Abstract. Traumatic brain injury (TBI) leads to permanent neurological impairment, and methylene blue (MB) exerts central nervous system neuroprotective effects. However, only one previous study has investigated the effectiveness of MB in a controlled cortical impact injury model of TBI. In addition, the specific mechanisms underlying the effect of MB against TBI remain to be elucidated. Therefore, the present study investigated the neuroprotective effect of MB on TBI and the possible mechanisms involved. In a mouse model of TBI, the animals were randomly divided into sham, vehicle (normal saline) or MB groups. The treatment time-points were 24 and 72 h (acute phase of TBI), and 14 days (chronic phase of TBI) post-TBI. The brain water content (BWC), and levels of neuronal death, and autophagy were determined during the acute phase, and neurological deficit, injury volume and microglial activation were assessed at all time-points. The injured hemisphere BWC was significantly increased 24 h post-TBI, and this was attenuated following treatment with MB. There was a significantly higher number of surviving neurons in the MB group, compared with the Vehicle group at 24 and 72 h post-TBI. In the acute phase, the MB-treated animals exhibited significantly upregulated expression of Beclin 1 and increased LC3-II to LC3-I ratios, compared with the vehicle group, indicating an increased rate of autophagy. Neurological functional deficits, measured using the modified neurological severity score, were significantly lower in the acute phase in the MB-treated animals and cerebral lesion volumes in the MB-treated animals were significantly lower, compared with the other groups at all time-points. Microglia were activated 24 h after TBI, peaked at 72 h and persisted until 14 days after TBI. Although the number of Iba-1-positive cells in the vehicle and MB groups 24 h post-TBI were not significantly different, marked microglial inhibition was observed in the MB group 72 h and 14 days after TBI. These results indicated that MB exerts a neuroprotective effect by increasing autophagy, decreasing brain edema and inhibiting microglial activation.

Introduction

It is estimated that >10,000,000 individuals suffer traumatic brain injury (TBI) annually worldwide (1). TBI contributes to significantly to mortality rates, and several survivors are subject to complications, including neurological deficit and motor dysfunction (2).

Although survival rates following head injury have significantly improved in the last few decades due to advances in emergency and intensive care medicine, no effective proactive treatment options exist for augmenting functional recovery or for preventing long-term neuropsychiatric complications post-TBI (3). An increasing number of studies have demonstrated that the extent of brain damage post-TBI depends, not only on the initial trauma, but also on secondary injury caused by neuroinflammation, cerebral edema and breakdown of the blood-brain barrier (4). Secondary brain
injury occurs between a duration of a few hours and several months, ultimately leading to neuronal death or neurodegeneration (2). Due these consequences, investigations have focused on the mechanisms or pathways of secondary brain injury to attempt to identify novel drugs for its inhibition or attenuation.

Methylene blue (MB) is an inexpensive, United States Food and Drug Administration-approved drug, which has been used safely in humans for >120 years (5). It is used for diagnostic procedures and for the treatment of methemoglobinemia, malaria, cyanide poisoning, carbon monoxide poisoning (6), cardiopulmonary bypass (7) and septic shock (8).

Previous studies have demonstrated that MB also exerts beneficial effects on the central nervous system. For example, it may exert neuroprotective activity against Parkinson’s disease (9), amyotrophic lateral sclerosis (10) and ischemic stroke (11). A previous study investigated the effectiveness of MB in a rat model of controlled cortical impact (CCI) injury and revealed that MB minimized lesion volume, behavioral deficits and neuronal degeneration (12). However, the specific mechanisms of MB against TBI remain to be elucidated. Therefore, the present study investigated the neuroprotective effect of MB on TBI and the possible mechanisms involved, focusing on the association between MB and the mechanisms of secondary brain injury, including neuroinflammation, brain edema and autophagy.

Materials and methods

Animals. Mature male Institute for Cancer Research mice (25-30 g; Zhejiang Academy of Medical Sciences, Hangzhou, China) were used in the present study. A total of 210 mice were used in total (70/group). The animals were maintained on a 12 h light/dark cycle with access to food and water ad libitum. The study was approved by the Ethics Committee of Zhejiang University (Hangzhou, China), and all experimental procedures were approved by Zhejiang University Institutional Animal Care and Use Committee and conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (13).

Model of TBI. The procedure used for creating the CCI model of TBI was as described previously (14). Briefly, the mice were anesthetized with pentobarbital sodium (50 mg/kg intraperitoneally) and mounted in a stereotaxic frame. Following a midline incision and retraction of the skin, a 4 mm diameter craniotomy was performed over the right frontoparietal cortex using a 5 mm trephine, between the bregma and lambda, without damaging the underlying dura. The bone flap was removed and the CCI was produced perpendicular to the brain surface (12° from the vertical) using a Benchmark CCI Stereotaxic Impactor (Benchmark Deluxe™; myNeuroLab, St. Louis, MO, USA) fitted with a 2.5 mm tip (3.0 m/sec; 100 ms dwell time; 2 mm depth). The same surgeon performed all CCI surgeries for each cohort of mice. The core body temperature of the mice was maintained at 36.5-37°C during surgery using a rectal thermometer coupled to a heating pad. The bone flap was immediately replaced and sealed, and the scalp was sutured. The mice in the sham group underwent identical surgical procedures, but without CCI. All animals were returned to their cages when fully recovered from anesthesia in a heated chamber.

Experimental design. The mice were randomly assigned into either sham, vehicle (normal saline) or MB groups. Either normal saline or MB (1 mg/kg; 10mg/ml, cat. no. 0517-0301-10, American Regent, Inc. Shirley, NY, USA) was infused intraperitoneally for 1 h following TBI, and at 0.5 mg/kg 6 h following TBI. A total of 1 mg/kg MB or the same volume of saline was administered daily for the next 3 days. The MB dosage and treatment time-points were determined based on a previous study (15) and preliminary experiments.

Evaluation of neurological deficits. The modified neurological severity score (mNSS) was used to examine the effects of MB on neurological deficits at all time-points following TBI. Neurological function was graded on a scale of 0-18, as previously described (Table I) (16).

Brain water content (BWC). At 24 h and 72 h post-TBI, the mice were sacrificed and the brains were removed and divided into right and left hemispheres, cerebellum and brainstem, which were weighed immediately to obtain the wet weight (ww). The samples were dried at 80°C for 72 h, following which the dry weight (dw) was measured. The BWC was calculated as follows: (ww - dw) / ww x 100% (17).

Hematoxylin and eosin (HE) staining and lesion volume assessment. The mice were anesthetized with pentobarbital sodium (50 mg/kg intraperitoneally; 1%; Sigma-Aldrich, St. Louis, MO, USA), and then sacrificed by spinal dislocation at 24 h, 72 h, and 14 days post-TBI for HE staining (Sigma-Aldrich) and immunohistochemistry. Briefly, the mice were deeply anesthetized, as described above, and were transcardially perfused with 20 ml saline solution (4°C), followed by 100 ml 10% formalin phosphate-buffered saline solution (pH 7.4) for 30 min (Wuhan Jiahuaüen Chemical Co. Ltd., Wuhan, China). The brains were then removed and post-fixed in the same perfusate at 4°C for 72 h, sectioned coronally from 1 mm anterior to the lesion site to the posterior margin of the lesion, and paraffin-embedded (Sigma-Aldrich). The area of the lesion was determined in 10 sections at 300 µm intervals throughout the entire lesion (4 µm thickness per section). The sections were HE-stained using standard methods (0.5% hematoxylin for 5 min at 25°C; 0.5% eosin for 1 min at 25°C), imaged with a stereomicroscope using bright-field illumination (Olympus BX51; Olympus, Tokyo, Japan) and analyzed using ImageTool software 3.0 (University of Texas Health Science Center, Texas, TX, USA). The brain injury volume (mm³) was calculated as follows: ∑(An + An + 1) x d / 2, where A is the area of apparent brain tissue loss and d is the distance between sections, as previously described (14,18), with minor modifications.

Immunohistochemistry. The samples were treated, as described above. Coronal sections (4 µm thick) were obtained using a microtome (Leica CM1900; Lyca Microsystems GmbH, Wetzlar, Germany) and heated in an oven at 60°C overnight for deparaffinization. Antigen retrieval was
performed in citrate buffer (pH 6.0; 0.01 mmol/L) containing citric acid (cat. no. 791725) and sodium citrate tribasic dihydrate (cat. no. 6132-04-3; Sigma-Aldrich) at 95˚C for 10 min. The slides were incubated with fresh 3% hydrogen peroxide (Sigma-Aldrich) for 10 min at room temperature and were washed in deionized water three times every 5 min. The sections were then incubated with polyclonal primary antibodies against ionized calcium-binding adapter molecule 1 (Iba-1; rabbit anti-mouse; 1:1000; cat. no. 019-19741; Wako, Carpinteria, CA, USA), neuronal nuclear antigen (NeuN; rabbit anti-mouse; 1:200; cat. no. ABN78A4; EMD Millipore, Billerica, MA, USA), or Beclin 1 (1:500; rabbit anti-mouse; cat. no. ab62557; Abcam, Cambridge, MA, USA) overnight at 4˚C. Following rinsing with PBS, the slides were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG for 30 min at room temperature, followed by reacting with fresh diaminobenzidine solution (99%; Sigma-Aldrich). The sections were hematoxylin-stained, dehydrated and mounted. Images were captured using a light microscope (Olympus BX51; Olympus) and immunofluorescence data were analyzed using Image-Pro Plus (Media Cybernetics, Inc., Rockville, MD, USA).

**Western blotting.** Western blotting was performed, as described previously (19). Briefly, the cortical samples were homogenized and centrifuged at 14,000 x g for 20 min at 4˚C. The resulting supernatants were centrifuged for 10 min and the protein content was measured using a bicinchoninic acid kit (Beyotime Institute of Biotechnology, Haimen, China). Equal quantities of protein (60 µg) from each sample were resuspended in loading buffer, denatured at 100˚C for 3-5 min, separated using 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Hangzhou Fu De Biological Technology Co., Ltd., Hangzhou, China) at a constant current and transferred onto polyvinylidene fluoride membranes (Hangzhou Fu De Biological Technology Co., Ltd.). The membranes were blocked with non-fat dry milk for 2 h and were subsequently incubated overnight at 4˚C with polyclonal primary antibodies against microtubule-associated protein light chain 3 (LC3; 1:1,000; rabbit anti-mouse; cat. no. 2775; Cell Signaling Technology, Inc., MA, USA), Beclin 1 (1:500; Table I. Modified neurological severity scores (16).

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Motor assessments</strong></td>
<td></td>
</tr>
<tr>
<td>Raising mice by tail (normal, 0; maximum, 3)</td>
<td></td>
</tr>
<tr>
<td>Flexion of forelimb</td>
<td>1</td>
</tr>
<tr>
<td>Flexion of hindlimb</td>
<td>1</td>
</tr>
<tr>
<td>Head movement &gt;10° to vertical axis within 30 sec</td>
<td>1</td>
</tr>
<tr>
<td>Putting mice on the floor (normal, 0; maximum, 3)</td>
<td></td>
</tr>
<tr>
<td>Normal walk</td>
<td>0</td>
</tr>
<tr>
<td>Unable to walk straight</td>
<td>1</td>
</tr>
<tr>
<td>Circling toward paretic side</td>
<td>2</td>
</tr>
<tr>
<td>Falls down to paretic side</td>
<td>3</td>
</tr>
<tr>
<td><strong>Sensory assessments</strong></td>
<td></td>
</tr>
<tr>
<td>Placing test (visual and tactile test)</td>
<td>1</td>
</tr>
<tr>
<td>Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Beam balance test (normal, 0; maximum, 6)</strong></td>
<td></td>
</tr>
<tr>
<td>Balances with steady posture</td>
<td>0</td>
</tr>
<tr>
<td>Grasps side of beam</td>
<td>1</td>
</tr>
<tr>
<td>Hugs beam but one limb falls from beam</td>
<td>2</td>
</tr>
<tr>
<td>Hugs beam and two limbs fall from beam, or spins on beam (&gt;60 sec)</td>
<td>3</td>
</tr>
<tr>
<td>Attempts to balance on beam but falls off (&gt;40 sec)</td>
<td>4</td>
</tr>
<tr>
<td>Attempts to balance on beam but falls off (&gt;20 sec)</td>
<td>5</td>
</tr>
<tr>
<td>Falls off; no attempt to balance or hang on to beam within 20 sec</td>
<td>6</td>
</tr>
<tr>
<td><strong>Reflex absence and abnormal movements (normal, 0; maximum, 4)</strong></td>
<td></td>
</tr>
<tr>
<td>Pinna reflex (head shake when auditory meatus is touched)</td>
<td>1</td>
</tr>
<tr>
<td>Corneal reflex (eye blink when cornea is lightly touched with cotton)</td>
<td>1</td>
</tr>
<tr>
<td>Startle reflex (motor response to a brief knocking noise)</td>
<td>1</td>
</tr>
<tr>
<td>Seizures, myoclonus, myodystony</td>
<td>1</td>
</tr>
</tbody>
</table>

Maximum points 18. One point is awarded for the inability to perform a task or for lack of a tested reflex (pinna reflex, corneal reflex, startle reflex). 13-18, severe injury; 7-12, moderate injury; 1-6, mild injury.
rabbit ant-mouse; cat. no. ab63557; Abcam) and β-actin (1:2,000; rabbit ant-mouse; cat. no. sc-130656; Santa Cruz Biotechnology, Dallas, TX, USA). The membranes then were processed with HRP-conjugated secondary antibodies for 1 h at room temperature and protein bands were visualized on X-ray film (Kodak, Rochester, NY, USA) and quantified using ImageJ software 1.48U (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. All data are expressed as the mean ± standard deviation and were analyzed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). Two experimental groups were compared using Student’s t-test and differences between the groups were analyzed using one-way analysis of variance, followed by the Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

MB alleviates cerebral edema. The injured hemisphere BWC was significantly increased 24 h post-TBI, whereas treatment with MB attenuated this increase (sham, 78.5%; vehicle, 80.7%; MB, 79.5%; P<0.05; Fig. 1A). However, the contralateral hemisphere, cerebellum and brain stem BWCs were not significantly different among the groups (data not shown). At 72 h post-TBI, the BWC decreased, however, no the vehicle (78.9%) and MB (78.6%) groups demonstrated no statistically significant different (P>0.05; Fig. 1A), which indicated that the CCI-induced cerebral edema began to subside 72 h post-TBI.

MB reduces neurological functional deficits. TBI resulted in neurological functional deficits (Fig. 1B-D), determined using mNSS. Compared with the vehicle group, the mNSS scores were significantly lower in the MB group 24 and 72 h post-TBI, which indicated that MB significantly reduced the neurological deficits (P<0.05). However, no significant differences were identified in the scores between the two groups 14 days post-TBI (P>0.05), suggesting that functional compensation occurred in the two groups. Notably, a significant difference in neurological function remained between the vehicle and sham groups (P<0.05), which was not observed between the MB group and the sham group (P>0.05).

MB reduces cerebral lesion volume and prevents neuronal death. The cerebral lesion volumes was assessed to confirm the neuroprotective effect of MB in TBI. The lesion volumes in the MB group lesion were significantly smaller, compared with those of the vehicle group at 24 h (2.49, vs, 2.98 mm³), 72 h (2.54, vs, 3.29 mm³) and 14 days (1.44 vs, 2.24 mm³; P<0.05), as shown in Fig. 2). The NeuN staining confirmed the effect of MB in preventing neuronal death and revealed the presence of a significantly higher number of neurons in the MB group, compared with the vehicle group at 24 h (1,014, vs, 815 cells/mm²) and 72 h (976, vs, 719 cells/mm²; P<0.05; Fig. 3A-C), which indicated that MB prevented neuronal death at the site of injury.

MB inhibits microglial activation. Since neuroinflammation is important in the pathogenesis of TBI, the effect of MB on microglial activation was assessed using Iba-1...
immunostaining. The number of microglia was low at 24 h post-TBI, however, activation was observed at 72 h, which persisted until 14 days post-TBI (Fig. 2). Although the numbers of Iba-1-positive cells in the MB and vehicle groups at 24 h post‑TBI were not significantly different (292, vs. 320 cells/mm²; P>0.05; Fig. 2), marked inhibition of microglial activation was observed in the MB-treated mice, compared with the vehicle mice at 72 h (644, vs. 1,053) and 14 days (557, vs. 762 cells/mm²) post-TBI (P<0.05; Fig. 2).

The analysis using ImagePro Plus for identification of the Iba-1 optical densities of the two groups yielded similar results at 24 h, (12,025, vs. 13,503; P>0.05), 72 h (27,838, vs. 50,107; P<0.05) and at 14 days (13,202, vs. 19,725; P<0.05; Fig. 2) following TBI.

MB increases autophagy. MB promotes autophagy in ex vivo slice cultures or primary neurons (20). To examine the possible mechanism underlying the action of MB against TBI, the effect of MB on autophagy was assessed using Beclin 1 immunostaining. Intense Beclin 1 staining was observed in the cytoplasm, which was more prominent in the MB group (Fig. 3D and E, compared with the vehicle group. Based on the increased expression of Beclin 1 and increased LC3-II/LC3-I ratio, the level of autophagy was higher in the MB group, compared with the vehicle group at 24 h (Beclin 1, 4.4, vs. 3.2-fold; LC3-II/LC3-I, 1.29, vs. 0.63) and 72 h (Beclin 1, 2.8, vs. 2.0-fold; LC3-II/LC3-I, 1.06, vs. 0.56) post-TBI (P<0.05; Fig. 4A-F). This suggested that MB increased autophagy, resulting in high levels of autophagic traffic in the acute phase of TBI.

Discussion

TBI is a major cause of mortality worldwide, and survivors may suffer permanent neurological impairment, including reduced cognitive function (21), neurodegenerative disorders, including Alzheimer's disease (22), and brain tumors (23). TBI can be divided into primary and secondary injury. Primary injury mechanisms result from initial mechanical damage, leading to neuronal, axonal and glial death, and blood vessel breakage (24). The long-term consequences of TBI predominantly result from the mechanisms of secondary injury (25), in cases where the primary tissue damage cannot be treated. Therefore, inhibiting or reducing secondary brain injury is key in the treatment of TBI for improving injury outcomes. The results of the present study demonstrated that MB attenuated TBI-induced edema, neuroinflammation and neuronal death, increased autophagy and reduced cerebral lesion volume.

In the present study, treatment with 1.5 mg/kg intraperitoneal MB on the first day of injury attenuated the TBI-induced increase in the BWC of the injured hemisphere. It is important to note that TBI-associated mortality is generally caused by cerebral edema and increased intracranial pressure (26). In the present study, the BWC of the MB group was significantly decreased 24 h post-TBI, although the differences between the MB and vehicle groups were not significant. However, even a small increase in BWC can lead to significantly increased intracranial pressure and poorer outcomes (27). These data indicated that MB may improve neurological outcome and survival rates following moderate to severe TBI. Post-traumatic brain edema is considered to be generated by vasogenic edema.
and cytotoxic edema (28). The former commonly occurs within a few hours following injury, followed by the latter, which may persist for weeks (29). No statistical difference was observed in the BWC between the two groups 72 h post-injury, which indicated that MB may have reduced vasogenic edema in the CCI model. However, the specific mechanisms of MB in decreasing cerebral edema require further investigation.

TBI initiates a neuroinflammatory cascade, which is characterized by microglial activation and increased production of proinflammatory cytokines (30). Microglia release neurotrophic factors, including nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3, which promote neuronal growth and survival (31). The detrimental effects of prolonged microglial activation, accompanied by a neuroinflammatory cascade, may counter these benefits (32). Microglial activation persists for several years following the initiating TBI, in which persistent microglial activation is correlated with the degree of cognitive impairment (33) and may predispose to the development of neurodegenerative disorders, including Alzheimer's disease (32). Nitric oxide (NO), a signaling molecule that freely diffuses across the plasmalemma and hundreds of micrometers between cells, is generated via enzymatic L-arginine cleavage by NO synthase (NOS). By binding to the heme group of soluble guanylate cyclase (sGC), NO increases the conversion of

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Figure 3. Methylene blue prevents neuronal death and promotes autophagy at the perilesional area following TBI. (A and B) Representative images of NeuN-stained coronal tissue sections 24 and 72 h post-TBI. (Scale bar=50 µm). (C) Quantitative analysis revealed a significant increase in the number of NeuN-positive cells 24 and 72 h post-TBI following treatment with MB. The data are presented as the mean ± standard deviation (n=6; *P<0.05). (D and E) Representative images of Beclin 1-stained coronal tissue sections 24 and 72 h post-TBI demonstrating marked cytoplasmic staining in the MB-treated cells (Scale bar=50 µm). MB, methylene blue; TBI, traumatic brain injury; NS, normal saline.
guanidine triphosphate to cyclic guanidine monophosphate (cGMP), acting as an early chemoattractant and microglial activator (34,35). MB eliminates cGMP immunoreactivity at lesions and inhibits microglial accumulation (34). Other possible mechanisms of MB in microglial inhibition include binding to peroxynitrites, to NO itself, or inhibiting NOS activity (36). Using a superoxide dismutase 1 gene mutation G93A (SOD1G93A) model of amyotrophic lateral sclerosis, Dibaj et al. (10) demonstrated that MB inhibits the microglial reaction in control and in SOD1G93A mice. However, compared with the present study, no alterations in microglial activity were observed in the systemically MB-treated mice, compared with the controls. In the present study, microglial activation was observed following TBI, which peaked at 73 h and persisted until 14 days post-TBI. Intraperitoneal MB significantly inhibited the microglial activation at 72 h and 14 days post-TBI. In addition to the model and experimental design differences, the different usage of MB may explain the contradictory results.

Autophagy degrades long-lived stable proteins, and entire organelles can be recycled under stress conditions or during development (37). Autophagy is important for homeostasis and serves as a cellular mechanism for eliminating destructive organelles and recycling components for reuse. Diskin et al. (38) first reported in 2005, that TBI may stimulate autophagy. Using a mouse model of closed head injury, Beclin 1, required for initiating autophagy, was observed to increase in the neurons and activated astrocytes at the site of injury, providing indirect evidence that autophagy is increased following TBI. The effects of rapamycin, an inducer of autophagy, has been assessed in the same model, revealing that the increase in autophagy produced beneficial neurological effects and increased the numbers of surviving neurons at the site of injury (39). In the present study, upregulation of Beclin 1 and an increased LC3-II/LC3-I ratio was observed 24 and 72 h post-TBI in the MB- and vehicle-treated mice, indicating the activation of autophagy. However, the difference between the two groups was statistically significant, as were the number of surviving neuron numbers in the MB group. These data indicated that MB intervention promoted autophagy and may assist in preventing neuronal death following TBI. To date, few studies have focused on the association between MB and autophagy. Using cell models and organotypic brain slice cultures from a mouse model of frontotemporal dementia, Congdon et al. (20) confirmed that MB alters the expression levels of LC3-II, Beclin 1 and p62, suggesting that MB is a potent inducer of autophagy. Wang et al. (40) reported that MB inhibits the activity of heat shock protein 70 prior to promoting autophagy. The present study demonstrated that MB promoted autophagy in the CCI model of TBI. This is the first report, to the best of our knowledge, to demonstrate the effect of MB on autophagy in TBI, however, the specific mechanism remains to be elucidated.

In conclusion, the present study demonstrated that MB exerted a neuroprotective effect by increasing autophagy, decreasing brain edema and inhibiting microglial activation, which reduced cerebral lesion volume, neuronal death and neurological deficit. Notably, the US FDA has approved MB as a drug (12) and, at low doses, no side effects have been identified (9). The present study did not examine the molecular mechanisms underlying the effects of MB in the regulation of autophagy and microglial activation, and further investigation of these possible mechanisms is required. In addition, investigations to determine a suitable dose and application duration, and to determine the side effects of MB in treatment of TBI is required.

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