Shikonin regulates autophagy via the AMPK/mTOR pathway and reduces apoptosis of human umbilical cord mesenchymal stem cells to improve survival in tissues surrounding brain contusion

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Abstract. Shikonin has been reported to regulate autophagy via the AMP-activated protein kinase (AMPK)/mTOR signalling pathway and decrease apoptosis in transplanted human umbilical cord mesenchymal stem cells (HUMSCs). In the present study, HUMSCs were exposed to oxygen glucose deprivation (OGD) in vitro for 12 h, and TUNEL fluorescence staining was used to detect apoptosis. Differences in autophagy and AMPK/mTOR pathway-related protein expression following treatment with shikonin were quantitatively analyzed by western blotting. Green fluorescent protein-labelled stem cells were implanted into traumatic brain injury-model mice and the survival of HUMSCs was observed after 7 days. Shikonin increased the number of cells in brain tissue surrounding the contusion 7 days after transplantation. Furthermore, shikonin treatment decreased apoptosis, increased the expression of autophagy-related proteins, increased phosphorylated AMPK expression and downregulated phosphorylated mTOR expression. In addition, the autophagy inhibitor 3-methyladenine attenuated these effects and aggravated apoptosis. Subsequently, shikonin upregulated autophagy and protected HUMSCs in the area surrounding contused brain tissue. Shikonin may regulate autophagy via the AMPK/mTOR signalling pathway and protect transplanted HUMSCs from apoptosis induced by hypoxia/ischemia.

Introduction

Traumatic brain injury (TBI) represents a global health problem (1). The pathophysiology of brain injury following craniocerebral trauma is complex and is characterized by initial and secondary damage for several days following trauma (2). Among the numerous causes of morbidity and mortality associated with trauma, the incidence of TBI is becoming the most important cause, resulting in severe disability and mortality (3) and functional impairment, which can affect the quality of life (4,5).

At present, the treatment strategies for brain trauma mainly include the control of secondary injury and promotion of neurorehabilitation through training and drug administration (6). However, this type of intervention is not enough to fully restore neural system function, so new strategies should be discovered (1,6). In the last decade, some studies on stem cell transplantation as a TBI replacement therapy (7,8), in animal models (9) and in the clinic have produced promising results (10,11). The benefits of stem cell transplantation are multifaceted. Firstly, the ability of stem cells to undergo neural differentiation and long-distance migration to the injury site allows them to directly replace dead or dying cells (12,13). Secondly, the presence of stem cells in lesions indirectly affects the microenvironment. By secreting growth factors, stem cells can promote the proliferation of nerve cells and neurotransmitter transmission (14,15). However, transplanted stem cells have relatively poor survival, and their limited survival following brain injury and early death of transplanted cells limits bone marrow mesenchymal stem cell-based treatment (16,17). In order to fully use the therapeutic potential of stem cells, it is crucial to determine the cause of their early death and develop strategies to improve their survival rate.

Previous studies reported that autophagy has a protective effect on mesenchymal stem cells (MSCs) under stress conditions, such as ischemia stroke (18,19). Autophagy is a process of cellular self-protection that supports the homeostasis of cells under external and internal environmental stresses, including undernutrition, infection, presence of cell debris and protein aggregation (20,21). Autophagy and apoptosis are the two main pathways involved in cell protection from external stress in various cell injury models, and autophagy seems to be beneficial in the setting of traumatic brain injury (22,23). Therefore, in this study, we aimed to determine the mechanisms of action of shikonin on HUMSCs and to assess their potential for autophagy induction during traumatic brain injury.
stimuli. These two processes are usually co-regulated but result in opposite cell outcomes (22). Autophagy is a self-digesting process during which a double-membrane vesicle, known as the autophagosome, is produced around the targeted organelle. Autophagosomes degrade their cargo via a lysosomal mechanism (23). The autophagy process can maintain cellular fitness, nutrition, and energy levels during starvation or exposure to external stress (24). Conversely, the programmed cell death of damaged or senescent cells is known as apoptosis (24). Previous studies demonstrated that external stress stimuli may trigger one of these two processes, depending on the cellular environment (25,26). The inhibition of one of these pathways can lead to the activation of the other. Triggering autophagy during programmed cell death can reduce apoptosis and prolong cell survival, whereas reducing autophagy in normal cells can increase apoptosis (27,28). The regulation of autophagy may therefore provide a new mechanism to prevent cell apoptosis under stress conditions.

Shikonin is a natural naphthoquinone derivative isolated from the root of plant comfrey, and which possesses some anti-tumour, anti-inflammatory (29-31), antibacterial (32) and antithrombotic (33) activities. A study has reported that shikonin can inhibit tumour cell proliferation and metastasis by inhibiting the phosphorylation of mTOR (34). Shikonin can increase autophagy in A375 cells with 1.2 $\mu$M in 24 h (35). The present investigation illustrates the protective effect and underlying mechanism of shikonin in human umbilical MSCs (HUMSCs) in a hypoxic-ischemic state in vitro and in vivo.

Materials and methods

Materials. Shikonin was purchased from MedChemExpress (cat. no. HY-N0822) and was dissolved in DMSO to prepare a stock solution (10 mg/ml; stored at -20°C) and diluted to 1.2, 1.6, 2, 4, and 8 $\mu$M for use. 3-methyladenine (3-MA), an inhibitor of the PI3k/AMPK pathway used for creating an inhibition model of autophagy, was purchased from MedChemExpress (cat. no. HY-19312) and dissolved in double distilled water to prepare stock solutions (5 mM and 1,000 $\mu$M stored at 4°C). DMEM and fetal bovine serum (FBS) were purchased from HyClone; GE Healthcare Life Sciences. Trypan blue stain (0.4%; cat. no. T10282) was purchased from Invitrogen; Thermo Fisher Scientific, Inc. A DeTie microplate reader was used to measure the absorbance at a wavelength of 450 nm. Following CCK-8, the optimal processing time and drug concentration was obtained. All cells were divided into three groups: OGD, OGD + shikonin, OGD + shikonin and 3-MA. OGD groups were treated only with OGD. In group OGD + shikonin, shikonin was used to treat cells with OGD. In group OGD + shikonin + 3-MA, shikonin and 3-MA (5 mM for 24 h before OGD) were used at same time.

Animal model and tissue. A total of 18 male C57BL/6 mice (15-20 g; 9-12-week-old) were obtained from the Animal Experimental Center of Wenzhou Medical University. Mice were divided into three groups as follows: TBI, TBI + shikonin and TBI + shikonin + 3-MA groups (6 mice per group). Treatments were administered for 3 consecutive days before the TBI model was established. In mice, 50 mg/kg shikonin (36) was orally administered with 3-MA (1.5 mg/100 g), as appropriate. TBI was modelled using an Impact One™ Stereotaxic Impactor (Leica Microsystems, Inc.) at a depth of 1.0 mm and speed of 3 m/sec (37) in anesthetized mice. Animal anesthesia was performed using a Reyward small animal anesthesia machine and isoflurane inhalation at 3-4%. Once the gas completely filled the induction box (~1 min), the animals were placed into it. Once the animals were completely anesthetized (~2-3 min; animals
were turned over and did not attempt to resume prone position),
anesthesia was maintained using 1-1.5% isoflurane, and the
gas flow rate was about 300-500 ml/min. GFP-HUMSCs
were transplanted 24 h after TBI. The experimental mice
were sacrificed seven days later. In each group, three mice
cortical tissue surrounding the trauma was obtained and
stored at -80 °C immediately. The other three, the brains were
fixed with 4% PFA for 24 h with 4°C. Then the sections were
dehydrated with 30% sucrose for 48 h with 4°C, embedded
in optimal cutting temperature compound (OCT) and frozen,
then sectioned at 10 µm. Animal experiments lasted a total
of 11 days. All animal health, behaviour and animal welfare
(including regular cleaning of cages, replacement of food
and drinking water and no more than 5 mice in one cage) were
monitored at the Animal Experiment Center of Wenzhou
Medical University. The experimental mice were sacrificed
by decapitation following an overdose of isoflurane. The study
was approved by the Ethics Committee of The First Affiliated
Hospital of Wenzhou Medical University (Wenzhou, China).
All procedures were in compliance with the Animal Care and
Use Committee of Wenzhou Medical University.

Cells transplantation. GFP-HUMSCs were trypsinized with
0.05% trypsin solution for 3 min at 37 °C before transplanta-
tion. Then, HUMSCs were transferred from the culture dish to
the test tube, washed with PBS for 3 times, and then injected
into the TBI modelled area with a stereotaxic instrument and
a micro injection needle (1x10 ^6 cells in 3 µl at a delivery rate
of 1 µl/min).

Trypan blue staining. Trypan blue staining was performed after
shikonin treatment. HUMSCs were detached by trypsin-EDTA, and resuspended in medium. Subsequently, 200 µl cell suspension was supplemented with 0.4% trypsin blue (ratio cell suspension:trypsin blue; 9:1) and incubated for 5 min at room temperature. Under the microscope, dead cells were stained blue, whereas living cells were transparent and colour-
less. ImageJ (v. 1.6.0; National Institutes of Health) was used to
count the number of normal cells and dead cells in the figure.
The rate of living cells was calculated by GraphPad Prism 7.0.

TUNEL immunofluorescence for HUMSC. To prepare TUNEL
detection solution, TdT enzyme was added with fluorescent
labelling solution at a 1:9 dilution. The HUMSCs were seeded
on the glass cover in 24-well plate, and use PBS to wash redu-
dant medium. The cell culture plate was then washed twice
with TBS-Tween-20 (TBS-T) and incubated with secondary
antibody (dissolved in 1% BSA; 1:5,000) at room tempera-
ture for 1 h. Membranes were washed three times with
TBS-T and exposed to Bio-Rad ChemiDoc XRS (Bio-Rad Laboratories, Inc.) under West Femto ECL Substrate (Beijing
Solarbio Science & Technology Co., Ltd.) and analysed with
Image Lab 3.0 (Bio-Rad Laboratories, Inc.).

Autophagy analysis. After preparing cell protein samples, western blotting was used to verify the increase in autophagy.
Autophagy is mediated by LC3BII, and increases when LC3BII
is converted to LC3BII (38). Therefore, LC3BII and LC3BII, as
well as the expression of their downstream products in each
group, were used to analyze autophagy.

Statistical analysis. The data were expressed as the
means ± standard deviation. One-way ANOVA with Dunnett's
post hoc test was used to determine the statistical significance
of the observed differences. Statistical analysis was performed
using and GraphPad Prism 7.0 (GraphPad Software, Inc.).
P<0.05 was considered to indicate a statistically significant
difference.

Results

Cell proliferation. HUMSCs were used to study the effect of
shikonin (Fig. 1A) on cell proliferation. The results from the
CCK-8 assay demonstrated that the optimal condition under
OGD is 0.8 µM shikonin treatment for the 12 h (Fig. 1B).
In normal culture conditions, no significant cell death was
observed after 24-h treatment with shikonin at different
concentrations according to results from trypan blue staining.
(Fig. 1C shows cell density at various concentrations under
light microscope following shikonin treatment, 1D trypan blue
staining and 1E quantification of trypan blue staining).

Shikonin protects HUMSCs from hypoxia-ischemia-induced
apoptosis. Previous studies demonstrated that the maximum
time for hypoxia-induced early apoptosis of mesenchymal
stem cells occurs at 24 h (39). The present study investigated
whether shikonin could reverse this process in HUMSCs.
Since 12 h was the optimal time for drug administration,
HUMSCs were exposed to OGD for 12 h together with 0.8 µM
shikonin, and apoptosis was determined using the TUNEL
assay (Fig. 2D). The number of cells stained with TUNEL
divided by the number of cells stained with DAPI can reflect the
degree of apoptosis. Shikonin significantly inhibited TUNEL
signal, and the TUNEL/DAPI ratio of the shikonin group was

Western blotting (WB). Brain tissues were mashed on the
ice, lysed with RIPA buffer for 30 min on ice and sonicated
(30% energy, 2 sec duration, 2 sec interval, total time 30 sec).
Centrifuge at 12,000 rpm for 10 min at 4°C. Transfer the
supernatant to a new tube. PMSF (1:100) was added, and a
BCA kit was used to determine protein concentration. Cells
were washed with PBS, lysed with RIPA buffer for 30 min
on ice and sonicated (30% energy, 2 sec duration, 2 sec
interval, total time 30 sec). PMSF (1:100) was added, and a
Figure 1. Toxicity and effect of shikonin on HUMSCs. (A) Chemical structure of shikonin. (B) Cell proliferation assessed with the Cell Counting Kit-8 assay. (C) HUMSCs were treated with shikonin for 24 h under normal conditions and were imaged with a light microscope (magnification, x200). (D) Cells under the same conditions were stained with trypan blue for 3 min and observed under a light microscope (magnification, x200). (E) Quantitative analysis of cell proliferation activity from trypan blue stainin. HUMSC, human umbilical mesenchymal stem cell.

Figure 2. Shikonin reduced hypoxia-induced apoptosis and promoted autophagy in HUMSCs. Shikonin (0.8 µM) was added to the media and cells were placed in an OGD incubator. (A) Western blotting and quantitative data of (B) LC3BII and (C) Beclin 1 in HUMSCs exposed to OGD for 12 h. (D) TUNEL staining and (E) statistical analysis of TUNEL-positive HUMSCs. Data are presented as the means ± standard deviation. n=3. ***P<0.001. HUMSC, human umbilical mesenchymal stem cell; OGD, oxygen glucose deprivation; LC3, microtubule-associated protein 1A/1B-light chain 3; LC3BI, cytosolic LC3; LC3BII, LC3-phosphatidylethanolamine conjugate.
significantly lower compared with the OGD group (P<0.001; Fig. 2E). In the present study, shikonin promoted autophagy at the concentration of 0.8 µM used to decrease HUMSC apoptosis in vitro. The conversion rate of the cytosolic form of LC3 [LC3BII; ratio of LC3BII to LC3-phosphatidylethanolamine conjugate (LC3BI); P<0.01; Fig. 2A and B] and the expression of Beclin-1 (shikonin + OGD vs. OGD group; P<0.01; Fig. 2A and C) were also measured. The results from western blotting demonstrated that shikonin-treated cells had significantly increased autophagy compared with OGD-treated cells. Shikonin treatment increases autophagy and reduces apoptosis by activating the AMPK/mTOR signalling pathway. The AMPK/mTOR signalling pathway is an important regulator of autophagy in numerous cell types (39,40). The present study investigated whether this signalling pathway could mediate the anti-apoptotic effect of shikonin in HUMSCs (Figs. 3 and 4). The results from western blotting demonstrated that ATG5 and p-AMPK expression was lower in the OGD group compared with the OGD + shikonin group; however, p62 and p-mTOR expression was higher in the OGD group compared with the OGD + shikonin group. Conversely, shikonin exposure resulted in a significant increase in the ratio p-AMPK/AMPK (shikonin + OGD vs. OGD group; P<0.01; Fig. 3A and D) and p62 expression (shikonin + OGD vs. shikonin + OGD + 3-MA group; P<0.01; Fig. 3A and C), and an increase in ATG5 (shikonin + OGD vs. shikonin + OGD + 3-MA group; P<0.01; Fig. 3A and B) and p-mTOR expression (shikonin + OGD vs. shikonin + OGD + 3-MA group; P<0.05; Fig. 3A and E). In addition, the results from TUNEL assay demonstrated that the number of TUNEL-positive cells following treatment with 3-MA and shikonin was significantly higher compared with shikonin-treated cells (shikonin + OGD vs. OGD group, P<0.01; shikonin + OGD vs. shikonin + OGD + 3-MA group, P<0.01; Fig. 4).

Shikonin increases the survival rate of transplanted cells in the tissues surrounding the brain contusion. HUMSCs with GFP protein gene emitting green fluorescence were found in brain tissue surrounding the TBI, suggesting that transplanted HUMSCs were able to survive and migrate to the site of injury (41). In addition, the GFP from HUMSCs was significantly increased 7 days after transplantation in the shikonin-pretreated group compared with HUMSCs alone (shikonin + TBI vs. TBI group, P<0.01; shikonin + TBI vs. shikonin + TBI + 3-MA group, P<0.05; Fig. 5A). These findings suggested that shikonin may improve the number and cell viability of HUMSCs in the transplanted area.

Expression of autophagy- and apoptosis-related proteins in tissues surrounding trauma. The expression of autophagy- and apoptosis-related proteins in the tissues surrounding the

Figure 3. Shikonin enhances autophagy in HUMSCs in the hypoxic-ischemic state by regulating the AMPK/mTOR pathway. (A) Western blotting results demonstrated that (B) ATG5 and (D) p-AMPK were upregulated with shikonin treatment but downregulated after treatment with the AMPK inhibitor 3-MA. (C) p62 and (E) phosphorylation of mTOR levels were downregulated after shikonin treatment and upregulated when 3-MA was used. n=3. Data are presented as the means ± standard deviation. *P<0.05 and **P<0.01. HUMSC, human umbilical mesenchymal stem cell; OGD, oxygen glucose deprivation; ATG5, autophagy protein 5; AMPK, AMP-activate protein kinase; 3-MA, 3-methyladenine; P-AMPK, phosho-AMPK; P-mTOR, phosho-mTOR.
Figure 4. Shikonin enhanced human umbilical mesenchymal stem cell survival in a hypoxic-ischemic state. (A) Representative images of cells stained by TUNEL assay and counterstained with DAPI. Shikonin reduced cell death after OGD, but 3-MA reversed the effects. (B) Results from three independent experiments. *P<0.01. OGD, oxygen glucose deprivation; 3-MA, 3-methyladenine.

Figure 5. Shikonin increased the survival rate of transplanted cells in tissues surrounding the brain contusion. (A) Transplanted HUMSCs can survive in host tissues, as shown by transplantation of shikonin + GFP-HUMSCs, where the use of shikonin for pretreatment increased the survival rate of the transplanted HUMSCs. (B) Quantification of the number of surviving HUMSCs in shikonin-pretreated TBI mice 7 days after transplantation. n=3. *P<0.05 and **P<0.01. HUMSC, human umbilical mesenchymal stem cell; GFP, green fluorescent protein; TBI, traumatic brain injury; 3-MA, 3-methyladenine.

Brain injury 12 h after HUMSC transplantation was analyzed using western blotting. The results demonstrated that Beclin-1 (shikonin + TBI vs. TBI group; P<0.01; Fig. 6A and B), Bcl-2/Bax ratio (shikonin + TBI vs. TBI group; P<0.01; Fig. 6H)
and Bcl-2 (shikonin + TBI vs. TBI group; P<0.05; Fig. 6E and F) expression, and LC3B (shikonin + TBI vs. TBI group; P<0.01; Fig. 6A and C) conversion in the shikonin + TBI group were higher compared with the TBI group. However, expression of Bax (shikonin + TBI vs. TBI group; P<0.05; Fig. 6E and G) and caspase-3 (shikonin + TBI vs. TBI group; P<0.05; Fig. 6E, D and H) was decreased. In mice transplanted with HUMSCs and co-treated with 3-MA and shikonin, these effects were reversed (shikonin + TBI vs. shikonin + TBI + 3-MA group; P<0.05; Fig. 6).

Discussion

Acute TBI is associated with severe complications, including tissue ischemia, excitotoxicity and overproduction of free radicals, leading to the release of inflammatory molecules, and to axonal and neuroendothelial cell damage (42,43). Stem cell transplantation represents a novel treatment that could have a crucial role in the prognosis of patients with TBI (1). In the acute phase of TBI, there is severe cerebral ischemia in the region of brain contusion. At this point, the transplanted stem cells in this region are in a hypoxic and hypotrophic state, eventually resulting in severe apoptosis, which impairs the therapeutic benefits of stem cell therapy (42-44). Therefore, improving the survival rate of transplanted stem cells in this environment is crucial. In the present study, shikonin-induced autophagy served an important role in protecting mesenchymal stem cells from hypoxia-ischemia-induced apoptosis through AMPK/mTOR-related signalling pathway, which may allow MSCs to become resistant to the fluctuation of growth factors and nutrient deprivation in the ischemic microenvironment, particularly following TBI injury.

Although human and rodent brains share some physiological similarities, there are significant differences in the structure and function of their brain, which may cause rodents to respond differently to trauma (45). Furthermore, although the Glasgow coma score is an important indicator in TBI clinical experiments (45,46), it is not usable in animals. Therefore, since computerized tomography, Magnetic resonance imaging and other imaging methods can be used to directly evaluate the prognosis of TBI, these methods may be able to confirm the results of the present study. Previous studies demonstrated that shikonin can improve the survival of chondrocytes, reduce chondrocyte apoptosis and promote tissue repair after spinal injury (29). These studies indicated that shikonin would be a relatively effective inhibitor of apoptosis. It has been demonstrated that shikonin can increase the expression of Beclin-1 and reduce the conversion rate of LC3-II, and the lesion volume (35,36). Previous studies reported an endogenous protective mechanism of tissue ischemia and an increase in AMPK during tissue ischemia, indicating that this pathway is active during tissue ischemia (47-49). The present study demonstrated that shikonin could regulate autophagy by inhibiting the AMPK/mTOR pathway and reduce apoptosis and increase the survival rate of HMSCs, providing therefore a protective effect against contused peripheral ischemic tissue in stem cell-based therapies.

Decreased cell viability under ischemic stress is a barrier to stem cell therapy. Autophagy is a cellular response that is critical for the survival of cells under metabolic stress and energy starvation (7,20). Autophagy is a catabolic process that transfers cytoplasmic components to lysosomes for their degradation (50). Hypoxia and ischemia are sources of cellular stress (51). By eliminating cellular energy supplies and
de destructing organelles damaged by free radicals, autophagy serves a vital role in cell survival (52,53). Previous studies on shikonin reported that it increases autophagy (35,54). In the present study, shikonin reduced the apoptosis of HUMSCs under hypoxic-ischemic conditions, which was accompanied by an increase in autophagy. Furthermore, the protective effect of shikonin was significantly decreased with the addition of the autophagy inhibitor 3-MA, which confirmed that shikonin exerted an anti-apoptotic effect by regulating autophagy in HUMSCs.

AMPK is a stress-signalling kinase that is a key regulator of energy production and depletion pathways, thus protecting cells from hypoxia and cell death (18,40). One of the main mechanisms of AMPK action is the regulation of autophagy under stress conditions (48). Under hypoxia/SD or other energy shortages, AMPK may act as a sensor of cellular energy change, activated by a lower ATP/AMP ratio (18,40). Furthermore, mTOR is a major downstream target of AMPK and can stimulate autophagy by inactivating the autophagy pathway inhibitor mTOR complex 1 (55). It was therefore hypothesized that the AMPK/mTOR signalling pathway could play a positive regulatory role in hypoxia, starvation and other energy stress events (55). The present study demonstrated that under hypoxic-ischemic conditions, the expression of p-AMPK was increased by shikonin, whereas the expression of p-mTOR was decreased. The opposite effects on p-AMPK and p-mTOR were observed when the AMPK inhibitor 3-MA was used together with shikonin. These results suggested that the regulation of autophagy via the AMPK/mTOR signalling pathway and, ultimately, the inhibition of apoptosis, may be considered as a potential mechanism through which shikonin inhibits apoptosis in HUMSCs.

In conclusion, shikonin promoted the survival of HUMSCs under TBI conditions. The results from the present study indicated that shikonin may regulate autophagy via the AMPK/mTOR signalling pathway and protect HUMSCs from apoptosis induced by hypoxia/ischemia. These results provided a basis for the clinical application of stem cell therapy in brain trauma treatment strategies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

QZ and LR designed the study. XZ and KWu performed cell culture and TUNEL detection. XZ, LH and ZS performed the western blot analysis. KWu and JR performed CCK-8 assays and trypan blue staining. KWa and LH performed animal experiments and immunofluorescence. XZ, LH, LR and QZ performed data analysis and statistics. XZ, LH and LR confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Wenzhou Medical University (Wenzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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