Neurotensin-induced hypothermia improves neurologic outcome after hypoxic-ischemia*

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Objective: **External cooling is commonly used to force induction of mild hypothermia but requires equipment, has a slow onset of action, and must be prolonged to provide permanent neurologic benefits after hypoxic-ischemia. It is unknown whether the method for inducing mild hypothermia affects neurologic outcome after near-drowning. The objective of the study was to induce mild hypothermia with neurotensin analog NT77 or external cooling in a rat model of near-drowning. We hypothesize that NT77 would be more effective for improving neurologic outcome than external cooling of the same duration.**

Design: **Rats were randomized to a normothermic control, neurotensin-induced hypothermia, brief external cooling, or prolonged external cooling group after asphyxial cardiac arrest.**

Setting: **Laboratory investigation.**

Subjects: **Forty-eight rats.**

Interventions: **Mild hypothermia was induced by external cooling for 4 hrs (brief external cooling) or 24 hrs (prolonged external cooling) or by neurotensin-induced hypothermia administration 30 mins after asphyxial cardiac arrest in rats.**

Measurements: **Outcome was assessed by a neurologic deficit score, the Morris water maze, and CA1 hippocampus histology 15 days after resuscitation.**

Main Results: **Neurologic deficit score at 72 hrs after asphyxial cardiac arrest was lower with neurotensin-induced hypothermia (score, 0) and prolonged external cooling (score, 0) vs. normothermic control (score, 20) and brief external cooling (score, 18; $p < .05$). Latency time in the Morris water maze 15 days after asphyxial cardiac arrest was decreased with neurotensin-induced hypothermia (14 ± 11 secs) and prolonged external cooling (18 ± 9 secs) vs. normothermic control (74 ± 17 secs) and brief external cooling (78 ± 18 secs, $p < .05$). There was less ischemic neuronal damage with neurotensin-induced hypothermia (28 ± 24%) and prolonged external cooling (21 ± 14%) vs. normothermic control (61 ± 32%) and brief external cooling (51 ± 32%).**

Conclusions: **Neurotensin-induced hypothermia improved neurologic outcome after asphyxial cardiac arrest in rats vs. brief external cooling but was comparable to prolonged external cooling. (Crit Care Med 2004; 32:806–810)**

Mild (33–35°C) hypothermia induced in laboratory animals after cardiac arrest and stroke has been shown to improve cerebral outcome (1–4). The results of two clinical trials of mild prolonged hypothermia induced after resuscitation from cardiac arrest have also been encouraging (5, 6). External and endovascular cooling are the most common methods for inducing mild hypothermia after cerebral ischemia. External cooling in large animals and humans without general anesthesia, aside from requiring equipment, may take hours to achieve mild hypothermia because of competing thermoregulatory mechanisms (7). These delays in attaining mild brain hypothermia after cerebral ischemia may negate some of hypothermia's therapeutic effect on cerebral outcome (8). In addition, hypothermia induced by external cooling must be prolonged to have permanent cerebral resuscitative benefits (2). These limitations have created the need to find alternative methods for inducing therapeutic mild hypothermia after cerebral ischemia.

Hibernation in mammals is a physiologic state with significant reductions in metabolism and body temperature (9, 10). These physiologic changes provide significant resistance to cerebral ischemia (11). Homeostatic control of body temperature during hibernation is maintained at a lower set point, a property characterized as regulated hypothermia (12, 13). Regulated hypothermia also has the theoretical advantage of inducing mild hypothermia more quickly and with less stress since the normal counterregulatory mechanism of shivering as well as increased catecholamine and cortisol release is not prominent during the initial cooling process (7).

The trigger that initiates hibernation has not been well defined. However, neurotensin concentrations have been shown to be elevated during hibernation (14). The primary effects of neurotensin in the brain include thermoregulation and antinociception (15). Neurotensin, an endogenous tridecapeptide, has neurotensin-specific receptors throughout the central nervous system of mammals, including rats and humans (16). Neurotensin induces hypothermia by activation of neurotensin receptors in the brain (17). Neurotensin is normally degraded rapidly by circulating peptidases found in the blood and thus must be injected directly in the brain to induce hypothermia. However, the replacement of tyrosine with Neo-Trp at the 11th position of the chemically active 8–13 fragment of neurotensin led to the development of new neurotensin analogs that can be administered intravenously, rapidly cross the blood brain barrier, and maintain mild hypothermia for hours (18). These drugs may provide a practical method for rapidly inducing hypothermia within minutes

***See also p. 897.**

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DOI: 10.1097/01.CCM.0000114998.00860.FD

without the need for general anesthesia. In addition, when neurotensin degrades, the brain temperature returns to normal without the need for external application of heat.

Drowning victims die primarily by asphyxiation (19). If respiratory function is not restored in approximately 4–5 mins, the victims suffer asphyxial cardiac arrest. Many of these previously young and healthy victims can be resuscitated from asphyxial cardiac arrest (near-drowning) but suffer devastating, permanent brain injuries (20). It is likely that the hypercarbia and hypoxia that occur before cardiac arrest in near-drowning contribute to this brain injury (21, 22). The outcome model of asphyxial cardiac arrest in rats reproduces the pathophysiology of near-drowning and can be useful for evaluating neurologic outcome after this form of brain injury (23).

There is a significant gap in the literature on research directed at developing therapies for patients who have suffered brain damage from near-drowning. The purpose of this study is to determine whether the method (external cooling vs. neurotensin) for inducing hypothermia after resuscitation from asphyxial cardiac arrest will alter neurologic and histologic outcome. We hypothesize that neurotensin-induced hypothermia will reduce brain injury after resuscitation from asphyxial cardiac arrest and improve neurologic outcome. We also hypothesize that the neurologic improvement with neurotensin will provide neurologic benefits comparable to prolonged external cooling.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee approved the protocol, and the care and handling of animals were in accord with the National Institutes of Health guidelines. The study was prospectively randomized in a blinded fashion and was conducted in a university research laboratory. Forty-eight rats were randomized to a normothermic control (NC, n = 12), brief external cooling (BC, n = 12), neurotensin-induced hypothermia (NT), and prolonged external cooling (PC, n = 12) group. Rats in the NC group had brain temperature maintained at 37°C during asphyxial cardiac arrest and reperfusion. Rats in the BC group had ice and cool air applied to the head to induce mild hypothermia (33–34°C) beginning 30 mins after resuscitation and maintained for 4 hrs. Rats in the NT group were given diphenhydramine (4 mg/kg intravenously) 30 mins and neurotensin (NT77 10 mg/kg intravenously over 1 hr) starting 40 mins after resuscitation. NT77 is a neurotensin analog that can induce mild hypothermia when administered intravenously (24). Diphenhydramine was given before NT to attenuate the 20–70 mm Hg decrease in blood pressure observed in pilot studies. The transient hypotension was suspected of being caused by histamine release and was attenuated by diphenhydramine. Rats receiving diphenhydramine alone did not demonstrate altered neurologic outcome compared with NC rats (data not shown). Rats in the PC group had hypothermia induced with ice and cool air applied to the head to induce mild hypothermia starting 30 mins after resuscitation and maintained for 24 hrs. Hypothermia was maintained in the external cooling groups by having the brain temperature telemetric signal control a servo-regulated system (Mini-Mitter, Sun River, OR) composed of a parrot brooder incubator (Brinsea, Titusville, FL) and cooling fan. The cooling fan was activated when the brain temperature increased above 33.5°C and turned off when the temperature decreased below 33.3°C. When the rat's brain temperature decreased below 33.3°C, the brooder was activated until the temperature increased to 33.3°C. The rate of cooling caused by NT was reproduced in the BC and PC groups by spraying the entire body with water, applying ice, and adjusting a cooling fan. All rats were prepared for asphyxial cardiac arrest and reperfusion as previously described (23). Briefly, rats were anesthetized with 4% isoflurane, intubated, and mechanically ventilated with a combination of 30% oxygen and 70% nitrous oxide. Titrated isoflurane anesthesia was maintained throughout the preparation phase. Catheters were placed in the left femoral vessels for the monitoring of mean arterial pressure, withdrawal of blood for arterial blood gases, and administration of intravenous medications. A telemetric temperature probe was stereotactically placed in the epidural space 5 days before asphyxial cardiac arrest, and brain temperature was monitored and maintained at 37 ± 0.1°C throughout the surgical preparation and cardiac arrest and for 30 mins after resuscitation. Rats were chemically paralyzed with vecuronium (1 mg/kg intravenously), and apneic asphyxia was induced by discontinuation of ventilation. Asphyxia led to cardiac arrest within approximately 4 mins in all rats, and asphyxia was maintained for 8 mins. Rats were resuscitated with epinephrine (0.005 mg/kg intravenously), sodium bicarbonate (1mEq/kg), mechanical ventilation with 100% oxygen, and chest compressions. Chest compressions were stopped when there was a return of spontaneous circulation (ROSC; mean arterial pressure ≥60 mm Hg) or no ROSC after 2 mins. Prior studies have demonstrated that resuscitation attempts >2 mins produced unreliable 72-hr survival required for neurologic testing (23). Rats were extubated 2 hrs after ROSC and returned to a temperature-controlled environment where the brain temperature was maintained according to therapeutic intervention. Rats had free access to food and water during recovery.

A neurologic deficit score (NDS) was performed daily for 15 days after ROSC by an investigator blinded to the treatment. Rats were tested for coordination (balance beam walk, placing test, depth perception, righting reflex) and for motor and sensory function as previously described (23). The NDS ranges from 0 (normal) to 100 (brain dead).

On days 11–15 after ROSC, rats were assessed for performance in a Morris water maze (25). The time required to locate the hidden platform (latency time) was recorded. Rats performed four swim trials on each day with a maximum swim time of 2 mins before terminating the trial, if they were unable to locate the platform. A 2-min rest period was provided between each trial. Rats were natural swimmers and attempted to escape from the maze because they wanted to avoid the cold-water insult.

After completion of Morris water maze testing, rats were reanesthetized with 4% isoflurane and mechanically ventilated. A needle was placed in the left ventricle and advanced into the ascending aorta, proximal to the carotid arteries. The descending aorta was cross-clamped, and 4% buffered paraformaldehyde was infused under a pressure of 100 cm water for a total of 100 mL. The rats were decapitated and the heads were placed in paraformaldehyde at 4°C for 24 hrs before the brains were removed. The brains were embedded in paraffin, and sections 7 μm thick were cut, stained with hematoxylin-eosin, and examined by light microscopy. The pathologist (KP) was blinded to the treatment groups. The total number of neurons (ischemic and normal) was counted in standardized sections of the hippocampus (CA1 region). A neuron was considered to show acute ischemic cell death if the cytoplasm was eosinophilic and the nucleus was pyknotic or karyorrhectic (26).

Data Analysis. Physiologic variables (arterial blood gas, glucose, heart rate, and mean arterial pressure), latency time, and percentage of ischemic neuronal damage were reported as mean and SD. ROSC was compared between groups by chi-square analysis. Neurologic deficit scores between groups were assessed by Kruskal-Wallis analysis. When an overall significant difference was found, a Dunn test was used to determine specific between-group differences. A Mann-Whitney U test was used to determine the between-group differences in physiologic variables, latency time (Morris water maze), and percentage of ischemic neuronal damage in the CA1 region

of the hippocampus. Data were entered and analyzed with SPSS software. For all analyses, alpha was set at a $p < .05$ level of significance.

RESULTS

Physiologic variables, including arterial blood gas, glucose, and mean arterial blood pressure, were similar between groups at baseline. The mean time to cardiac arrest from start of asphyxia (208 ± 22 secs) and duration of cardiopulmonary resuscitation (28 ± 15 secs) was similar between groups. There were no significant differences in ROSC between groups (NC, 10 of 12; BC, 10 of 12; NT, 11 of 12; PC, 11 of 12), and all resuscitated rats survived 15 days. There were no significant differences in arterial blood gases, glucose, and mean arterial pressure measurements up to 150 mins after ROSC between groups. The brain temperature tracings of the four groups are represented in Figure 1. Mild hypothermia was maintained for 236 ± 52 mins in the NT group, whereas the NC rats had a brain temperature of 37.1 ± 0.3°C throughout 72 hrs of monitoring after ROSC. Shivering was noted in the BC and PC groups starting 1 hr after resuscitation. No shivering was noted in the NC or NT groups at the same time points. The NDS at 24 hrs after ROSC in the NC group (22 median) was significantly higher (worse) than the BC (score, 10), NT (score, 8), and PC groups (score, 8) ($p < .05$ Kruskal-Wallis, Fig. 2). However, the NDS score in the BC group increased to 18 and was no different from NC (score, 20) at 72 hrs (Fig. 2). There was no difference in NDS between groups 15 days after ROSC (NC 2, BC 2, NT 0, and PC 0). The latency time in the NT (14 ± 11 secs) group was significantly better than the BC (78 ± 18 secs) or NC (74 ± 17 secs) groups 15 days after ROSC ($p < .05$ analysis of variance). There was no difference in latency time between the NT and PC group 15 days after ROSC (Fig. 3). Latency time was also lower in the NT vs. BC group on days 12–15 post-ROSC ($p < .05$ analysis of variance). The percentage of ischemic neuronal damage in the CA1 region of the hippocampus was less in the NT (28 ± 24%) vs. BC (51 ± 32%) and NC (C 61 ± 32%) groups ($p < .05$ analysis of variance). The percentage of ischemic neuronal damage in the PC (21 ± 14%) group was similar to the NT group (28 ± 24%).

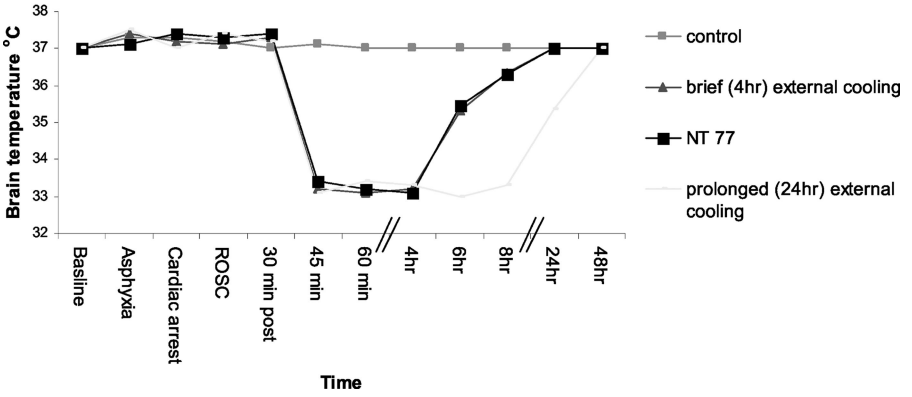

Figure 1. Brain temperature was measured telemetrically and recorded during surgical preparation and for 24 hrs after reperfusion from asphyxial cardiac arrest. *ROSC*, return of spontaneous circulation; *NT*, neurotensin.

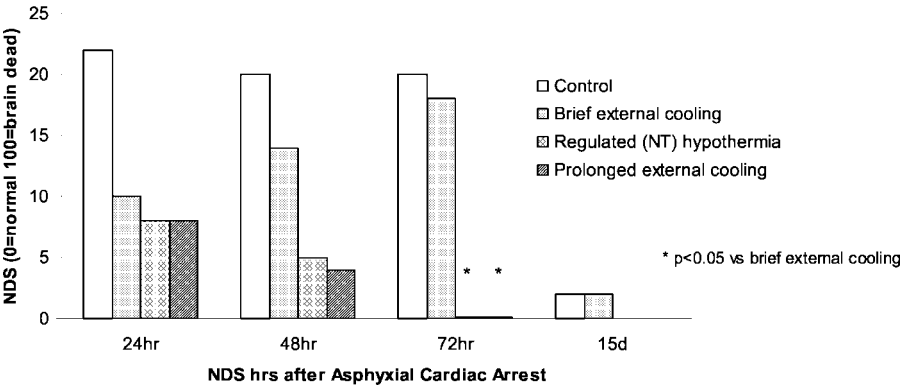

Figure 2. Neurologic deficit scores (*NDS*) after reperfusion from asphyxial cardiac arrest. *NT*, neurotensin.

DISCUSSION

A single dose of neurotensin analog NT77 after resuscitation from asphyxial cardiac arrest produced mild hypothermia for approximately 4 hrs. The rats had better neurologic outcome when compared with rats that received external cooling for 4 hrs. In addition, the performance in the Morris water maze and percentage of histologic damage in the NT77-treated group were similar to those values in the group that received prolonged (24 hrs) external cooling, suggesting that the neuroresuscitative benefits of NT may be prolonged.

Even though near-drowning is a major cause of brain injury, there is a paucity of basic science research for this disease process (20). The reason for this neglect is unclear, but the existence of no recognized animal model of near-drowning may be contributory. The prominent pathophysiological derangement of near-drowning is asphyxia (19). If the asphyxia is not immediately corrected, it produces cardiac arrest and hypoxic-ischemic brain injury. The outcome model of asphyxial cardiac arrest produces hypoxic-ischemic brain injury that is histologically similar to humans with asphyxia (27) and produces measurable deficits of function. NT77-induced hypothermia was shown to improve neurologic outcome after this global brain ischemic injury.

Neurologic deficit scores were better in the brief (4-hr) external cooling group compared with normothermic controls 24 hrs after resuscitation, but the benefit dissipated by 72 hrs. Colbourne and Corbett (2) reported similar findings of transient neurologic benefit with brief hypothermia. In contrast, rats with NT77-induced hypothermia had better NDS scores at 72 hrs and performed well in the Morris water maze 15 days after resuscitation with results comparable to the prolonged (24-hr) external cooling group. These behavioral improvements with NT77 administration corresponded to histologic changes in the CA1 region, the area responsible for the acquisition of new memory and learning in rats. The NT77 group did not shiver during the

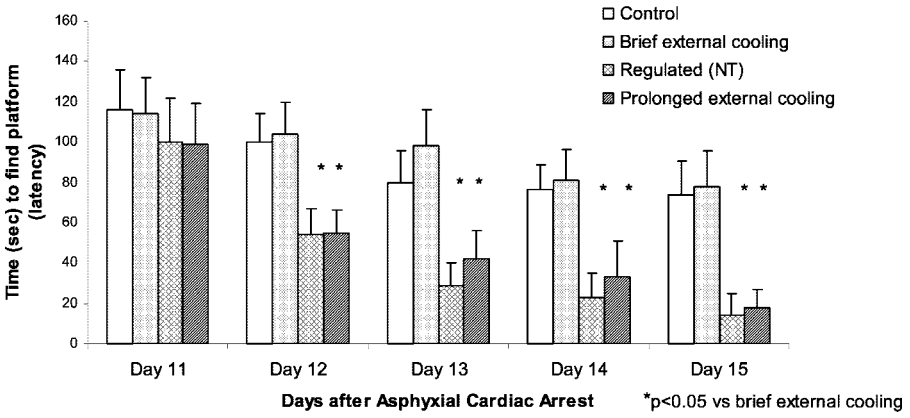

Figure 3. Performance (latency time) in the Morris maze 11–15 days after reperfusion from asphyxial cardiac arrest in rats. *NT*, neurotensin.

A single dose of NT77 administered after resuscitation from asphyxial cardiac arrest in rats produced mild hypothermia and improved neurologic outcome compared with brief external cooling.

induction of hypothermia. Forced external cooling produces shivering, increased metabolic rate, and release of cortisol and catecholamines (28). These physiologic stressors may contribute to delayed brain injury during brief external cooling and may be attenuated with NT77-induced hypothermia (29, 30). The reason why prolonged external cooling provides protection from this delayed deterioration is unknown. Mechanistic studies will be required to determine how NT77 and prolonged external cooling alter reperfusion injury.

There appears to be a therapeutic window after resuscitation from cerebral ischemia when mild hypothermia is effective (31). Prolonged external cooling in humans was shown to be effective after cardiac arrest (5, 6). However, the same results for mild therapeutic hypothermia were not demonstrated after head injury (32). Delays in the onset of therapeutic hypothermia in these patients in addition to a difference in pathophysiology may have contributed to no improvement in neurologic outcome (33). Once external cooling is initiated, temperature decreases very slowly, although other methods such as endovascular cooling may expedite the process (34, 35). The large body mass and normal counterregulatory mechanisms of thermoregulation may slow the efficacy of mechanical cooling for inducing hypothermia in humans (7). Future studies are required to determine whether NT77 can alter thermoregulatory mechanisms in larger animals and humans and possibly influence the rate of cooling.

External application of heat is required to return mammals to normal body temperature after mild hypothermia induced by external cooling. The brain is very sensitive to the application of heat after cerebral ischemia, and hyperthermia can worsen neurologic outcome (36). The hypothermic effect of NT77 resolves spontaneously at room temperature as the drug is metabolized. The externally cooled groups required additional warming with a heat lamp to rewarm at the same rate as the NT77 group. It is unknown how these differences in heat transfer during rewarming may have altered neurologic outcome, but this point would benefit from further investigation.

A rapid bolus administration of NT77 can cause transient hypotension although it does not seem to worsen neurologic outcome (37). However, diphenhydramine administered shortly before an infusion of NT77 prevented transient hypotension. The hypotensive mechanism of NT is unknown but is likely to be related to histamine release (38). Diphenhydramine alone has not been shown to provide hypoxic protection to the brain; however, the study design does not exclude the possibility that diphenhydramine could have acted synergistically with NT77 to improve neurologic outcome during reperfusion (39).

It cannot be stated with certainty that the hypothermic effect of NT77 was solely responsible for improved neurologic outcome after asphyxial cardiac arrest, because we were unable to remove the hypothermic effect of NT77 during reperfusion and still have survival. Pilot studies were conducted to maintain normothermic brain temperature during NT77 administration to address this question, and rats receiving NT during reperfusion required a significant amount of external warming to maintain normothermia. All of these rats died within 24 hrs, and gross examination of the organs did not reveal the cause of death. Histology was not performed on the brains because our previous experience with this model has shown that the brains look normal for 24–48 hrs after reperfusion. One possibility for these deaths is that an area of the brain not measured with the brain temperature probe became hyperthermic and contributed to the early deaths. However, we do not have a tool for continuously monitoring the temperature of multiple regions of the rat brain. No mechanisms were studied to determine how NT altered reperfusion injury. However, now that distinct differences in neurologic outcome have been demonstrated by different methods of inducing hypothermia, future studies are planned to determine how NT77-induced hypothermia alters reperfusion injury, especially free radical production. Finally, the end point after resuscitation in this study was relatively short. However, the delayed neurologic deterioration that occurred with brief external cooling by 72 hrs after resuscitation did not occur after NT77-induced hypothermia. In addition, performance in the Morris maze of the NT group was similar to the prolonged external cooling group 15 days after resuscitation. Studies extending for months or years will be needed to definitively demonstrate whether NT-induced hypothermia has permanent neurologic benefits after resuscitation from asphyxial cardiac arrest.

Induction of hypothermia with NT77 is relatively easy in rats because of their small body mass. Humans may not have as dramatic a decrease in body temperature with NT77; however, NT77 may provide a simple adjunct to mechanical methods of cooling (external cooling or endovascular) by reducing the physiologic response to a decrease in core tem-

perature. The cardiovascular properties of NT77 must be well defined in human studies before being considered for use in clinical trails of cerebral ischemia. The Brain Resuscitation Clinical Trials have demonstrated that deleterious cardiovascular effects of drugs on the heart can negate the potential cerebral benefits of novel therapies (40). Finally, an understanding of how NT77 causes hypothermia and alters reperfusion injury may lead to the development of a safe and effective method for improving neurologic outcome after near-drowning and other cerebral insults.

CONCLUSIONS

A single dose of NT77 administered after resuscitation from asphyxial cardiac arrest in rats produced mild hypothermia and improved neurologic outcome compared with brief external cooling. The neurologic benefits of NT77 after resuscitation in this rat model of near-drowning were comparable to those of prolonged external cooling. NT77 may be useful for improving neurologic outcome after a cerebral insult such as near-drowning.

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