Neuroprotective effects of deep hypothermia in refractory status epilepticus

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Abstract

Objective: Pharmacoresistance develops quickly during repetitive seizures, and refractory status epilepticus (RSE) remains a therapeutic challenge. The outcome of RSE is poor, with high mortality and morbidity. New treatments are needed. Deep hypothermia (20°C) is used clinically during reconstructive cardiac surgery and neurosurgery, and has proved safe and effective in those indications. We tested the hypothesis that deep hypothermia reduces RSE and its long-term consequences.

Methods: We used a model of SE induced by lithium and pilocarpine and refractory to midazolam. Several EEG measures were recorded in both hypothermic (n = 17) and normothermic (n = 20) animals. Neuronal injury (by Fluoro-Jade B), cell-mediated inflammation, and breakdown of the blood–brain barrier (BBB) (by immunohistochemistry) were studied 48 h following SE onset.

Results: Normothermic rats in RSE seized for 4.1 ± 1.1 h, and at 48 h they displayed extensive neuronal injury in many brain regions, including hippocampus, dentate gyrus, amygdala, entorhinal and pyriform cortices, thalamus, caudate/putamen, and the frontoparietal neocortex. Deep hypothermia (20°C) of 30 min duration terminated RSE within 12 min of initiation of hypothermia, reduced EEG power and seizure activity upon rewarming, and eliminated SE-induced neuronal injury in most animals. Normothermic rats showed widespread breakdown of the BBB, and extensive macrophage infiltration in areas of neuronal injury, which were completely absent in animals treated with hypothermia.

Interpretation: These results suggest that deep hypothermia may open a new therapeutic avenue for the treatment of RSE and for the prevention of its long-term consequences.

Introduction

While we have made considerable progress in treating epilepsy, status epilepticus (SE) remains a therapeutic challenge. SE has an incidence of 10–41/100,000.1,2 Mortality was over 50% in the VA Cooperative Study,3 27% in population-based studies in Virginia4 and 11–24% in other studies.5 Morbidity is considerable, particularly in the elderly. Almost a quarter of survivors experience deterioration in their functional outcome,6 with 10% requiring long-term care,7 6% developing an associated chronic encephalopathy,8 and 41% ultimately developing epilepsy.9

Drugs fail to stop SE in 31–53% of cases.3,10,11 During SE, pharmacoresistance develops progressively. The anticonvulsant potency of benzodiazepines can decrease 20-fold in 30 min of seizures.12 Phenytoin and barbiturates also lose potency, but more slowly.13 In clinical studies, early treatment of SE is much more effective than late treatment, suggesting pharmacoresistance. In the VA Cooperative Study,3 four treatments were randomly rotated; the first treatment was successful in 53% of patients, the third in 2% of patients. Seizure-induced trafficking of synaptic GABA_A and glutamate receptors may in part explain the development of time-dependent pharmacoresistance.14 Refractory SE (RSE) defined by refractoriness to at least two drugs, and super-refractory SE (SRSE), defined by failure to respond to adequate treatment for at least 24 h, have become commonplace in
Intensive Care Units, at an enormous cost and with very poor outcomes.15

We need an alternative treatment for RSE/SRSE, and our results suggest that hypothermia could be that treatment. Hypothermia acts by a completely different mechanism than anticonvulsant drugs, and may be able to stop RSE. Mild hypothermia reduces seizure activity in experimental animals,16–18 although seizures often recur upon rewarming19 and sometimes convulsive seizures stop but EEG continues to show seizure activity.20 Successful treatment of clinical SE with mild hypothermia has been reported.18,21,22 However, hypothermia failed to reduce the incidence of neonatal seizures following hypoxia-ischemia.23

Deep hypothermia has not been studied extensively for the treatment of RSE, in spite of the demonstration that cooling to 23°C stops kainate seizures better than cooling to 28°C19 and of reports of partial success in preventing seizure recurrence upon rewarming.20,24 However, it failed to prevent epileptogenesis following experimental SE.25 Recent developments in ICU technology have reduced the complications of hypothermia. Mild hypothermia has become routine treatment for neonatal hypoxic-ischemic encephalopathy,26 and traumatic brain injury.27 It has been used extensively for postcardiac arrest encephalopathy,28 although recent studies do not support its usefulness in children29 or adults.30

Deep hypothermia is routinely used to protect the brain or spinal cord when circulatory arrest is needed in cardiac surgery,31 and neurosurgery,32 although it has significant complications, including increased risks of bleeding, coagulopathy, and infection.33–35 Today, the technology for delivering mild hypothermia is available in most hospitals, and the technology for delivering deep hypothermia is available in major surgical centers. We studied deep hypothermia in the treatment of benzodiazepine-refractory experimental SE, and found that it is very effective in stopping seizures and reducing SE-associated neuronal injury, cell-mediated inflammation, and breakdown of the blood–brain barrier (BBB).

### Materials and Methods

#### Animals

Male Wistar rat (300–400 g; Charles River, MA) were used. Rats were housed in a temperature- and humidity-controlled room with 12 h light-dark cycles (7 AM–7 PM) and had free access to food and water. All experiments were conducted with the approval and in accordance with the regulations of the Institutional Animal care and Use Committee of West Los Angeles VA Medical Center.

#### Induction of SE and drug treatment

Rats were administered lithium chloride (3 mEq/kg, #L-0505; Sigma, St. Louis, MO) subcutaneously and, 16 h later, SE was induced with i.p. pilocarpine hydrochloride (60 mg/kg, #P6503; Sigma). Only lithium/pilocarpine-treated rats displaying behavioral/EEG seizures were used. All rats received scopolamine methyl bromide (1 mg/kg, #S8502; Sigma), a muscarinic antagonist that does not cross the BBB, at the same time as pilocarpine, to decrease peripheral cholinergic effects such as pulmonary secretions. The seizures occurred 8–20 min after pilocarpine injection, so that time from pilocarpine injection to midazolam treatment was ~30 min. The time between the second stage 3 or higher seizure and the onset of continuous polyspikes was both short and reproducible (1.28 ± 1.1 min, n = 16). The second stage 3 seizure was chosen as our time anchor to insure that all rats were in full SE. The animals subsequently received scopolamine (2 mg/kg i.p.; #S1013; Sigma) to remove the original seizure trigger without stopping SE, and midazolam (3 mg/kg; Caraco Pharmaceutical Laboratories Ltd, Detroit, MI) i.p. 12 min after the second major seizure (stage 3 or higher) to make sure that pharmacoresistance and self-sustaining seizures were well established. In this study, RSE was refractory to one drug (the usual clinical definition is two drugs), which is sufficient for proof-of-principle of the use of hypothermia, and minimizes the complex interactions between multiple drugs and temperature homeostasis.

#### Deep hypothermia

In some animals, cooling was initiated after midazolam injection (“early cooling”; n = 5) (Fig. 1). In other animals, cooling was not initiated for an additional 15 min to eliminate the rats that stop seizing after drug treatment and therefore were not pharmacoresistant (“delayed cooling”; n = 17). Animals were placed in rodent restraint bags (Harvard Apparatus, Holliston, MA) so that the nose and mouth extended beyond the bag. Holes were cut in the bag to allow connection of the EEG cables to the electrode connector and placement of the thermistor rectal probe (Yellow Springs Instr., Yellow Springs, OH). The animal was then placed in an ice bag and two more ice bags were placed on the sides. The body temperature was maintained at 20°C for 30 min. At the end of the cooling period, the animal, in the bag with cables and probes still in place, was transferred to a heating pad and allowed to warm up to normal body temperature. The cables and probes were then removed and the animal released into the normal housing cage.
Temperature monitoring

The body temperature was monitored in all animals using a rectal probe. Some animals had a dental drill hole in the skull at 4 mm anterior to bregma and 2 mm left of the medial suture for brain surface temperature measurement. A guide cannula was fixed in place over the opening 1–2 weeks before SE induction, to allow placement of a thermocouple temperature probe (Physitemp, Clifton, NJ). Analog output from the rectal probe and the thermocouple probe were fed into a BioPac MP150 analog to digital converter (BioPac Systems, Goleta, CA). The digital values were calibrated against a NIST traceable reference thermometer.

EEG monitoring

Under isoflurane anesthesia, the animals were implanted with stainless steel skull screws to serve as recording electrodes. Two electrodes were used for bipolar recording and were located 3 mm anterior to lambda and 4 mm left and right of the medial suture. The third electrode served as reference and was located 1 mm anterior to bregma and 1 mm to the right of the midline defined by the medial suture. The screw electrodes were connected to a tripolar connector (Plastics One, Roanoke, VA) and dental cement used to cover the electrodes so that just the connector was exposed. Animals were used 1–2 weeks after electrode implantation.

The BioPac Systems MP150 was used to record digital EEG using a BioPac UM100A preamplifier. Sampling rate was 200 Hz. Recording was started at pilocarpine injection and was continuous for 24 h which included an initial pilocarpine segment of EEG, the development of SE, drug treatment, cooling, warming, and the overnight recovery period.

The EEG were processed offline to detect seizures and spikes using Stellate Systems Harmony (Natus, Pleasanton, CA) software with default parameters: amplitude threshold 2.7, minimum frequency 3 Hz, maximum coefficient of variation 40% for seizure detection and a spike amplitude threshold of six for spike detection.

The outcome measures were the ratio of EEG power at time divided by the average baseline EEG power (before pilocarpine), the number of seizures per 24 h, the cumulative seizure time per 24 h (time spent seizing, subtracting post- and interictal time), the number of spikes per 24 h, the time spent in high amplitude (>2× prepilocarpine baseline) EEG discharge per 24 h, and the time needed for EEG amplitude to fall below two times the pre-pilocarpine EEG amplitude for at least 1 min, which in this experimental paradigm is close to the time of termination of SE.

Tissue preparation for neuronal injury and immunohistochemical studies

Some animals of the normothermic (n = 9) and hypothermic group (delayed hypothermia; n = 9) were anesthetized with an overdose of pentobarbital (100 mg/kg i.p.) 48 h after induction of SE. Then, the animals underwent

![Figure 1](image-url)
transcardiac perfusion with 4% phosphate-buffered formaldehyde (#P-6148; Sigma). Brains were kept in situ at 4°C overnight, after which they were removed and postfixed in the same perfusate for 2–3 h. Subsequently, brains were kept in Phosphate buffer (PB) 0.1 mol/L containing 30% sucrose for 48–72 h. Floating sections (30 μmol/L thickness) were obtained using a sliding microtome.

Fluoro-Jade B staining

Coronal sections were mounted, dried, incubated in potassium permanganate solution (0.06%; w/v) for 15 min, washed and incubated in Fluoro-Jade B staining solution for 30 min. After three rinses, slides were dried overnight at room temperature, cleared three times in xylene and coverslipped with Permount medium. In hippocampal CA1, the number of injured cells was counted by unbiased stereology using the optical dissector method. The first series of one in five sections were stained with Fluoro-Jade B, and the analysis was performed using a microscope (Olympus AX70, Center Valley, PA) with a motorized stage connected to a computer running the Stereo Investigator software (MBF Bioscience, Williston, VT). A counting frame of 45 × 45 μm was randomly positioned in a sampling grid of 70 × 120 μm. In the other areas, distribution of Fluoro-jade B-positive cells was scored as follows: 0, no injury; 1, 1–30 positive cells per field; 2, 31–60 positive cells per field; 3, 61–100 positive cells per field; 4, more than 100 positive cells per field.

Immunohistochemical studies

Macrophages/monocytes were revealed using an antigen retrieval procedure (#HK080-9K; BioGenex Laboratories, San Ramon, CA), in which the coronal floating sections were heated at 80°C for 35 min in 10 mmol/L citrate solution (pH 6.0) and cooled to room temperature. Sections were then rinsed in distilled water, washed in 0.1 mol/L PB for 10 min and incubated in a blocking buffer (5% donkey serum) in 0.1 mol/L PB at room temperature for 1 h. They were then incubated overnight at 4°C with the blocking buffer containing a primary antibody: mouse monoclonal anti-macrophages/monocytes (clone ED-1; Millipore, Billerica, MA; MAB1435; 1/300) or mouse monoclonal anti-T-cell (clone 15-6A1; Santa Cruz Biotechnology, Dallas, TX; sc-52711; 1/1000), then washed in PB three times and exposed to Alexa-Fluor 488-linked donkey anti-mouse (#A21202; Life Technologies, Carlsbad, CA) immunoglobulins diluted 1:200 with the blocking solution for 2 h at room temperature.

For the detection of rat IgG, coronal sections were incubated in the same blocking buffer for 1 h and then with Alexa Fluor 488 donkey anti-rat IgG (H+L) antibody (#A21208; Life Technologies) diluted 1:200 with the blocking solution for 2 h at room temperature.

Statistical analyses

EEG and cell injury data for normothermic and hypothermic groups were analyzed with the Mann–Whitney test.
(GraphPad version 6, La Jolla, CA). Comparison of cell survival was analyzed with Fischer exact test (GraphPad version 6). Statistical significance was defined as $P < 0.05$. In all graphs, data are presented as mean ± SEM.

**Results**

**Deep hypothermia reduces EEG power and stops seizures**

We first examined the therapeutic benefit of “early” cooling, initiated just after midazolam treatment. Temperature was brought down to 20°C and maintained as near to that temperature as possible for 30 min. Mean cooling time from 37°C to 20°C was 39.5 ± 2.67 min (mean ± SEM; $n = 5$). Mean rewarming time from 20°C to 36°C was 65.4 ± 11.8 min (mean ± SEM; $n = 5$). Rectal and brain surface temperatures were measured in the early treatment group, and were nearly identical, with less than half a degree of difference at any time point ($n = 5$ in each group). Only rectal temperature was measured in subsequent experiments. Cooling lowered EEG power in a dose-dependent fashion (Table 1). EEG power in normothermic animals decreased from pretreatment SE, probably in part due to midazolam, but remained significantly above baseline for 4.1 ± 1.1 h (mean ± SEM; $n = 9$) after midazolam injection, indicating continuation of SE. In hypothermic animals, cooling brought power down to baseline level in 23.8 ± 3.9 min (mean ± SEM; $n = 5$) and significantly lowered EEG power compared to normothermia at 30 min and beyond as described in a preliminary report.

In these animals, EEG power decreased below pre-pilocarpine baseline, and remained close to baseline values for the remaining of the 24 h, suggesting that SE had been terminated and did not return.

Altogether, these results showed that midazolam treatment followed by early cooling is a very efficient procedure to stop SE. However, the presence of false positives in the hypothermic groups (animals whose SE might have stopped from midazolam alone) could not be ruled out. In our second series of experiments (“delayed hypothermia”), the cooling was delayed 15 min after midazolam and only applied to rats which remained in SE at that time. Indeed, this study showed that midazolam alone stopped SE in 17 out of 70 animals (24.3%) and these animals were discarded. In EEG studies, normothermic ($n = 20$) and hypothermic ($n = 17$) groups were comparable until cooling was initiated: time from pilocarpine injection to treatment was similar (normothermic group: 35 ± 2.5 min; hypothermic group: 37 ± 2.5 min), as were relative EEG power at initiation of hypothermia [normothermic group: 19 ± 1, hypothermic group: 18 ± 1 times baseline, non-significant (NS)] and progression to polyspikes (normothermic group: 1.3 ± 0.4 min, hypothermic group: 1.1 ± 0.6 min). Cooling significantly lowered EEG power compared to normothermic rats at every time point between 15 and 135 min following the initiation of cooling. EEG power decreased below pre-pilocarpine baseline 30 min following initiation of cooling and slightly increased above this baseline at 135 min and beyond (Fig. 2). Mortality during SE or in the ensuing 48 h was 42% in normothermic rats (13/31), against 10% in hypothermic animals (2/22; early and delayed groups; $P = 0.0127$ by Fischer exact test, two-tailed). Delayed hypothermia ($n = 17$) reduced the number of seizures by 65% ($P < 0.001$), the number of spikes by 91% ($P < 0.0001$), cumulative seizure time by 60% ($P < 0.01$), the time needed to reach an EEG power of twice the pre-seizure baseline by 94% ($P < 0.0001$), the time in high-amplitude discharges by 55% ($P < 0.01$) during the first 24 h following the initiation of cooling (Table 2). The duration of individual seizures was not altered: 24 ± 1 sec in normothermics ($n = 20$) versus 26 ± 2 sec in hypothermics, ($n = 17$; NS). Time to terminate SE after initiation of hypothermia was 198 ± 41 min in normothermics ($n = 20$) and 12 ± 2 min in hypothermics ($n = 17$; $P < 0.0001$). The hypothermic group ($n = 17$) had 19.3 ± 5.2 seizures/24 h versus 55.5 ± 11 in normothermics ($n = 20$; $P < 0.001$). During rewarming, there were some spikes but no seizures in hypothermic animals. Dur-

**Table 2.** Effect of deep hypothermia on several EEG measures recorded during the first 24 h following initiation of cooling (mean ± SEM).

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| Measure                                      | Normothermic group ($n = 20$) | Hypothermic group ($n = 17$) | Reduction | P-value (Mann–Whitney) |
|---------------------------------------------|-------------------------------|------------------------------|-----------|------------------------|
| Number of computer-detected seizures/24 h  | 55.5 ± 11                     | 19.3 ± 5.2                   | 65%       | 0.0005                 |
| Cumulative seizure time (min)               | 24.4 ± 5.4                    | 9.6 ± 3.7                    | 60%       | 0.0016                 |
| Number of computer-detected spikes/24 h     | 4898.5 ± 861.9                | 441.5 ± 109.7                | 91%       | <0.0001                |
| Time in high amplitude discharge (h)        | 9.2 ± 1.2                     | 4.2 ± 1.4                    | 55%       | 0.001                 |
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ing the rewarming period of the hypothermic animals, normothermic animals had $12 \pm 3$ seizures (mean $\pm$ SEM; $n = 20$). However, 02:31–16:17 h after the end of rewarming, isolated seizures were found in eight out of 17 hypothermic animals ($4.1 \pm 1.4$ seizures; mean $\pm$ SEM), and in one animal, SE returned.

**Distribution of neuronal injury**

When animals were perfused 48 h after pilocarpine injection, and neuronal injury was studied with Fluoro-jade B, normothermic animals ($n = 9$) which had prolonged refractory SE showed neuronal injury in many areas, including CA1, CA3, frontoparietal cortex, piriform cortex, entorhinal cortex, caudoputamen, thalamus, and hilus of dentate gyrus (Fig. 3). When refractory SE was treated with delayed hypothermia, none of the brains showed significant neuronal injury in any region except for three out of 9 animals which, respectively, showed 1, 4, and 7 injured cells per field in the frontoparietal cortex (Fig. 3 and Table 3). The severity of CA1 injury was positively correlated with seizure severity, as measured by the ratio of EEG power at 45 min divided by the average baseline EEG power before pilocarpine ($P = 0.001$, Spearman correlation).

**Distribution of IgG-like immunoreactivity**

In normothermic animals ($n = 9$), 48 h after pilocarpine injection, antibodies against rat IgG revealed diffuse and cell staining of many areas, suggesting brain–blood barrier breakdown. All regions which showed neuronal injury also showed IgG-like immunoreactivity (LI). This sharply contrasted with the brains of hypothermia-treated animals ($n = 9$), which were devoid of IgG-LI (Fig. 4A and B).

**Distribution of monocytes-macrophages and T cells**

In the normothermic animals ($n = 9$), using ED-1 antibody, a marker of monocytes and macrophages, we observed 48 h after initiation of RSE many monocytes-macrophages in many areas, including CA1, CA3, frontoparietal cortex, piriform cortex, entorhinal cortex, caudoputamen, thalamus, and hilus of dentate gyrus. Hypothermia-treated animals showed no macrophages in the brain ($n = 9$; Fig. 4C–F). There were few T cells in either group (data not shown).

**Discussion**

Our results show that RSE was terminated by deep hypothermia of relatively brief duration in all animals,
and returned long after the rewarming period in only one of 17 animals. In hypothermic animals, EEG power was remained below preseizure baseline for 75 min after rewarming, probably reflecting a combination of postictal depression and midazolam-induced depression. This is in sharp contrast to normothermic animals, which retained an EEG power at least an order of magnitude above preseizure baseline for 4.1 ± 1.1 h after seizure onset. Many hours after return to normothermia, however, the EEG showed a return of isolated seizures in half of the animals. By then, midazolam would be expected to have been metabolized, but pilocarpine might still be present in sufficient amount to restart seizure activity.

Mild hypothermia has been shown to reduce seizure activity in experimental animals and in humans.16–18 Moderate hypothermia19,20 reduced the severity of SE and of SE-associated neuronal loss. Deep hypothermia had even greater effects.19 Anecdotal clinical reports suggest that mild hypothermia inhibits seizures.18,21,22 Cold saline perfusion suppressed a spike focus during electrocorticography.16 Deep hypothermia has not been used for RSE, but is routinely used to protect the brain or spinal cord when circulatory arrest is needed in cardiac surgery31 and neurosurgery.32 Many of the complications reported after deep hypothermia are the result of induced circulatory arrest, not of hypothermia itself.34,37

Deep hypothermia efficiently attenuated the long-term consequences of RSE, including neuronal injury, BBB disruption and cell-mediated inflammation. Hypothermia has been shown to be strongly neuroprotective in ischemia, trauma, and other pathological conditions.38 Still, it is remarkable that neuronal injury as measured by Fluoro-Jade B staining was completely prevented by hypothermia in most animals, since hypothermia was initiated on the average 31 min after seizure onset, and in this model of SE, signs of neuronal injury are already present after 30 min of seizures.39 However, midazolam treatment, while unable to stop SE by itself, may have contributed in part to the neuroprotection provided by hypothermia.

The therapeutic efficacy of hypothermia can be explained by its biological properties. Hypothermia reduces cerebral metabolic rate by 6–7% per degree Celsius, so that at 20°C, human cerebral oxygen consumption was measured at one-fifth of normothermic values.40 Hypothermia alters the function of ion pumps,41 intrinsic membrane properties and voltage-gated ion channels.42 It slows release of excitatory neurotransmitters43 and modifies gene expression.44 These actions reduce excitatory drive and would be expected to inhibit seizure activity as

| Table 3. Neuronal injury assessed by fluoro-jade B staining in rats subjected to refractory SE followed by normothermia or hypothermia. |
|---------------------------------------------------------------|
|                                                                 |
|                                                                 |
| Normothermic | Hypothermic |
| rats (n = 9) | rats (n = 9) |
|----------------------------------------------------------------|
| Frontoparietal cortex | 3 (1–4) [8] | 0 (1) [3] |
| Entorinal cortex | 4 (4) [8] | 0 (0) [0] |
| Piriform cortex | 4 (4) [8] | 0 (0) [0] |
| Amygdala | 4 (4) [8] | 0 (0) [0] |
| CA3 | 1 (1–2) [6] | 0 (0) [0] |
| Dentate gyrus, hilar | 1 (1–2) [8] | 0 (0) [0] |
| Thalamus | 4 (1–4) [8] | 0 (0) [0] |
| Caudoputamen | 4 (4) [6] | 0 (0) [0] |

The first numbers represent the median damage score. The numbers in parentheses indicate the range of neuronal injury. The numbers in brackets represent the numbers of animals in which neuronal injury could be observed. SE, status epilepticus.
observed in this study. They also activate several neuroprotective mechanisms: reduction in the cerebral demand for oxygen and glucose;\textsuperscript{45} preservation of ATP and energy stores and of tissue pH; reduction in the release of excitotoxins\textsuperscript{46} and of calcium influx into neurons,\textsuperscript{47} inhibition of early gene expression and stress response, induction of the expression of heat shock, and other stress proteins;\textsuperscript{48} and finally, inhibition of early molecular cascades involved in neuronal apoptosis.\textsuperscript{49} The neuroprotective role of hypothermia has been well-documented after cardiac arrest,\textsuperscript{28,50} stroke,\textsuperscript{51} neonatal hypoxic-ischemic encephalopathy,\textsuperscript{52,53} and traumatic brain injury.\textsuperscript{54} It has also been seen with seizure-associated neuronal injury.\textsuperscript{55}

In addition to its action on seizure activity and neuroprotective mechanisms, hypothermia can reduce BBB disruption and inflammation following traumatic brain injury.\textsuperscript{58} In the present study, hypothermia may have directly contributed to reduction of neuronal injury, BBB leakage and macrophage-mediated inflammation. However, these effects could also be explained by the rapid termination of RSE.

These results suggest that deep hypothermia may open a new therapeutic avenue for the treatment of RSE and for the prevention of its long-term consequences. The technology for carrying out mild hypothermia is available in most hospitals, and that for deep hypothermia exists in major surgical centers. In view of the high expense and poor prognosis of ICU pharmacotherapy of RSE, hypothermia might be considered as an alternative treatment of last resort of RSE and of SRSE.

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Author Contributions
Conception and design of the study was done by C. W., R. B., M. G., and J. N.; acquisition and analysis of data by C. W., R. B., M. G., and J. N.; or drafting the manuscript or figures by C. W., J. N., and R. B.

Conflict of Interest
None declared.

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