05$ vs. untreated control animals, § = $p<0.05$ vs. slow rewarming.

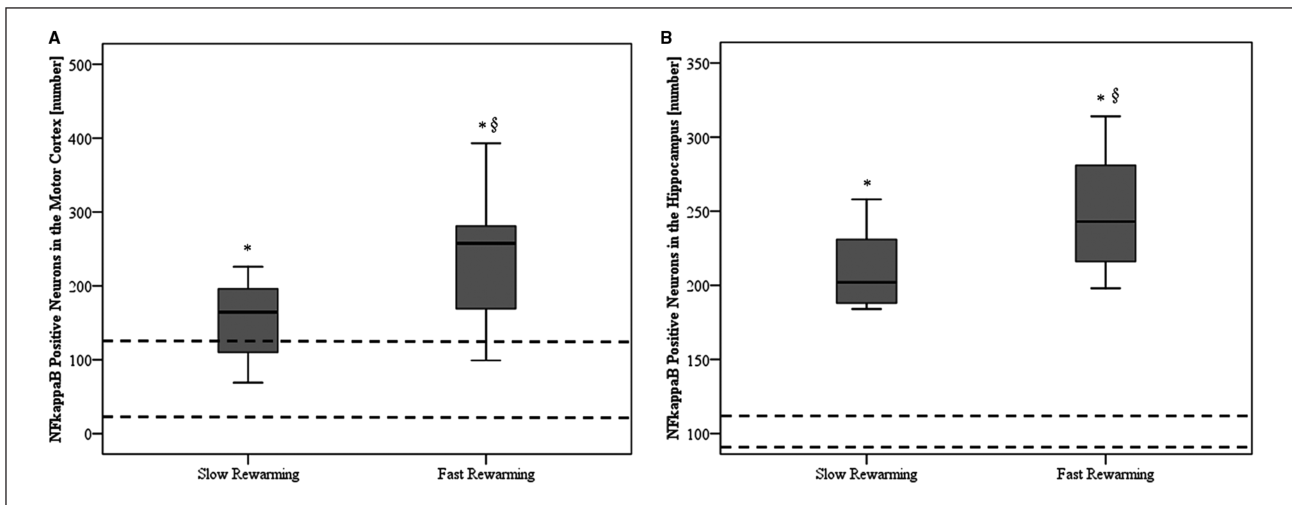

Figure 2. Number of NFκB positive neurons in the motor cortex and the hippocampus counted in five high-magnification fields (x400) on the first postoperative day after 45min of DHCA followed by slow or fast rewarming

In the motor cortex (2A) as well as in the hippocampus (2B), the number of NFκB positive neurons was elevated compared to untreated controls. There was a significant difference between the two rewarming groups for both brain regions. Data are presented as median ± percentile, dashed lines mark the 5 and 95% confidence interval of untreated control animals, * = $p<0.05$ vs. untreated control animals, § = $p<0.05$ vs. slow rewarming.

showed a correlation between duration of CPB and the magnitude of systemic inflammatory reaction[8, 9].

The increase in eosinophilic neurons in the fast rewarming group was accompanied by an accentuated NFκB expression. NFκB undoubtedly plays a crucial role in the inflammatory cascade, as it influences gene transcription and the subsequent release of pro-inflammatory cytokines[10]. This NFκB expression can be affected by temperature, with hypothermia attenuating NFκB activity through differing mechanisms, depending on the sort of ischemia (focal or global)[3, 11]. Our results suggest that not only temperature per se affects NFκB expression, but also rewarming plays an important role.

Finally, some important limitations of our study need to be discussed. This rodent recovery model was established to study the isolated effect of DHCA and CPB on cerebral outcomes. Therefore, we used young and healthy animals, without any co-morbidities, congenital heart

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disease or preoperative hypoxemia that, in the clinical setting of cardiac surgery using DHCA, were identified as risk factors for adverse cerebral outcome[13]. We did not perform a sternotomy, cardiac surgery or direct cardiac cannulation, thus, keeping the surgical trauma at a minimum in order to allow the survival of the animals. Finally, we chose to set the endpoint of our experiment at 24 hours following DHCA to allow the assessment of maximum histologic damage and NFκB expression in the brain. Therefore, further investigations of other parameters, such as neurologic performance, was not possible, leaving the question unanswered, whether the rewarming strategy would have an impact on long-term outcome.

In summary, fast rewarming after DHCA in young rats led to pronounced histologic damage and accentuated cerebral NFκB expression compared to slow rewarming. The adverse effect of fast rewarming in this study cannot be attributed to hyperthermia, suggesting factors beyond hyperthermia during rewarming as contributors to adverse cerebral outcome.

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