Original Article
Alantolactone plays neuroprotective roles in traumatic brain injury in rats via anti-inflammatory, anti-oxidative and anti-apoptosis pathways

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Abstract: Traumatic brain injury (TBI) is a common disease associated with a high rate of morbidity and mortality. Secondary brain injury following TBI triggers pathological, physiological, and biological reactions that lead to neurological dysfunctions. Alantolactone (ATL) is a well-known Chinese medicine that possesses strong anti-inflammatory properties, but its role in TBI remains poorly understood. The objective of this study was to evaluate the protective effect of ATL in a rat model of controlled cortical impact (CCI). We observed the neurological scores, brain water content, oxidative stress, neuroinflammation and apoptosis by performing an enzyme-linked immunosorbent assay, western blotting, quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR), immunohistochemical (IHC) staining and other methods after CCI. The neurological scores, brain water content, levels of oxidative stress and inflammatory cytokines, and apoptosis index were markedly decreased following the ATL treatment in rats after TBI. Moreover, the antioxidant and anti-inflammatory effects of ATL in TBI may be partially mediated by inhibition of the NF-κB pathway and suppression of Cyclooxygenase 2 (COX-2). In addition, ATL attenuated TBI-induced neuronal apoptosis by suppressing the cytochrome c/caspase-dependent apoptotic pathway. Thus, ATL could exert neuroprotection in rats in a TBI model. Importantly, ATL has great potential in the clinical treatment of TBI.

Keywords: Alantolactone, traumatic brain injury, inflammation, apoptosis

Introduction

Traumatic brain injury (TBI) is currently the main cause of death and disability among young adults worldwide, causing great economic and psychological burdens on society and the patients' families [1]. The pathological process of TBI includes primary and secondary injury [2]; the primary injury is the result of mechanical factors immediately after trauma. The brain damage and dysfunction observed after TBI are primarily attributed to the secondary injury [3]. The underlying mechanism of secondary injury in TBI mainly includes oxygen free radicals, neuroinflammation, brain edema formation and neuronal apoptosis [4, 5]. Oxidative stress commonly occurs after acute injury, such as trauma and ischemic or hemorrhagic stroke [6-8]. However, the brain is rich with lipids and is susceptible to oxidative stress; therefore, the use of antioxidants is a potential targeted therapy for TBI [9-11].

The inflammatory response after TBI is as another major secondary injury process that increases the permeability of the blood-brain barrier (BBB), brain edema and intracranial pressure and eventually leads to delayed neuronal death and neurological dysfunction [12, 13]. Interleukin (IL)-1β and IL-6, Prostaglandin E2 (PGE2), and necrosis factor-α (TNF-α) are crucial pro-inflammatory cytokines following TBI [14-17]. Targeting inflammation may serve as a promising treatment measure for TBI [18]. Unfortunately, most current research methods have failed to identify a single mechanism underlying secondary injury [19]. Therefore, new potential drugs that produce a combined therapeutic effect are urgently needed.
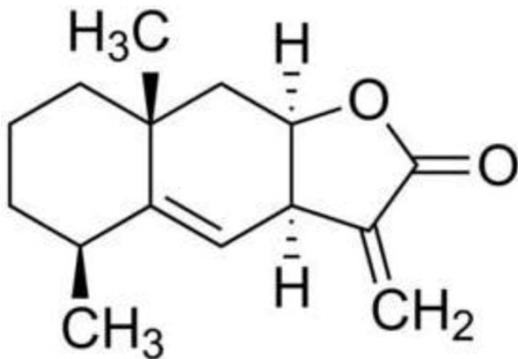
Figure 1. The chemical structure of Alantolactone. Molecular formula: C15H20O2. Molecular weight: 232.32 g/mol.

Alantolactone (ATL) is a sesquiterpene lactone compound that is isolated from the roots of Anula helenium and possesses a wide range of biological activities, including antibacterial, antifungal, hepatoprotective, antihelminthic, and antitumor activities. Muhammad Khan et al [20] found that ATL could exert antioxidant effects by inhibiting reactive oxygen species (ROS) and stabilizing Glutathione (GSH) in ATL antitumor studies. Cyclooxygenase (COX) catalyzes the first step in the production of prostaglandins by arachidonic acid, but reactive oxygen species are produced during this process [21]. In addition, COX-2, which is an inducible isoform, plays a key role in the regulation of PGE2 biosynthesis and is thought to play an important role in the inflammatory response after TBI [22]. In our previous studies, ATL could penetrate the BBB [23] and was more effective than celecoxib (a classical COX-2 inhibitor), which was demonstrated by both *in vitro* and *in vivo* studies. In addition, the precise effect of alantolactone on the NF-κB signaling pathway was demonstrated in our previous study [23]. Moreover, ATL has been shown to have a rapid onset without causing obvious damage to normal tissues and organs *in vivo* [24].

To the best of our knowledge, ATL has not been previously used for the treatment of TBI. In the current study, we examined whether ATL could have protective effects in TBI. Furthermore, the possible molecular mechanisms underlying the effect of ALT in TBI were found to be related to the COX-2 signaling pathway. Therefore, our results provide evidence supporting applications of ATL in TBI treatments.

Materials and methods

Plant materials and chemicals

Alantolactone (ATL, purity > 98%) was purchased from Tauto Biotech Co., LTD. (Shanghai, China). The chemical structure of alantolactone is shown in **Figure 1**. The malondialdehyde (MDA), superoxide dismutase (SOD), GSH, glutathione disulfide (GSSG), and Bradford protein assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies specific to COX-2, BAX, cleaved caspase-3, NF-κBp65 (p65), phospho-NF-κBp65 (p-p65), phospho-IκB-α (p-IκB-α), β-actin, and Lamin B1 and all secondary antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Inc., USA). The primary antibodies for COX IV and cytochrome c were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. The remaining reagents were used according to the analyses performed.

Animals and experimental grouping

Male Sprague-Dawley (SD) rats (pathogen-free grade males weighing 220-250 g) were purchased from the Animal Experimental Centre of Dalian Medical University, China (Certificate No. SCXK (Liao) 2008-0002). All animals had free access to sterilized food and water and were allowed to habituate for 7 days before the experiments. The study was approved by the Animal Research Ethics Committee of Dalian Medical University. All experimental procedures were performed according to the Guidance Suggestions for the Care and Use of Laboratory Animals published by the Ministry of Science and Technology of the People's Republic of China.

One hundred and fifty SD rats were randomly divided into the following five groups for the efficacy experiment, and each group was assessed at 2 time points (24 and 48 h): (a) Sham-operated control group (Sham): the rats only underwent a craniotomy, but their brain tissue was not damaged. After the operation, the rats received the same amount of vehicle by an intraperitoneal injection every 24 h (n = 30); (b) Sham-operated control + ATL group (Sham + ATL): After the operation, the rats received 20 mg/kg of ATL by an intraperitoneal injection

Table 1. Neurological severity scores

Items	Detailed description	Points (success/failure)
Exit circle	Ability to enter or exit a 30-cm circle (3 min)	0/1
Mono/hemiparesis	Paresis of contralateral upper or lower limb	0/1
Startle reflex	Innate reflex (flinching in response to a loud hand clap)	0/1
Seeking behavior	Physiological behavior as a sign of "interest" in the environment	0/1
Straight walk	Alertness/initiative/straight walking when placed on the floor	0/1
Round stick balance	Ability to balance on a round stick 5 mm in diameter for 10 s	0/1
Beam balance	Ability to balance on a beam 7 mm in width for 10 s	0/1
Beam walk (3 cm)	Ability to cross a beam (length × width, 30 × 3 cm)	0/1
Beam walk (2 cm)	Ability to cross a beam (length × width, 30 × 2 cm)	0/1
Beam walk (1 cm)	Ability to cross a beam (length × width, 30 × 1 cm)	0/1
Maximum score		10

N = 6 per group.

every 24 h (n = 30); (c) TBI control group (Control): After TBI, the rats received the same amount of vehicle by an intraperitoneal injection every 24 h (n = 30); (d) 10 mg/kg of ATL treatment group: After TBI, the rats received 10 mg/kg of ATL by an intraperitoneal injection every 24 h (n = 30); (e) 20 mg/kg of ATL treatment group: After TBI, the rats received 20 mg/kg of ATL by an intraperitoneal injection every 24 h (n = 30).

TBI model

The TBI model used in the present study was based on the controlled cortical impact (CCI) model with some modifications as previously described by Brody DL, *et al* [25]. Briefly, the rats were anesthetized with 4% chloral hydrate and maintained at 37.0 ± 0.5°C on a thermal mat throughout the surgical procedure. CCI was produced in the rat using a PCI3000 PinPoint Precision Cortical Impactor (Hatteras Instruments, Cary, NC, USA). After exposing the skull through a central skin incision and removing the soft tissue with a cotton tip, a circular craniotomy approximately 4 mm in diameter was performed in the middle of the right parietal bone approximately 0.5 mm from the sagittal, coronal, and lambdoid sutures, while the dura remained intact. The CCI parameters were as follows: impact tip diameter, 3 mm; velocity, 2 m/s; compression time, 85 ms; and compression distance, 1 mm. Using these impact parameters, we established a moderate injury model. The impact tip was wiped with alcohol after each impact. Finally, we sutured the wounds, woke the rats, and placed the rats on a heating

cushion to maintain normal body temperature for 30 to 60 min. The sham-operated rats underwent the same procedure without percussion.

Neurobehavioral score

The neurological score was assessed 24 h and 48 h after TBI using the neurological severity score (NSS) system. Two observers independently assessed the ability of each rat (N = 6) to perform 10 different tasks, and if the results were inconsistent, the results were obtained through discussion. Rats that failed to perform the tasks received one or no points (**Table 1**) [26]. The observers were blinded to all group information.

Brain water content

The water content in the brain was measured using the wet-dry weight method as previously described [27]. In brief, the contusion cortex and surrounding injured brain tissues were rapidly harvested, quickly weighed to obtain the wet weight, dried in an incubator at 120°C for 24 h and reweighed (dry weight). The brain water content was calculated by the following formula: ratios = [(wet weight-dry weight)/wet weight] × 100%.

Tissue processing

For the western blot analysis and quantitative real-time reverse transcription polymerase chain reaction, the rats were rapidly killed 24 h post-TBI, and their ipsilateral cortexes were collected. The tissue was positioned directly over

the center of the injury site and included both contusions and penumbra. The samples were immediately frozen in liquid nitrogen and stored at -80°C until use. For the immunohistochemistry analysis, the ipsilateral brain tissue was removed 24 h after TBI, immersed in 4% paraformaldehyde overnight and was measured by a fluorescence-activated cell sorter (FACS) using the Annexin V-FITC Apoptosis Detection Kit (Nanjing KeyGEN Biotech. CO., LTD.).

Malondialdehyde (MDA) and superoxide dismutase (SOD) assay

The samples were homogenized and centrifuged at 3,000 × g for 10 min. Subsequently, the supernatants were collected, and the MDA levels and SOD activity were analyzed. The level of MDA and SOD were measured using commercially available kits (Nanjing Jiancheng Biochemistry Co., Nanjing, China) according to the manufacturer's instructions. The MDA level is represented in nmol/mg protein, and the SOD activity is expressed as U/mg protein.

Enzyme-linked immunosorbent assay (ELISA)

The total protein was determined using a using a BCA protein assay kit (Beyotime Biotechnology, China). The levels of inflammatory cytokines in the brain tissue were quantified using ELISA kits specific to the rat (IL-1β, IL-6, PGE2, and TNF-α from Shanghai Saimo Biotechnology, Shanghai, China) according to the manufacturer's instructions. The inflammatory cytokine contents in the brain tissues are expressed as nanogram per gram protein.

Isolation of rat cortical neurons

To rapidly isolate cortical neurons from the injured side of the rat brain, we followed a previously described method with certain minor modifications [28-30]. Briefly, after TBI, the injured-side cortex was cut into fragments, and the cells were dissociated by incubation with 2 mg/mL papain in DMEM for 30 min at 37°C. To produce a purified population of cells, we used the immune adherence method. The cell suspensions were poured into anti-neural cell adhesion molecule (NCAM)-coated petri dishes (Millipore, USA) and placed on a shaker for 1 h; then, the adhered cells were collected. Trypan blue was used to exclude the non-viable cells.

Mitochondrial membrane potential (MMP) measurement

JC-1 probe (Invitrogen, CA, USA) was employed to measure mitochondrial depolarization in isolated neurons. Briefly, the cells were cultured in six-well plates after the indicated treatments, incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37°C for 20 min and rinsed twice with PBS. The mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using FACS Accuri C6 (Genetimes Technology Inc.). Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

Western blot analysis

The total and nuclear proteins were extracted from the ipsilateral cerebral cortical tissue using the Total and Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, China) according to the manufacturer's instructions. The mitochondrial and cytoplasmic proteins were extracted from the remaining ipsilateral tissue using the Tissue Mitochondria Isolation Kit for Tissue Protocols (Beyotime Biotechnology, China). These proteins were separated by electrophoresis on a 7.5-12% SDS-PAGE gel and probed with specific antibodies. The protein bands were detected by enhanced chemiluminescence. The protein concentrations were determined using a BCA protein assay kit (Beyotime Biotechnology, China). Similar experiments were performed at least three times.

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR)

The total RNA was extracted from brain tissue using TRIzol reagent according to the manufacturer's protocol (TaKaRa Bio, Dalian, China). cDNA was reverse-transcribed using the PrimeScript RT Reagent Kit (TaKaRa Bio, Dalian, China) according to the manufacturer's instructions. The Q-PCR reaction was performed following the manufacturer's protocol (TaKaRa Bio, Dalian, China), and the amplification was performed using a Mx3005P Real-Time PCR System (Agilent, CA, USA). The relative mRNA expression of each gene was normalized to the β-actin RNA levels. The primers were synthe-
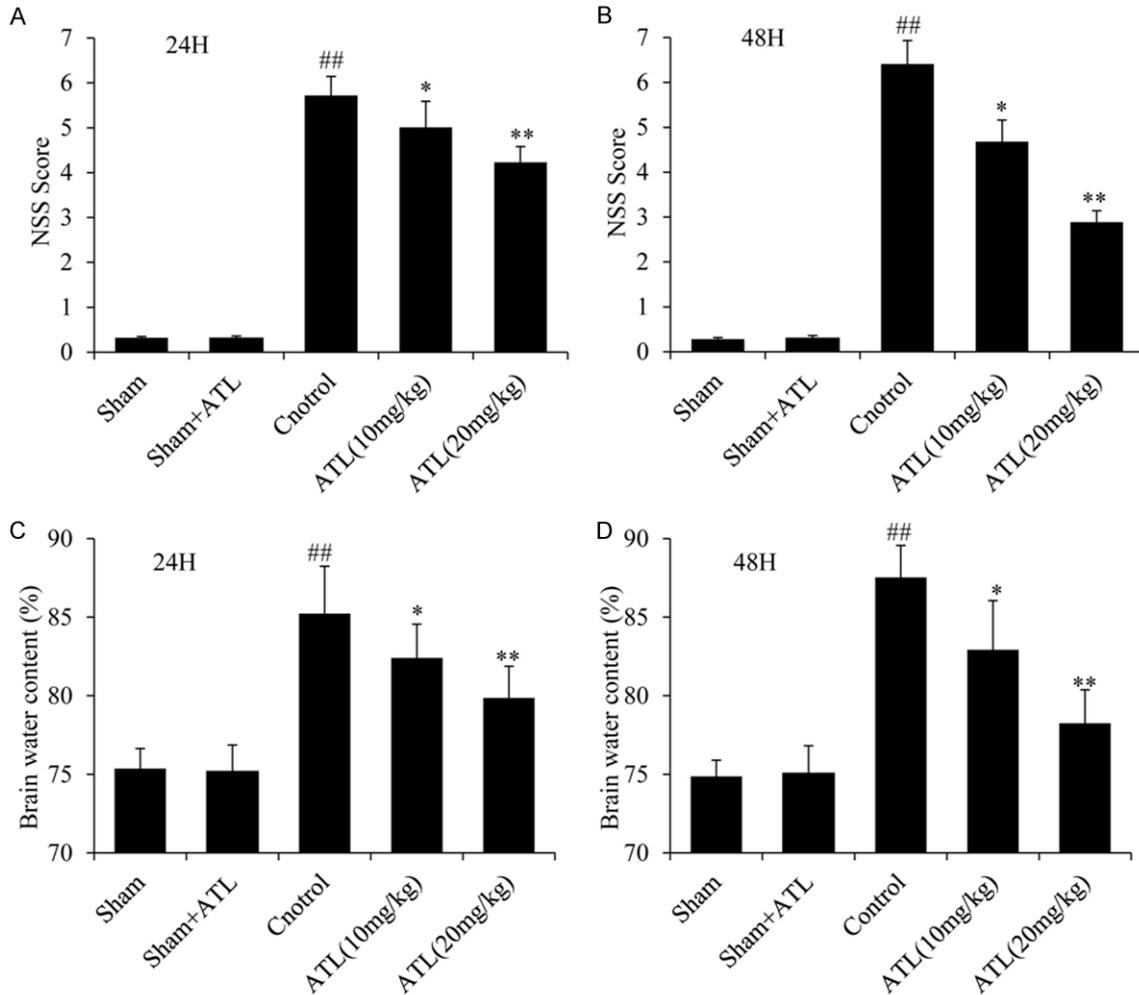
Figure 2. ATL decreases the NSS after TBI in rats and reduces the water content in the injured tissue at 24 h and 48 h. A and B: NSS Score. C and D: Brain water content. ##$P < 0.01$, vs. sham group. *$P < 0.05$, **$P < 0.01$, vs. control group.

sized by Invitrogen (Shanghai, China). The primers for COX-2 were as follows: 5'-ACCAACGCT-GCCACAACT-3 and 5'-GGTTGGAACAGCAAGGA-TTT-3'; the primers for β-actin were as follows: 5'-GGCACCCAGCACAATGAA-3' and 5'-TAGAAG-CATTTGCGGTGG-3'.

Immunohistochemical staining

The brain tissue samples were fixed with 10% neutral formalin and embedded in paraffin. For the immunohistochemical examination, the tissue sections (4 mm) were incubated with the COX-2 (1:50) and p-p65 NF-κB (1:50) antibodies and examined under a light microscope. The images were examined under a Leica DM 4000B microscope equipped with a digital camera.

Statistical analysis

Student's t-tests were performed to compare the values of the test and control samples *in vitro* and *in vivo*. All experiments were repeated three times. The data are represented as the mean ± standard deviation (SD). SPSS 17.0 software was used for all statistical analysis. ##$P < 0.01$, *$P < 0.05$ and **$P < 0.01$.

Results

ATL improved neurological function and alleviated cerebral edema after TBI

To evaluate whether ATL exerts a neuroprotection effect after TBI, we examined the NSS scores and brain water content 24 h and 48 h
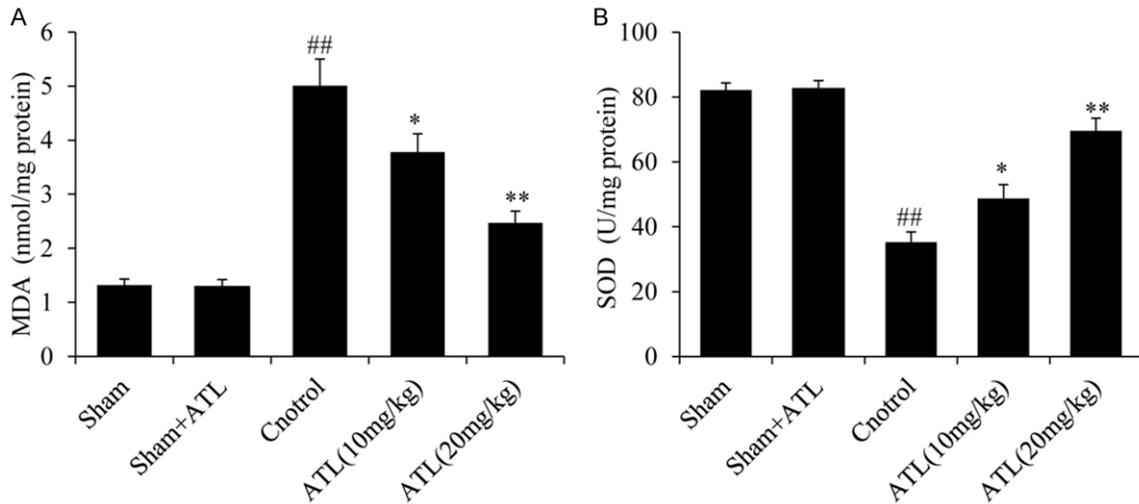
Figure 3. ATL reduces oxidative stress in brain tissue following TBI. A: The level of MDA in the cortex 24 h post-injury. B: The activities of SOD in the cortex 24 h post-injury. ##P < 0.01, vs. sham group. *P < 0.05, **P < 0.01, vs. control group.

Table 2. Reduced and oxidized glutathione levels in TBI rats (24 h post-injury)

Group	GSH (µmol/L)	GSSG (µmol/L)	GSH/GSSG
Sham	99.35 ± 5.32	25.76 ± 1.87	3.86 ± 0.15
Sham + ATL	99.42 ± 4.98	25.16 ± 2.13	3.92 ± 0.19
Control	58.82 ± 4.17##	54.51 ± 4.26##	1.08 ± 0.24##
ATL (10 mg/kg)	70.59 ± 6.19*	45.82 ± 3.25*	1.54 ± 0.32*
ATL (20 mg/kg)	87.49 ± 7.13**	32.93 ± 2.86**	2.66 ± 0.39**

Each value represents the mean ± SD (n = 6). ##P < 0.01 compared to the sham group; *P < 0.05, **P < 0.01 compared to the control group.

after TBI as shown in **Figure 2**. First, no differences were found in the NSS scores and brain water content between the sham and sham + ATL groups. However, the NSS scores and brain water content in the TBI control group were obviously higher than those in the sham group at the two time point (##P < 0.01). Furthermore, the ATL treatment significantly alleviated the neurological deficits and brain edema in a dose-dependent manner compared with those seen in the control group, (*P < 0.05, **P < 0.01). Thus, ATL might have a potential protective effect in rats and, hence, could be used in the subsequent trials.

ATL attenuated oxidative stress caused by TBI

To assess whether the neuroprotective effect of ATL was mediated by oxidative stress-related signaling pathways, the indicators of lipid peroxidation and antioxidant enzyme activity, including MDA level, SOD activity, GSH level,

GSSG level and GSH/GSSG ratio, were measured 24 h after TBI as shown in **Figure 3A**, **3B**) and **Table 2**. The TBI control group had a higher level of MDA and GSSG than the sham group (##P < 0.01). However, the levels of MDA and GSSG in the ATL treatment groups were significantly lower than those in the TBI control group (*P < 0.05, **P < 0.01). The activity of SOD, GSH and GSH/GSSH in the TBI control group was significantly lower after TBI than that after the sham injury (##P < 0.01), whereas the ATL treatment groups showed an obvious up-regulation of SOD, GSH and GSH/GSSH activity in a dose-dependent manner (*P < 0.05, **P < 0.01). Importantly, ATL had no effects on the sham group.

ATL induced significant reductions in multiplex cytokine levels after TBI

Inflammatory processes are considered major components of the secondary injury cascade following TBI [31]. As shown in **Figure 4**, the expression levels of the pro-inflammatory cytokines (i.e., IL-1β, IL-6, PGE2 and TNF-α) were low in the brains of rats in the sham group. The levels of those cytokines were markedly increased following TBI compared with those in the sham group (##P < 0.01); however, the administration of ATL significantly decreased the cytokine expression levels compared with those in
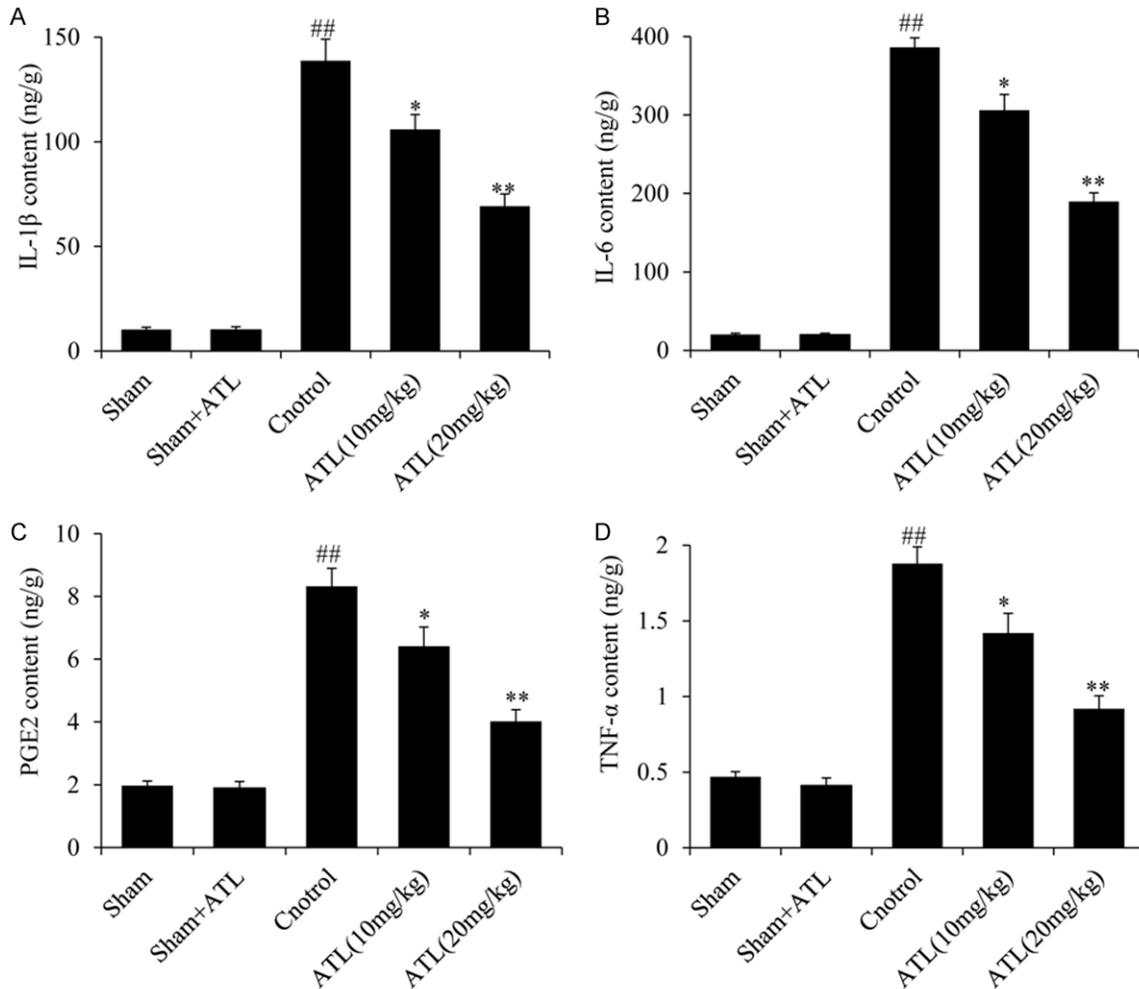
Figure 4. ATL ameliorates TBI-induced inflammation. ELISA analysis of the expression levels of pro-inflammatory genes, including (A) IL-1β, (B) IL-6, (C) PGE2 and (D) TNF-α 24 h after TBI. ##P < 0.01, vs. sham group. *P < 0.05, **P < 0.01, vs. control group.

the TBI control group (*P < 0.05, **P < 0.01). The above-mentioned indicators did not significantly differ between the sham and sham + ATL groups.

ATL inhibited the COX-2 signaling pathway in TBI

As mentioned above, ATL can significantly inhibit the expression of PGE2 and other inflammatory factors in TBI, and COX-2 is an inducible enzyme responsible for prostaglandin production at sites of inflammation [32, 33]. We immediately detected the COX-2 protein and molecular levels in TBI (**Figure 5**). As shown in **Figure 5A**, **5B** and **5D**, according to the western blot and IHC analyses, the expression levels of COX-2 were low in the sham group. Following

TBI, the expression levels of COX-2 in the cortex of rats in the TBI control group were significantly higher than those in the sham group (##P < 0.01). The expression levels of COX-2 were markedly lower in the ATL treatment group than those in the TBI control group (**P < 0.01). Furthermore, the RT-qPCR results were consistent. As shown in **Figure 5C**, the treatment with ATL significantly down-regulated the COX-2 mRNA levels in the ATL treatment group (**P < 0.01).

ATL inhibited the activation of the NF-κB signaling pathway and its translocation into the nucleus after TBI

The above-mentioned results demonstrate that ATL effectively suppressed the COX-2 gene ex-

Figure 5. ATL inhibits COX-2 expression in brain tissue 24 h after TBI. A and B: COX-2 expression by western blot assays. C: mRNA expression by RT-qPCR. D: COX-2 protein expression by immunohistochemical analysis (Original magnification, 400 ×). ##$P < 0.01$, vs. sham group. **$P < 0.01$, vs. control group.
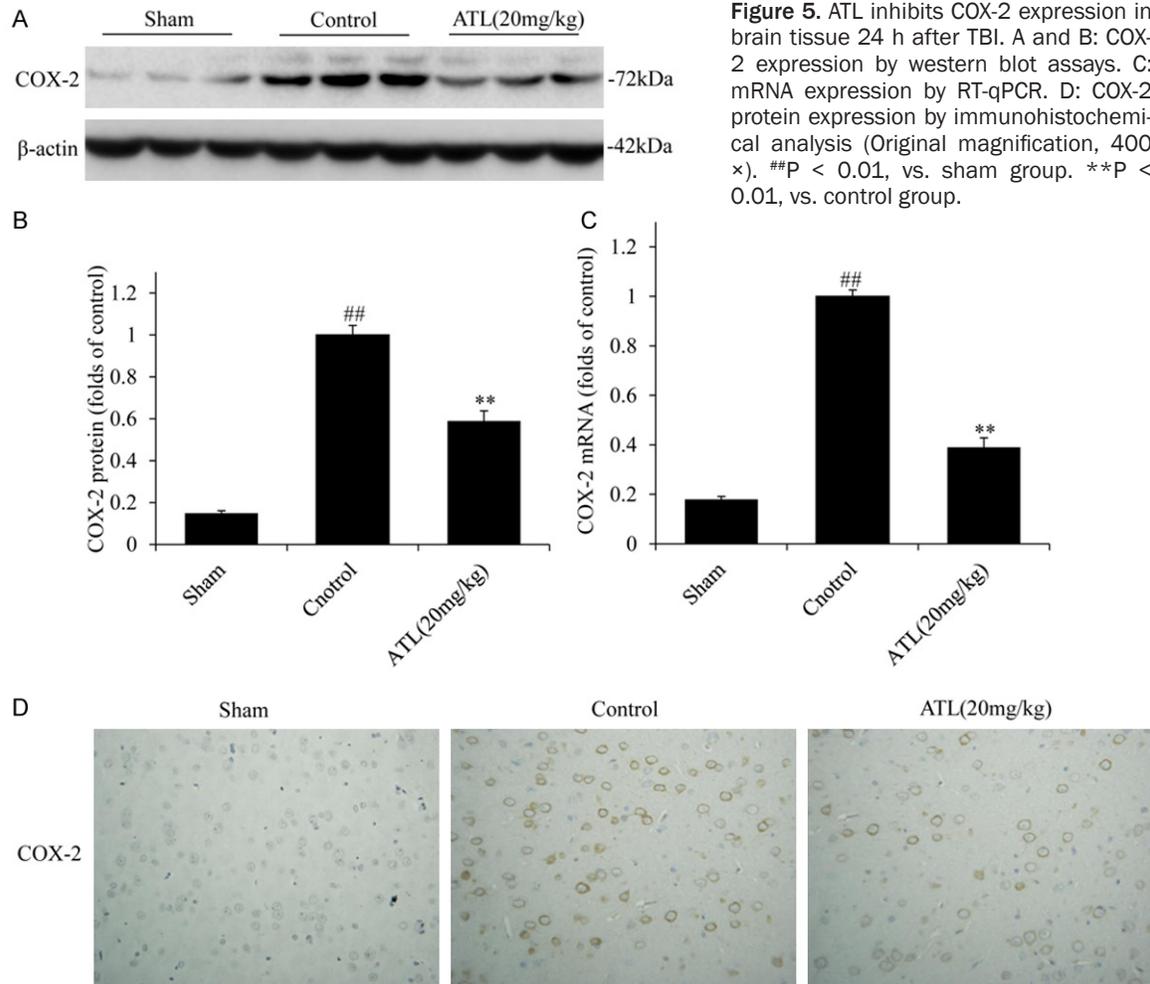
pression after TBI; however, COX-2 gene expression is strictly and specifically controlled by the binding of many transcription factors to the corresponding sites of its promoters, such as NF-κB [34, 35]. The activation of the NF-κB signaling pathway is closely related to the overexpression of COX-2 [36, 37]. As shown in **Figure 6**, according to the western blot and IHC analyses, the expression levels of p-p65 and p-IκB-α in the TBI control group were higher than those in sham group. However, these protein contents were obviously decreased in the ATL treatment group compared with those in the TBI control group (**$P < 0.01$). Then, we extracted the nuclear protein and performed a western blot analysis (**Figure 6E** and **6F**); the expression levels of NF-κB and p65 were significantly up-regulated in the TBI control group. Meanwhile, the protein content of p65 was markedly decreased in the ATL treatment group compared with that in the TBI control group (**$P < 0.01$).

ATL attenuated neuronal apoptosis in brain tissue after TBI

To determine whether ATL could inhibit neuronal apoptosis after TBI, we first detected the apoptosis-related proteins by performing a western blot analysis. As shown in **Figure 7A-C**, the protein levels of pro-apoptotic Bax and cleaved caspase-3 were markedly increased in the TBI control group compared with those in the sham group. However, these protein levels were significantly reduced after the treatment with ATL. Subsequently, we detected the changes in the mitochondrial membrane potential (**Figure 7D**). The MMP was obviously reduced in the TBI control group and significantly increased following the treatment with ATL. In addition, we extracted the cytosolic and mitochondrial proteins and performed a western blot analysis (**Figure 7E-H**). Relative to the sham groups, the cytosolic and mitochondrial cyt c levels increased and decreased, respec-

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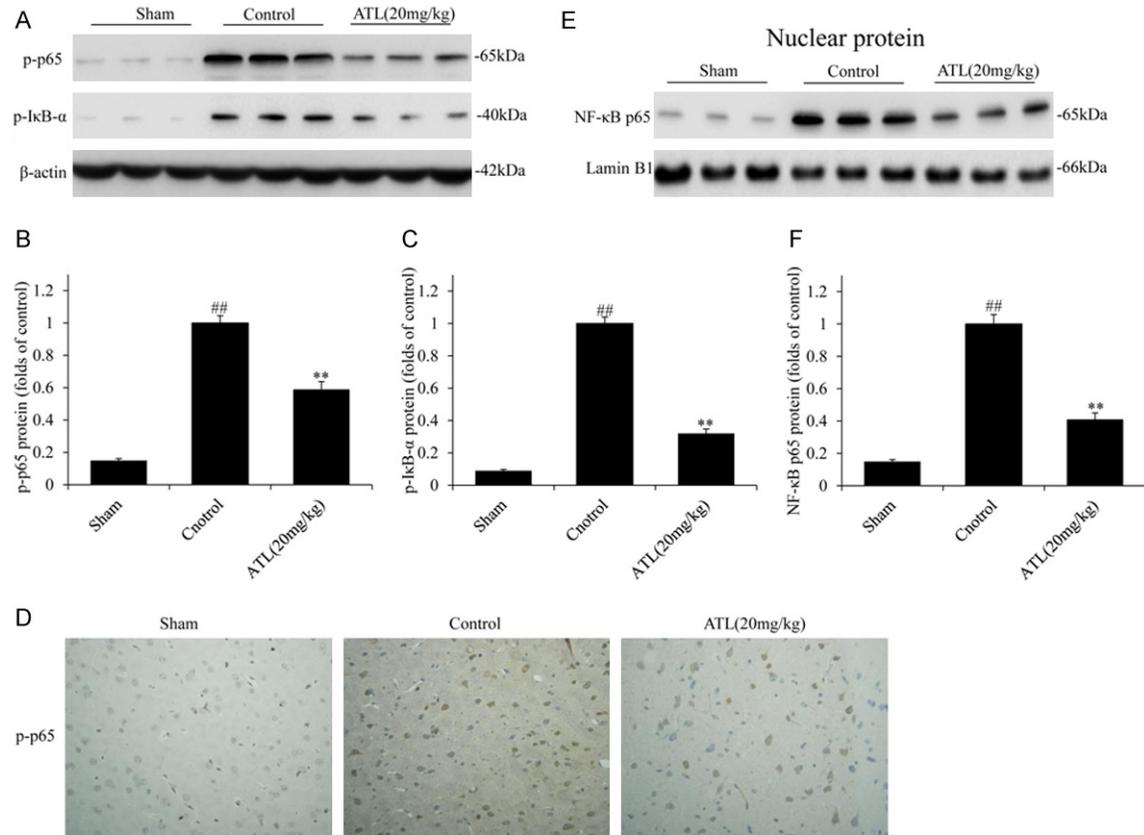
Figure 6. ATL inhibits the activation of the NF-κB signaling pathway and its translocation into the nucleus 24 h after TBI. A-C: The total protein expression of p-p65 and p-IκB-α by WB assays. D: The protein expression of p-p65 and p-IκB-α by immunohistochemical analysis (Original magnification, 400 ×). E and F: The nuclear protein expression of NF-κB p65 by WB assays. ##P < 0.01, vs. sham group. **P < 0.01, vs. control group.

tively, after TBI, while the cytosolic and mitochondrial cyt c levels markedly decreased and increased, respectively, after the treatment with ATL in a dose-dependent manner. Thus, following TBI, ATL could attenuate neuronal apoptosis by inhibiting cyt c release in the mitochondrion and suppressing caspase activation in the cytoplasm.

Discussion

Secondary brain injury after TBI occurs in a series of pathological processes, and oxidative stress, the inflammatory response and cell apoptosis are crucial mechanisms that mediate the subsequent histopathology and neurobehavioral deficits [38, 39]. In our study, we used a rat model to evaluate the protective effects of ATL after TBI and the potential molecular mechanisms and obtained the following results: (a) the administration of ATL improved the neurological function and alleviated cerebral edema after TBI; (b) ATL inhibited TBI-

induced oxidative stress, which was represented by the level of MDA and GSH and the activity of SOD; (c) ATL suppressed the production of pro-inflammatory cytokines after TBI by inhibiting the NF-κB/COX-2 signaling pathway; and (d) ATL attenuated neuronal apoptosis after TBI by suppressing the cytochrome c/caspase-dependent apoptotic pathway.

The brain is particularly vulnerable to oxidative stress attacks after TBI, and brain tissue produces more toxicants than other organs [40]. Due to oxidative stress following TBI, peroxidized enzymes become activated, destroying membrane phospholipids and mitochondrial functions, and several cellular components, such as RNA, DNAs, lipids, carbohydrates, and proteins, are harmed. Ultimately, neuronal cells and brain tissue suffer irreversible damage [41]. To inhibit the oxidative stress response after TBI, key enzymes related to oxidative stress have become the focus of current research studies. SOD and GSH are the most
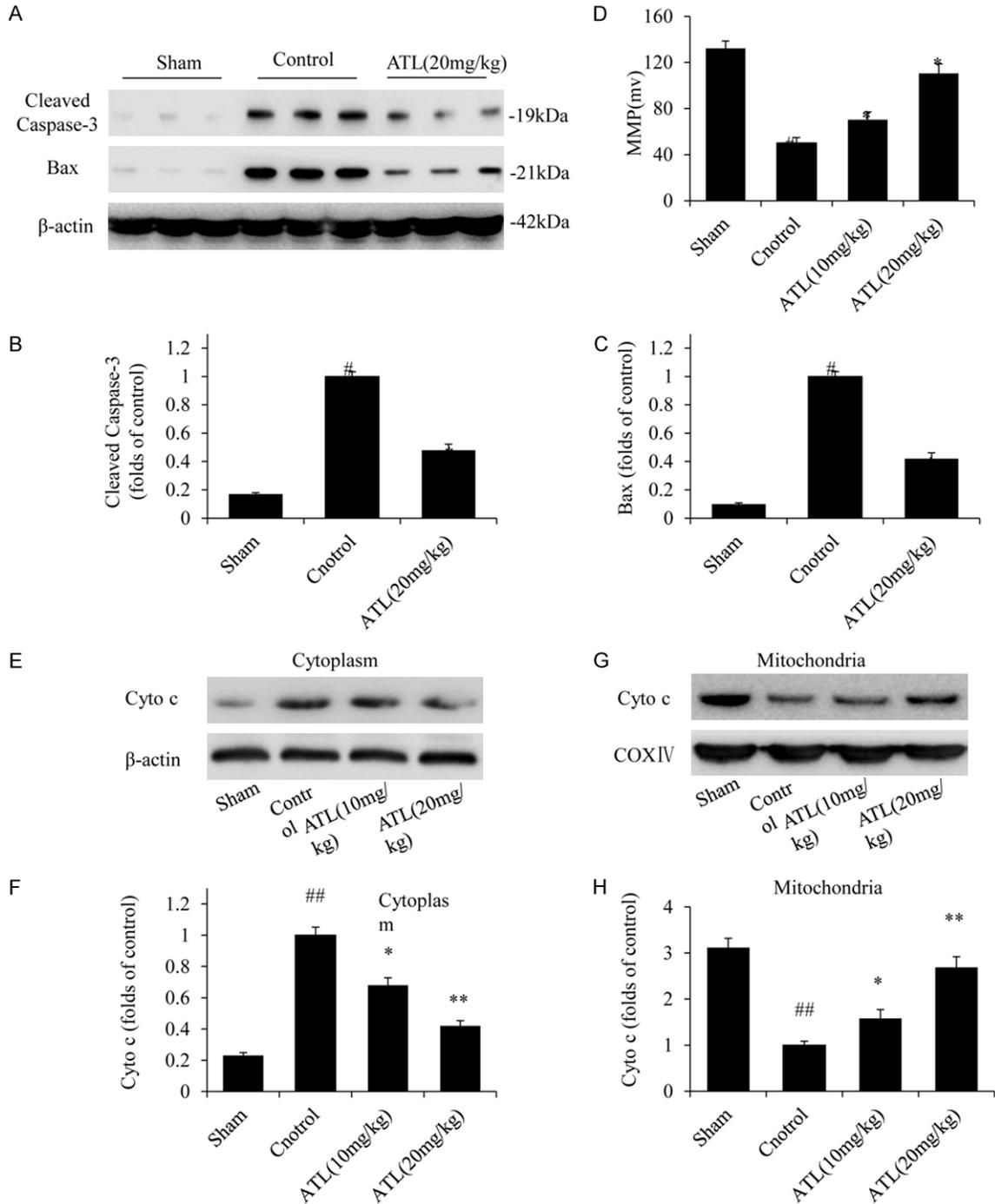
Figure 7. ATL attenuates TBI-induced neuronal apoptosis 24 h post-injury. A-C: The protein expression of cleaved caspase-3 and Bax by western blot assays. D: MMP. E and F: The cytoplasmic protein expression of cyt c by western blot assays. G and H: The mitochondrial protein expression of cyt c by western blot assays. ##P < 0.01, vs. sham group. *P < 0.05, **P < 0.01, vs. control group.

important antioxidants that prevent the brain from oxidative injury. SOD can catalyze the conversion of superoxide to hydrogen peroxide [42], and GSH is among the smallest antioxidants in body fluids [43]. MDA, which is a prod-

uct of lipid peroxidation, is an important marker of oxidative damage. The content of MDA in the tissue increases as the level of oxidative stress increases [44]. Following oxidative stress, GSH can combine with free radicals and convert to

GSSG. The GSH/GSSG ratio is an effective indicator used to monitor antioxidant activity. In the present study, ATL significantly improved the SOD activities, GSH level, and GSH/GSSG ratio in brain tissues after TBI. Meanwhile, ATL evidently diminished the MDA and GSSG levels.

The inflammatory responses induced following TBI are important therapeutic targets for reducing tissue damage following trauma [45]. IL-1β, IL-6, PGE2 and TNF-α are key pro-inflammatory cytokines after acute injuries, and these cytokines were up-regulated at the early stage, which initiated a continuous regional inflammatory response. In our study, ATL could markedly reduce the levels of these pro-inflammatory cytokines during the early period of TBI.

COX-2 expression is strictly and specifically controlled by the binding of transfactors to the corresponding sites of its promoters. However, the transcription factor NF-κB is involved in the regulation of a variety of inflammatory mediators, which is a cis-acting element of the COX-2 transcriptional regulatory sequence. NF-κB consists of homo- and heterodimers of different Rel family proteins (p65, RelB, c-Rel, p52, and p50) and is sequestered by binding to the inhibitory subunit I-κB in most non-stimulated cells [46]. The NF-κB pathway is mainly activated by the degradation of the phosphorylation of the inhibitory protein IκB, thereby liberating free NF-κB dimers to translocate to the nucleus [47]. Thus, activated NF-κB could bind to the COX-2 promoter region and initiate COX-2 transcription. In the present study, ATL significantly suppressed the expression of total p-p65, p-IκB-α, and nuclear p65. Thus, ATL suppressed COX-2 expression by inhibiting the activation of the NF-κB pathway.

Apoptosis is an important pathophysiological component during secondary damage in TBI [48, 49]. Apoptosis of neurons and oligodendrocytes in brain tissues causes neural functional deficits [50, 51]. Caspase-3 is activated during apoptosis in many types of central nervous system (CNS) cells, and its activation appears to be an important apoptotic event in the CNS [52, 53]. The Bcl-2 family proteins are the key step in the regulation of apoptosis in the nervous system. The pro-apoptotic Bax modulates intrinsic apoptotic cell death [54, 55]. In our study, ATL significantly inhibited the expression of cleaved caspase-3 and Bax after TBI. To further determine the possible mechanism underlying the apoptosis inhibition effect of ATL in TBI, we first detected whether the mitochondrial membrane was damaged; then, the increased release of cytochrome c from the mitochondria to the cytoplasm was observed, and increased apoptosis via activating caspase signaling cascades was confirmed. ATL inhibited the decrease in the mitochondrial membrane potential, and the cytosolic and mitochondrial cyt c levels were markedly decreased and increased, respectively, after the treatment with ATL, in a dose-dependent manner. Therefore, ATL attenuated TBI-induced neuronal apoptosis by inhibiting the mitochondrial apoptotic pathway.

In conclusion, ATL could have a potential neuroprotective effect in TBI and should be further explored for suitability in clinical applications for the treatment of TBI.

Acknowledgements

Disclosure of conflict of interest

None.

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