Autophagy Inhibitor 3-MA Weakens Neuroprotective Effects of Posttraumatic Brain Injury Moderate Hypothermia

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■ OBJECTIVE: **The role of autophagy in moderate hypothermia in posttraumatic brain injury (post-TBI) remains elusive. In this study, we evaluated the protective role of autophagy in post-TBI moderate hypothermia.**

■ METHODS: **Adult male Sprague-Dawley rats were randomly divided into 3 groups ($n = 36$/group): TBI with hypothermia group (sham), TBI with hypothermia and a single intracerebroventricular injection of saline (saline, 5 μL), and TBI with hypothermia and a single intracerebroventricular injection of 3-methyladenine (600 nmol, diluted in 0.9% saline to a final volume of 5 μL). All rats, except those in the behavioral tests, were killed at 24 hours after fluid percussion TBI. Immunohistochemistry staining, western blot, and transmission electron microscopy were performed to assess changes in apoptosis and autophagy after injection of 3-methyladenine. Motor function (beam-walk test) and spatial learning/memory (Morris water maze) were assessed on postoperative days 1—5 and 11—15, respectively.**

■ RESULTS: **Our results showed downregulation of the expression level of microtubule-associated protein 1 light chain 3 and Beclin-1, aggravation of behavioral outcome, and increase of apoptosis.**

■ CONCLUSION: **Our results suggest that the autophagy pathway is involved in the neuroprotective effect of post-TBI hypothermia and negative modulation of apoptosis may be 1 possible mechanism.**

INTRODUCTION

Traumatic brain injury (TBI) is a major cause of morbidity and mortality. Secondary brain injury, including cerebral ischemia/hypoxia, disrupted blood-brain barrier, dysfunction of ionic channels, vasogenic and cytotoxic edema, and inflammation, which contribute to delayed tissue damage and cell death, may be possible mechanisms of long-term motor and cognitive disabilities.[1-7] During the process, morphologic features of apoptosis and necrosis occur in a more delayed fashion.[8] Autophagy, a lysosome-dependent cellular degradation pathway, is the chief machinery for bulk degradation of superfluous or aberrant cytoplasmic components and has also been implicated both clinically and experimentally in the delayed response to TBI.[9] Autophagy can coexist or occur sequentially with apoptosis and the Bcl-2-Beclin-1 complex may serve as a point of crosstalk between the apoptotic and autophagic signaling pathways.[10,11] However, the role of autophagy in TBI remains controversial.[11,12]

Several clinical and experimental studies have shown the neuroprotective effects of mild to moderate hypothermia, including attenuation of cell death, inhibition of neurologic injury, reduction of infarct size, and improvement of neurologic outcome.[13-18] In our previous studies, we found that posttraumatic moderate

Key words
- Apoptosis
- Autophagy
- Behavioral assessment
- Moderate hypothermia
- Traumatic brain injury

Abbreviations and Acronyms
3-MA: 3-methyladenine
AIs: Autolysosomes
Aps: Autophagosomes
ICV: Intracerebroventricular
LC3: Protein light chain-3
MABP: Mean arterial blood pressure
PBS: Phosphate-buffered saline
PI3K: Phosphatidylinositol 3-kinase
TBI: Traumatic brain injury
TBST: TBS+Tween-20
TEM: Transmission electron microscopy
TUNEL: Terminal deoxyribonucleotidyl transferase-mediated biotin-16-dUTP nick-end labeling

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hypothermia significantly increases autophagy within the ipsilateral cortex and hippocampus CA1 region after fluid percussion TBI.[19,20] However, whether autophagy participates in the neuroprotective effect of moderate hypothermia is still elusive.

Based on these findings, the present study was designed to define the role of autophagy in post-TBI hypothermia, using the autophagy inhibitor 3-methyladenine (3-MA).

METHODS

Animals

Adult male Sprague-Dawley rats (320–380 g) were used. 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. 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 the period.

Experimental Design

Rats were randomly divided into 3 groups: TBI with hypothermia and intracerebroventricular (ICV) cannulation (sham, 32°C, $n = 36$), TBI with hypothermia and a single ICV injection of saline (saline, 32°C, $n = 36$), and TBI with hypothermia and a single ICV injection of 3-MA (3-MA, 32°C, n=36). All animals were subjected to the surgical procedure 24 hours before lateral fluid percussion injury and ICV injection. Rats in the sham group received TBI, post-TBI moderate hypothermia, and ICV cannulation without drug administration; rats in the saline group received TBI, post-TBI moderate hypothermia, and ICV cannulation with saline administration (5 μL); rats in the 3-MA group received TBI, post-TBI moderate hypothermia, and ICV cannulation with 3-MA administration (Sigma, St Louis, Missouri, USA; 600 nmol, diluted in 0.9% saline to a final volume of 5 μL). All rats, except those in the behavioral tests, were killed 24 hours after fluid percussion TBI. For terminal deoxyribonucleotidyl transferase-mediated biotin-16-dUTP nick-end labeling (TUNEL) staining and immunohistochemical analysis, rats were perfused transcardially by 4% paraformaldehyde in phosphate-buffered saline (PBS) 24 hours after TBI. Brains were removed and further fixed at 4°C overnight. Specimens were mounted in optimal cutting temperature compound; for electron microscopy, rats were perfused transcardially with 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 mmol/L cacodylate buffer (pH 7.4) and then brains were postfixed in the same fixatives for 24 hours.

Surgical Preparation

The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3.3 mL/kg). They were 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 the bregma and the lambda). Two skull screws (2.1 mm diameter, 6.0 mm long) were placed in burr holes, 1 mm rostral to the bregma and 1 mm caudal to the lambda. All animals were stereotaxically implanted with a stainless steel cannula into the right lateral ventricle of the brain (ventral-dorsal 3.5 mm, anterior-posterior 0.8 mm, medial-lateral 1.5 mm). The cannula was made as described previously.[21] The cannula was made from a Novofine 30-G needle (Novo Nordisk, Bagsvaerd, Denmark) and a PE-10 catheter (0.28 mm inside diameter, 0.61 mm outside diameter). The needle was cut to a length of 11 mm and its end was connected to a PE-10 catheter (35 mm long). The cannula was connected to a microsyringe and filled with saline before implantation. Correct cannula placement into the lateral ventricle was verified by the outflow of cerebrospinal fluid after disconnecting the syringe from the cannula. The cannula was heat sealed to prevent leakage of cerebrospinal fluid.

A rigid plastic injury tube (modified Luer-Loc needle hub, 2.6 mm inside diameter [Becton Dickinson, Mountain View, California, USA]) was secured over the exposed intact dura using cyanoacrylate adhesive. Then the injury tube and ICV cannulation were 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.

ICV Administration and Lateral Fluid Percussion Brain Injury

Rats were subjected to TBI 24 hours after the surgical procedure to minimize the possible confounding factors of surgery. A fluid percussion device (VCU Biomedical Engineering, Richmond, Virginia, USA) was used to create TBI, as described in detail previously.[22,23] On the day of TBI, rats were anesthetized with 10% chloral hydrate (3.3 mL/kg, intraperitoneally) and endotracheally intubated for mechanical ventilation. Then the suture was opened and bone wax was removed. A single ICV injection of saline (5 μL) or 3-MA (Sigma, St louis, Missouri, USA; 600 nmol, diluted in 0.9% saline to a final volume of 5 μL) was initiated in the saline group and the 3-MA group, respectively. The cannula was sealed by heat after injection. Then the injury tube was connected to the fluid percussion cylinder and a fluid pressure pulse was applied for 10 milliseconds directly onto the exposed dura to produce moderate TBI (2.1–2.2 atm). 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 injury was delivered within 10 seconds after disconnecting from the ventilator. Immediately after TBI, rats were ventilated with a 2:1 nitrous oxide/oxygen mixture without isoflurane and the rectal and temporalis muscle temperatures were recorded. Then the needle hub, screws, ICV cannula, and the dental cement were removed from the skull. The burr hole used for drug administration was filled with bone wax and covered with dental cement. Then the scalp was closed with sutures. Rats were extubated as soon as spontaneous breathing was observed. The rats in the sham TBI group were subjected to the same anesthetic and surgical procedures as the rats in the other groups but without drug administration. Throughout the procedure, the mean arterial blood pressure (MABP) was monitored continuously and blood gases were measured 15 minutes before and after fluid percussion 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

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