Attenuation of Cell Death in Injured Cortex After Post-Traumatic Brain Injury Moderate Hypothermia: Possible Involvement of Autophagy Pathway

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OBJECTIVE: **Multiple mechanisms participated in the cell death after fluid percussion traumatic brain injury (TBI). In the present study, we evaluated the effect on cell death in the injured cortex after fluid percussion TBI and investigated a possible role of autophagy.**

METHODS: **TBI model was induced by a fluid percussion TBI device. Moderate hypothermia (32°C) was achieved by partial immersion in a water bath (0°C) under general anesthesia for 4 hours. All rats were killed at 6 or 24 hours after TBI.**

RESULTS: **Cleaved caspase 3 evaluated with Western blotting and terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate-biotin nick end labeling-positive cells in injured cortex were significantly increased 6 hours after fluid percussion TBI and were further up-regulated 24 hours after TBI, dramatic up-regulation of Beclin-1 and protein light chain-3 expression levels was also observed. Further up-regulation of biomarkers of autophagy, attenuation of caspase 3 up-regulation and reduction of cell death was observed after 4 hours of hypothermia. Immunofluorescence analysis for cell localization demonstrated that protein light chain-3- and Beclin-1-positive cells included neurons and glial cells in the injured cortex after TBI and hypothermic treatment. By ultrastructural observation, autolysosomes in injured cortex were significantly increased at 6 and 24 hours after TBI and were further up-regulated after TBI hypothermic treatment.**

CONCLUSIONS: **These data suggest that hypothermic treatment could attenuate TBI-induced cell death in this fluid percussion TBI model, possibly through activation of autophagy pathway.**

INTRODUCTION

Traumatic brain injury (TBI) is a major cause of morbidity and mortality. There are 2 waves of cell death after TBI. In the first wave, cell death results from necrosis caused by membrane disruption, irreversible metabolic disturbance, and/or excitotoxicity immediately after mechanical trauma due to impact or penetration. During the second wave (6–48 hours after TBI), morphologic features of apoptosis and necrosis occur in a more delayed fashion (15, 22). Autophagy occurs in both waves, with unknown effects after TBI (8, 9, 21, 23, 26, 46).

Several clinical works and experimental studies have shown the neuroprotective effects of mild-to-moderate hypothermia, including inhibition of neurological injury, reduction of infarct size, and improvement of neurological outcome (7, 16). However, the changes in autophagy after hypothermia and the interactions autophagy and other cell death mechanisms remain unclear.

In present study, we investigated the effect of hypothermia on cell death in injured cortex and investigated the possible role of autophagy in the process.

Key words
- Apoptosis
- Autophagy
- Necrosis
- Hypothermia
- Traumatic brain injury

Abbreviations and Acronyms
AMP: Adenosine monophosphate
AMPK: Adenosine monophosphate-activated protein kinase
ATP: Adenosine triphosphate
BH: Bcl-2-homology
GFAP: glial fibrillary acidic protein
LC3: protein light chain-3
NeuN: neurone-specific nuclear protein
PARP: poly-ADP-ribose polymerase
PBS: phosphate-buffered saline
SNG: sham injury with normothermia group
TBI: Traumatic brain injury
THG: TBI with hypothermia group
TNG: TBI with normothermia group
TUNEL: Terminal deoxynucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick end labeling

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METHODS

Animals and Groups

Adult male Sprague-Dawley rats (320–380 g) obtained from the Center of Experimental Animal in Shanghai Jiao Tong University, School of Medicine were randomly divided into 3 groups, as previously described: sham injury with normothermia (SNG, 37°C; n = 72); TBI with normothermia (TNG, 37°C; n = 72); and TBI with hypothermia (THG, 32°C; n = 72) (15). All animal procedures were approved by the animal care and experimental committee of the School of Medicine of Shanghai Jiaotong University. Rats were housed in individual cages in a temperature and humidity-controlled animal facility with a 12-hour light/dark cycle. The rats were housed in the animal facility for at least 7 days before surgery, and they were given free access to food and water during this period.

Surgical Preparation

The rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (3.3 mL/kg) and were then mounted in a stereotaxic frame, an incision was made along the midline of the scalp, and a 4.8-mm diameter craniectomy was performed on the left parietal bone (midway between bregma and lambda). A rigid plastic injury tube (modified Leur-loc needle hub, 2.6-mm inside diameter) was secured over the exposed, intact dura using a cyanoacrylate adhesive. Two skull screws (2.1-mm diameter, 6.0-mm length) were placed in burr holes, 1 mm rostral to bregma and 1 mm caudal to lambda. The injury tube was secured to the skull with dental cement. Bone wax was used to cover the open needle hub connector after the dental cement hardened (5 minutes). The scalp was closed by sutures, and the animals were returned to their cages for recovery.

Lateral Fluid Percussion Brain Injury

A fluid percussion device (VCU Biomedical Engineering, Richmond, Virginia, USA) was used to cause TBI as described in detail previously (10, 28). The rats were subjected to TBI 24 hours after the surgical procedure to minimize the possible confounding factors of surgery. In brief, the device consisted of a Plexiglas cylindrical reservoir filled with 37°C isotonic saline. One end of the reservoir had a rubber-covered Plexiglas piston mounted on O-rings, and the opposite end had a pressure transducer housing with a 2.6-mm inside diameter male needle hub opening.

On the day of TBI, rats were anesthetized with 10% chloral hydrate (3.3 mL/kg, intraperitoneally) and endotracheally intubated for mechanical ventilation. The resulting pressure pulse was measured in atmospheres using an extracranial transducer (Statham PA 85-100; Gloud, Oxnard, California, USA) and recorded on a storage oscilloscope (Tektronix 5111; Tektronix, Beaverton, Oregon, USA). The suture was opened, and bone wax was removed. The rats were disconnected from the ventilator, and the injury tube was connected to the fluid percussion cylinder. Then, a fluid pressure pulse was applied for 10 milliseconds directly onto the exposed dura to produce moderate TBI (2.1–2.2 atm). The injury was delivered within 10 seconds after disconnecting from the ventilator.

After the initial observation, the rats were ventilated with a 2:1 nitrous oxide to oxygen mixture and the rectal and temporalis muscle temperatures were recorded. Then, the needle hub, screws, and dental cement were removed from the skull and the scalp was sutured closed. The rats were extubated as soon as spontaneous breathing was observed. The rats in the SNG were subjected to the same anesthetic and surgical procedures as the rats in the other groups but without being subjected to injury.

Manipulation of Temperature

The frontal cortex brain temperature was monitored with a digital electronic thermometer (model DP 80; Omega Engineering, Stamford, Connecticut, USA) and a 0.15-mm-diameter temperature probe (model HYP-033-1-T-G-60-SMP-M; Omega Engineering) inserted 4.0 mm ventral to the surface of the skull. The probe was removed before fluid percussion injury and replaced immediately after injury. Rectal temperatures were measured with an electronic thermometer with analogue display (model 43 TE; YSI, Yellow Springs, Ohio, USA) and a temperature probe (series 400; YSI). A brain temperature of 32°C was achieved by immersing the body of the anesthetized rat in ice-cold water. The skin and fur of all animals were protected from direct contact with the water by placing the animal in a plastic bag (head exposed) before immersion. Animals were removed from the water bath when the brain temperature was reduced to within 2°C of the target temperature. It took approximately 30 minutes to reach the target brain temperatures, which were maintained for 4 hours under general anesthesia at room temperature by intermittent application of ice packs as needed. Gradual rewarming to normothermia levels (37°C) was done during a 90-minute period to avoid rapid rewarming that may have influenced the secondary injury processes.

Throughout the procedure, the mean arterial blood pressure was monitored continuously and blood gases were measured 15 minutes before injury, 30 minutes after injury, and once every hour for up to 4 hours after TBI. All physiologic variables, with the exception of temperature, were within normal ranges, including mean arterial blood pressure (115–125 mmHg), pO_2 blood gas levels (120–150 mmHg), pCO_2 blood gas levels (35–45 mmHg), and blood pH (7.38–7.45). The brain and rectal temperatures of rats in THG were significantly decreased compared with those in SNG and TNG.

Hematoxylin and Eosin Staining

Rats were subjected to deep anesthesia by 10% chloral hydrate. At 6 or 24 hours after TBI, the rats were perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed, further fixed at 4°C overnight, and immersed in 30% sucrose/PBS at 4°C overnight. Specimens were mounted in opti-mum cutting temperature compound (OCT). Serial sections were obtained using a cryostat and stained with toluidine blue for 30 minutes and then 2–3 drops of glacial acetic acid were added. Once the nucleus and granulation were clearly visible, the sections were mounted in Permount or Histoclad. Images of the ipsilateral cortex were captured at ×40 (low magnification) and ×200 (high magnification) using a microscope (Nikon Labophot; Nikon USA, Melville, New York, USA). Sections were cut in a microtome and adhered to glass slides with poly-lysine. Hematoxylin and eosin -stained specimens were examined by 2 pathologists (who were blinded to sample identity), who