Original Article
Effect of BQ123 in early brain injuries caused by subarachnoid hemorrhage in rats

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Abstract: Objective: We discussed the treatment effect of BQ-123 on subarachnoid hemorrhage (SAH) in rats and analyzed the working mechanism. Method: SD rats were randomly divided into sham operation group, SAH group, high-dose BQ-123 group and low-dose BQ-123 group. Double hemorrhage injection was used to produce SAH model in rats and morphological changes were observed under the optical microscope and electron microscope. Hippocampal expressions of phosphatidylinositol 3-kinase (PI3-K), protein kinase B (PKB/Akt) and mammalian target of rapamycin (mTOR) were detected by immunohistochemistry and *Real-time PCR*. Learning and memory capacities of rats were determined by the shuttle box paradigm. Result: Compared with the sham operation group, SAH group showed severe neuronal damage in the hippocampus, with an upregulation of phosphorylated PI3-K, Akt and mTOR. SAH group had less surviving neurons and a decline in learning and memory capacities ($P<0.05$). Compared with SAH group, the BQ-123 treatment groups showed alleviated neuronal damage, upregulated PI3-K and Akt, downregulated mTOR, more surviving neurons and improved learning and memory capacities. These changes were more obvious in the high-dose group ($P<0.05$). Conclusion: BQ-123 has a good therapeutic effect in early brain injury after SAH, possibly by regulating the PI3K/Akt and inhibing mTOR activity.

Keywords: Subarachnoid hemorrhage, cell apoptosis, phosphatidylinositol 3-kinase, protein kinase B, mammalian target of rapamycin

Introduction

Subarachnoid hemorrhage (SAH) is a common neurosurgical disease. Although the mortality has decreased with technological advance, many patients still suffer from the neurological disorders, especially cognitive deficit caused by SAH. Cognitive deficit following SAH is related to cerebral vasospasm, disrupted homeostasis in cerebral ischemia and hypoxia, signal transduction disorders and cell apoptosis [1, 2]. Phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway is a classical signal transduction cascade responsible for different pathophysiological conditions. Once activated, PI3K can catalyze the production of PI(3,4,5)P3 from PI(4,5)P2. As a result, the signal is transmitted to Akt, the downstream effector of PI3K, leading to the phosphorylation of Akt. This will downregulate the expression of mTOR acting in the downstream and influence autophagy, proliferation and apoptosis of eukaryotic cells. Therefore, this pathway plays an important role in the diseases of the central nervous system [3, 4]. BQ-123 is an endothelin A receptor (ET_A) antagonist with high specific affinity that reduces vasospasm, inhibits sympathetic nervous system and renin-angiotensin system. Some studies have demonstrated the neuroprotective effect of BQ-123 in SAH [5], but the working mechanism is unknown. We built the SAH model in rats and administered different doses of BQ-123. The treatment effect of BQ-123 was evaluated by detecting the expressions of PI3-K, Akt and mTOR and the apoptotic cells in the hippocampus, in addition to the shuttle box test.

Materials and method

Grouping and modeling

One hundred and twenty male SD rats of clean grade weighing 350-450 g were purchased from Vital River Laboratory Animal Technology Co. Ltd. (license No. SCXK (Beijing) 2009-003). The rats were randomly divided into sham operation group, SAH group, low-dose BQ-123 group and high-dose BQ-123 group. Four time points were set, namely, 6 h, 24 h, 72 h and 144 h.

Double hemorrhage injection was used [6] (0.3 ml of autologous arterial blood was injected to the cisterna magna twice at an interval of 48 h). For the sham operation group, 0.3 ml of normal saline was injected twice to the cisterna magna. For the BQ-123 treatment groups, BQ-123 was injected to the tail vein 30 min before modeling (BQ-123 was diluted by normal saline at 10 ug/ml). The dose administered to the low-dose group and the high-dose group was 50 ug/kg and 75 ug/kg, respectively. The same dose was administered again 48 h after modeling. The SAH model produced was evaluated by the following criteria: ① Exudation of a small amount of bloody cerebrospinal fluid at the injection site during the second administration, which indicated the same position of injection as before; ② Clear presence of bloody fluid in the cisterna magna while stripping the brain tissues. During modeling, 7 rats died in the SAH group, and 1 rat from the SAH group was excluded because of failure to meet the criteria. In each of the two BQ-123 treatment groups, 6 rats died and 1 rat was excluded. Therefore, 30 rats in the sham operation group, 22 rats in the SAH group, 23 rats in the high-dose group and 23 rats in the low-dose group were included.

Shuttle box test: ZH-CSC shuttle box system was used. The rats were placed into the shuttle box at different time points and the test started from 9:00. After 5 min of adaptation, the stimulus (a tone) was given for 5 s, followed subsequently by an electric shock for 20 s. Another round of training began after an interval of 10 s. If the rats succeeded in escaping to the safe zone within 5 s after the stimulus, it was considered active avoidance reaction and the present round of training stopped. Otherwise, 1.5 mA AC electric shock was given for 20 s. If the rats escaped to the safe zone after the second

electric shock, it was considered passive avoidance reaction. If the rats still failed, the result was defined as negative for both active and passive avoidance reaction. Each rat received 30 electric shocks, and the passive avoidance latency (PAL) and the number of active avoidance reactions were recorded. The percentage of active avoidance reactions to the total number of trainings was the active avoidance reaction rate (AARR). The higher the AARR and the lower the PAL, the stronger the learning capacity was.

Morphological observations of hippocampal neurons under the optic microscope: Respective rats in each time points in sham group, five rats in SAH group in 6 h and 24 h, four rats in 72 h and 144 h, respectively five rats in two BQ123 group in 6 h, 24 h, 72 h and four rats in 144 h were decapitated under anesthesia. Brain tissues were harvested after perfusion using 4% paraformaldehyde. The scope of resection was from optic chiasma to transverse cerebral fissure, followed by conventional paraffin embedding. The tissues were cut into coronal sections (thickness 5 μm), which were subjected to HE staining. The hippocampal CA1 region was observed under the optical microscope with the micrometer (400×). The number of survival neurons in each field of vision was determined (survival neurons were defined as those having intact membrane, nuclei and nucleoli). The hippocampal CA1 region was equally divided into 3 parts, and the same position was selected for each part. The average percentage of surviving neurons in each field of vision was calculated (%) using Motic-6.0 image analysis system.

Observation under the electron microscope: Two rats in sham group at each time points and respectively one rat in SAH group and BQ123 group at each time point were decapitated for electron microscope observation. Cardiac perfusion was performed using fixing solution (2.5% glutaraldehyde, 2% paraformaldehyde in PBS buffer). The rats were sacrificed by cervical dislocation, and the hippocampal tissues were harvested and cut into 1 mm×1 mm×1 mm blocks. The tissues were fixed in 40 ml/L glutaraldehyde, washed twice with 0.1 mol/L cacodylic acid buffer, and fixed in 40 ml/L osmium tetroxide. After that, the tissues were washed with buffer, dehydrated in an acetone gradient and embedded in epoxy resin. A micro-

tome was used to prepare ultra-thin sections, which were double stained with uraylacetate and lead citrate. Finally the sections were observed under the 80 kV transmission electron microscope (H-7650, Hitachi) with images taken.

Immunohistochemical analysis and real-time PCR (RT-PCR) detection of PI3-K, Akt and mTOR

Immunohistochemical analysis: The animals number in each group was the same as used for light microscopy, and brain tissues were harvested and prepared into sections using the same method as in HE staining. After dewaxing and addition of deionized water, the cells were incubated with digestion solution at 37°C in an incubator for 20 min. The cells were washed with PBS for three times, 5 min each time. Endogenous peroxidase was eliminated by incubating the cells with 3% hydrogen peroxide for 15 min. After washing with PBS, the cells were incubated with rabbit polyclonal anti-rat antibodies to PI3-K, Akt and mTOR (1:300) at 4°C overnight. Next the cells were rewarmed at 37°C for 45 min, washed with PBS and incubated with biotinylated secondary antibodies at 37°C for 40 min. The cells were washed again with PBS and color development was performed by adding DAB substrate. The procedures of counterstaining with hematoxylin, dehydration, transparentization and sealing were performed routinely. Under the optical microscope. Quantitative analysis of positive rate: Each specimen was extracted 4 slices, each slice of the CA1 area average would be divided into 3 parts, each part is in the 4 visual fields of a same site. Finally, the Motic-6.0 image acquisition and image analysis system were used to analyze the positive cells in each group (absorbance, A).

Real time PCR analysis: Brain tissues were harvested and prepared into sections using RT-PCR, and total RNA was isolated. The nerve cell were rinsed twice with PBS and treated with the RNAisoPlus. Total RNA was precipitated by centrifugation at 12,000 g for 5 min at 4°C, washed twice with ethanol. The RNA was phenol-chloroform extracted and ethanol precipitated. The supernatant was discarded, and the precipitate was collected, dried (without heating) and dissolved in 30 ul of DEPC-treated water. OD260/280 value was measured and

RNA concentration was calculated. The PCR reaction was carried at 50°C for 2 min and at 95°C for 10 min, followed by running for 45 cycles at 95°C for 15 s and 60°C for 1 min. Real Time One Step PCR was carried out under the following conditions: For PI3-K, forward primer TGGGATGTATTTGAAGCACCT, reverse primer TGATGAGCAGGGTTTAGAGGA; for Akt, forward primer AGGAATACAAAAATTACCT-GGATGC, reverse primer ACGATTGGGTAGTTC-GGCATT; for mOTR, forward primer GCAATG-ATGAAGCCCTGGAGT, reverse primer TTCTCCG-CTCTGAACAAGGCT. All primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 software. Analysis of repeated measures design ANOVA and SNK-q analysis method were used. All data were reported as mean ± standard deviation ($\overline{x} \pm s$), with $P < 0.05$ indicating significant difference.

Result

Morphological observations

Optical microscope: In the sham operation group, hippocampal neurons showed regular arrangement and normal morphology; the cell body had normal size, with large, round nuclei and distinct nucleoli. In SAH group, the neurons showed degeneration and edema, with obscure cell contour; some cells were apoptotic, showing karyolysis, nuclear fragmentation or disappearance. Compared with the sham operation group, neuron density decreased obviously in the SAH group at each time point ($P < 0.05$). BQ-123 treatment groups had less severe morphological changes. More surviving neurons were observed in the field of vision. Compared with the SAH group, the treatment groups had a significant increase in neuron density at each time point ($P < 0.05$), especially in the high-dose group (**Figure 1**).

Electron microscope: Vascular endothelial cells of the sham operation group had normal structure and uniform thickness of basilar membrane. In SAH group, the vascular endothelial cells were swollen, with compressed and stenotic lumen, tortuous vessel wall, disrupted connections, loose, obscure or even ruptured basilar membrane. Vascular endothelial cells
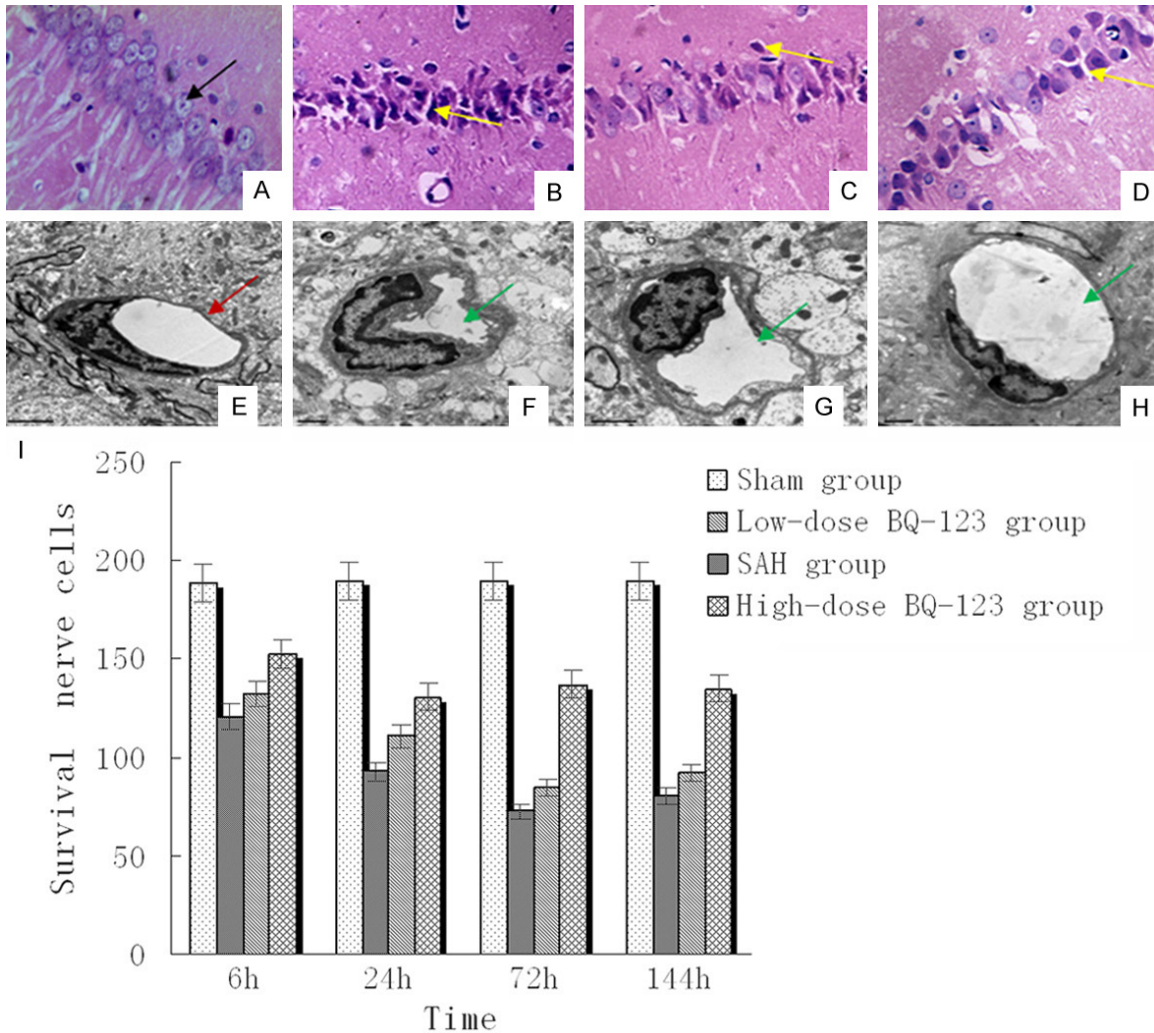
Figure 1. Morphological changes of neurons in the hippocampal CA1 region in each group at 24 h. A-D: Morphological changes of neurons in the sham operation group, SAH group, low-dose BQ-123 group, and high-dose BQ-123 group, respectively (HE staining 40×10). Arrow (black): the normal nerve cells, the arrow (yellow): dead nerve cells; E-H: Ultrastructural changes of hippocampal neurons in the sham operation group, SAH group, low-dose BQ-123 group, and high-dose BQ-123 group, respectively (electron microscope ×2,0000); Black arrows indicate the normal nerve cells; Yellow arrows indicate the dead nerve cells; I: Comparison of surviving neurons in the hippocampal CA1 region; The single factor variance analysis results showed that the number of SAH group at each time point of survival of nerve cells were lower than sham operation group (P<0.05); BQ-123 group at each time point the number of surviving nerve cells were higher than that of group SAH (P<0.05); high dose group BQ-123 at each time point the number of surviving nerve cells were higher than that of low dose group BQ-123 (P<0.05).

of BQ-123 treatment groups showed less aggregation, with intact membrane and distinct and uniform thickness basilar membrane (Figure 1).

Result of shuttle box test

Compared with sham group, rats in the SAH group showed slow reaction to stimuli, with a reduction in AARR and prolonged PAL (P=0.000). Compared with SAH group, rats in BQ-123 groups had quick reaction with higher AA-

RR and shorter PAL (P=0.000). The above changes were more significant in the high-dose group (Tables 1, 2).

Expressions of PI3-K and Akt by immunohisto-chemistry and RT-PCR

Immunohistochemistry results: PI3-K and Akt were mainly expressed in the nuclei, showing as small brown particles (Figure 2). Some positive cells for PI3-K and Akt were seen in the sham group. At each time point, SAH group had

Table 1. Comparison of AARR across the groups at each time point ($\bar{x} \pm s$)

Group	6 h	24 h	72 h	144 h
Sham group	73.67±1.63	74.17±2.14	75.67±1.86	76.83±1.72
SAH group	40.33±1.63*	48.17±2.32*	53.00±1.55*	59.17±2.04*
Low-dose BQ-123 group	43.78±1.29△	52.53±1.83△	55.13±1.52△	64.27±2.16△
High-dose BQ-123 group	45.83±1.72△,▲	55.12±1.47△,▲	60.17±2.14△,▲	65.67±2.25△,▲

Note: repeated measures design ANOVA. *$P<0.05$ compared with sham group; △$P<0.05$ compared with SAH group; ▲$P<0.05$ compared with low-dose BQ-123 group.

Table 2. Comparison of PAL across the groups at each time point ($\bar{x} \pm s$)

Group	6 h	24 h	72 h	144 h
Sham group	16.33±1.21	16.67±1.03	16.67±1.21	17.33±0.82
SAH group	50.83±1.47*	40.17±1.60*	34.17±1.72*	28.33±1.50*
Low-dose BQ-123 group	48.12±1.55△	37.50±1.52△	29.83±1.60△	24.83±1.38△
High-dose BQ-123 group	46.00±1.41△,▲	35.83±1.17△,▲	27.67±1.37△,▲	20.83±1.47△,▲

Note: repeated measures design ANOVA; *$P<0.05$ compared with sham group; △$P<0.05$ compared with SAH group; ▲$P<0.05$ compared with low-dose BQ-123 group.

higher immunopositive reaction of PI3-K and Akt than the sham group, which reached the peak at 24 h (P<0.05). The positive immunoreactivity of PI3-K and Akt was enhanced in the BQ-123 treatment groups as compared with SAH group (P<0.05) (**Figure 2**).

RT-PCR results: Using GAPDH as a reference gene to Sham group PI3-K mRNA and Akt mRNA was 1. The relative expression levels of mRNA and Akt mRNA PI3-K genes in each group were obtained according to the formula (ΔCt=the average value of Ct gene-housekeeping genes of average Ct value, ΔΔCt=the ΔCt of the experimental group - the ΔCt of the control group, relative expression =2-ΔΔCt) provided by statistical Ct values of PI3-K mRNA and Akt mRNA in each group. PI3-K mRNA and Akt mRNA expressions of SAH group at each time point were upregulated, reaching the peak at 24 h (P<0.05). Compared with SAH group, PI3-K mRNA and Akt mRNA expressions of the BQ-123 treatment groups were further increased, especially in the high-dose group (P<0.05) (**Figure 2**).

mTOR expression by immunohistochemistry and RT-PCR

mTOR was mainly expressed in the nuclei, showing as small brown particles (**Figure 2**). Occasionally the mTOR was detected in the sham group with positive cells stained brown. Compared with the sham group, the positive

immunoreactivity of mTOR in the SAH group was significantly enhanced, reaching the peak at 24 h (P<0.05). Two BQ-123 treatment groups had a decline in positive immunoreactivity of mTOR at each time point as compared with the SAH group (P<0.05). According to RT-PCR results, mTOR mRNA was significantly upregulated at each time point in SAH group as compared with the sham group, reaching the peak at 24 h (P<0.05). mTOR mRNA was downregulated greatly at each time point in the BQ-123 treatment groups as compared with SAH group, especially in the high-dose group (P<0.05) (**Figure 2**).

Discussion

China has witnessed a constant rise of incidence of SAH in recent years, which is about 10.5/100000 people every year [7]. In addition to high mortality, over 60% of SAH patients suffer from cognitive deficit and mental disorders, which bring physical and psychological burden to the patients [8]. Moreover, these sequelae may evolve into dementia. Study [9, 10] has shown that cerebral ischemia, hypoxia, disrupted cellular metabolism, mitochondrial damage, free radical accumulation and neuronal loss caused by SAH are the primary reasons for neurological impairment. The longer the duration of ischemia and hypoxia, the more severe the neurological impairment will be. BQ-123 is an endothelin A receptor (ET$_A$) antagonist
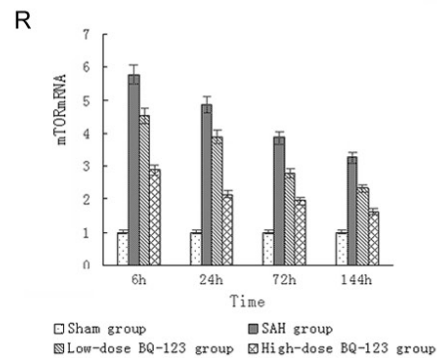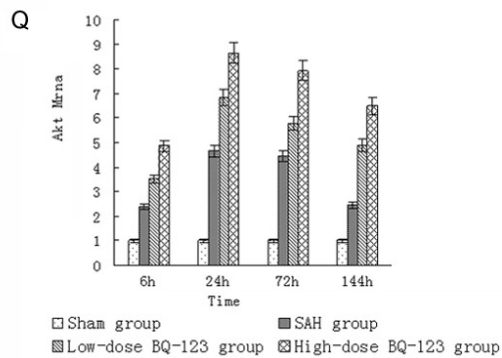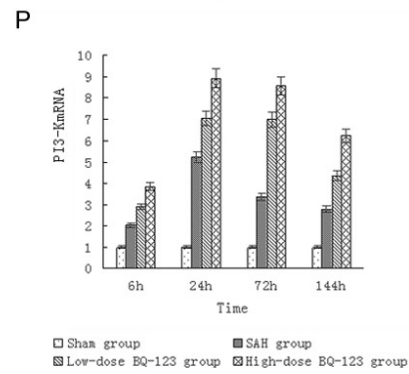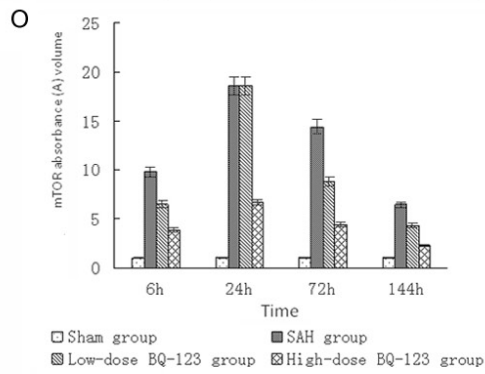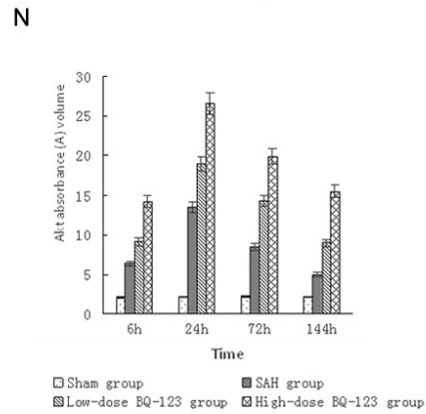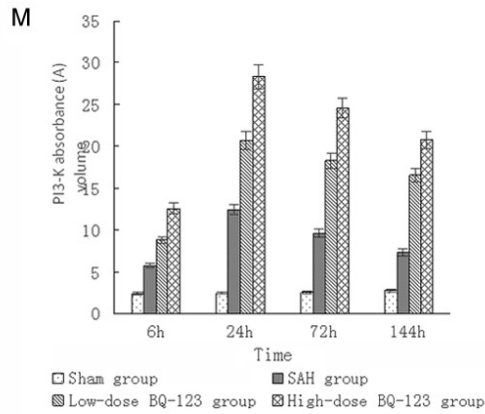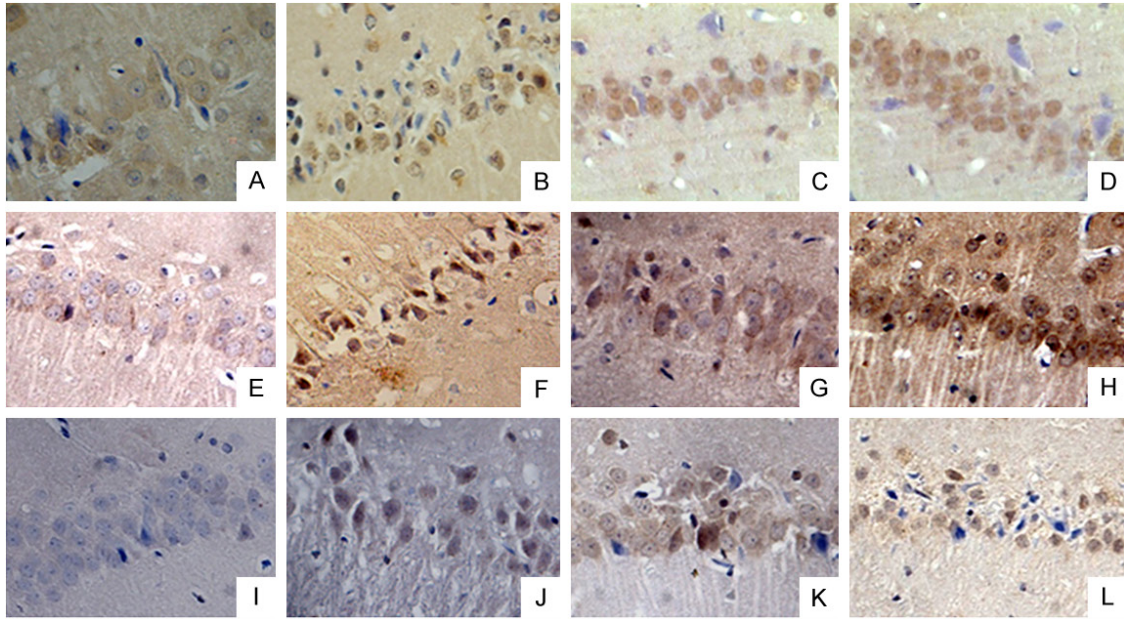
Figure 2. Immunohistochemical detection of PI3-K, Akt and mTOR in each group at 24 h. A-D: PI3-K expressions in the sham operation group, SAH group, low-dose BQ-123 group, and high-dose BQ-123 group, respectively (40×10). E-H: Akt expressions in the sham operation group, SAH group, low-dose BQ-123 group, and high-dose BQ-123 group, respectively (40×10). I-L: mTOR expressions in the sham operation group, SAH group, low-dose BQ-123 group, and high-dose BQ-123 group, respectively (40×10). M-O: Comparison of immune his to chemical results in hippocampus of rats with PI3-K, Akt and mTOR immune absorbance; single factor variance analysis showed that in the SAH group at each time point PI3-K, Akt and mTOR immune was higher than that of sham operation group (P<0.05); BQ-123 group at different time points of PI3-K and Akt immune absorbance were higher than in SAH group. MTOR immune was lower than that of SAH group (P<0.05); high dose BQ-123 group at different time points of PI3-K and Akt immune absorbance were higher than that in the low-dose BQ-123 group, mTOR was lower than the low dose group BQ-123 immune (P<0.05). P-R: Comparison of RT-PCR results in hippocampus of rats with PI3-K mRNA, Akt mRNA and mTOR mRNA levels; single factor variance analysis showed that in the SAH group at each time point of PI3-K mRNA, Akt mRNA and mTOR mRNA level was higher than that of sham operation group (P<0.05); BQ-123 group at each time point of the PI3-K mRNA and Akt mRNA were significantly higher in SAH group, the mTOR mRNA level was lower than SAH group (P<0.05); high dose BQ-123 group at each time point of the PI3-K mRNA and Akt mRNA were significantly higher in low dose BQ-123 group, the mTOR mRNA level was lower than the low dose group BQ-123 (P<0.05).

with high specific affinity. The D-Asp-L-Pro-D-Val-DTrp structure reduces the half-life and accelerates the metabolism of BQ-123 inside the organisms. Pharmacological studies discover that BQ-l23 binds to ET_A and inhibits vasospasm, improves cerebral perfusion pressure and blood flow and thus exerts a neuroprotective effect [11]. This study indicated that BQ-123 alleviated the damage of vascular endothelial cells in the hippocampal region, reducing vasospasm and increasing the number of survival neurons. This was consistent with the improvement of learning and memory capacity in rats. As in Josko and Itoh, we also demonstrated the treatment effect of BQ-123 on neurological impairment after SAH [12, 13].

PI3-K/Akt signaling is one of the important intracellular signal pathways that promote cell survival. The activation of PI3-K can induce the activity of protein kinases in the downstream, including PKB/Akt, PKC and PKA. It is generally believed that PI3-K activation has a positive regulatory effect on the central nervous system, which works by influencing the synthesis of transcriptional factors such as NF-κB and p53. Moreover, PI3-K can cause deactivation of glycogen synthase kinase-3 (GSK-3), BAD and caspase-9, thus preventing cell apoptosis. For example, Li et al. [14] used MCAO method to build the SAH model in rats and detected the expressions of p53 and Akt by immunohistochemistry and Western Blot. It was found that p53 was upregulated significantly after the use of PI3-K inhibitor LY294002, with severe loss of hippocampal neurons. This indicated the brain protection effect of PI3K/Akt signaling pathway by regulating p53 expression after SAH.

Zhang [15] found that ischemic preconditioning improved neurological impairment in animal models and reduced the area of damaged brain tissues. This was also related to the PI3K/Akt signaling pathway and downregulation of GSK3. Long-term potentiation (LTP) and long-term depression (LTD) are both related to learning and memory capacities. Inhibiting PI3-K activity can greatly induce the activity of adenosine A1, causing damage to synaptic plasticity, LTP-related damage and impairment of spatial learning [16]. Traditional Chinese medicine ShenxiongHuayu Capsule can improve learning and memory performance of demented animals, which is related to the activation of PI3K/Akt signaling pathway [17]. According to our results, both two BQ-123 treatments alleviated neuronal damage in the hippocampus and upregulated PI3-K and Akt, leading to higher AARR and shorter PAL. These changes were more obvious under higher dose. It was further proved that BQ-123 worked by regulating the PI3K/Akt signaling activity, though the reasons were unknown. Some researchers show that BQ-123 can increase endogenous NO content of the tissues, reduce calcium overload and maintain microenvironment stability [18].

mTOR is a highly conservative Ser/Thr (S/T) protein kinase, which can induce phosphorylation of downstream target proteins, thus influencing gene transcription and protein expression and regulating autophagy, proliferation and apoptosis of cells [19]. Xu [20] built the SAH model in SD rats, which were given rapamycin and later detected for phospho-mTOR by Western Blot. Results showed that trace

amount of rapamycin reduced the phosphorylation level of mTOR and alleviated cerebral edema and necrosis. This indicates the activation of mTOR by SAH and inhibiting mTOR can mitigate post-SAH brain injuries. Another study shows that estrogen has a brain protection effect by inhibiting mTOR activity [20]. mTOR is an important downstream effector of PI3K/Aktsignaling activity. The activation of PI3K/Akt signaling not only promotes the expression of mTOR, but also negatively regulates mTOR by regulating tumor suppressor TSC1 and tuberous sclerosis complex 2 (TSC2) [21]. In our study, mTOR expression was consistent with Akt expression, both reaching the peak at 24 h. After BQ-123 administration, Akt was upregulated in a dose-dependent manner, whereas mTOR was downregulated. This indicates the negative regulatory effect of BQ-123 by inducing the activity of PI3K/Akt signaling activity after SAH.

We proved that BQ-123 alleviated post-SAP brain injuries and promoted recovery of nerve functions. The mechanism may be that BQ-123 partially enhances the activation of PI3K/Akt signaling and inhibits mTOR activity, thus reducing neuronal loss. This finding sheds new light into SAH treatment based on BQ-123.

Disclosure of conflict of interest

None.

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