


Effects of 3-Methyladenine on Microglia Autophagy and Neuronal Apoptosis After Radiation-Induced Brain Injury

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Abstract

Objective: To determine the effect of the autophagy inhibitor, 3-methyladenine (3-MA), on cognitive function changes, microglia activity, neuronal apoptosis, and inflammation in rats following radiation-induced brain injury.

Methods: The following groups were established: control, model, and 3-MA. A rat model of radiation-induced brain injury was generated with a medium dose of X-rays. A Morris water maze was used to observe the cognitive function of the rats. H&E staining was used to observe the pathological changes in the hippocampus. The morphological and quantitative changes of neuronal nuclear (NeuN)-positive neurons and Iba-1-positive microglia in the ipsilateral hippocampus were analyzed by immunohistochemistry. Western blot analysis was done to measure the changes of NeuN ionized calcium binding adapter molecule 1 (Iba-1) and apoptosis-related proteins. Immunofluorescence staining of Iba-1 and Microtubule-associated protein light chain 3 (LC3) was done to evaluate the changes in microglia autophagy. TUNEL staining was used to detect apoptosis in the hippocampus. Enzyme-Linked Immunosorbent Assay was used to detect the levels of TNF- α and IL-6 as a measure of the inflammatory response in the hippocampus.

Results: After irradiation, the nucleus of the neurons in the hippocampus was constricted, the pyramidal tract structure was disordered, neuronal apoptosis was increased ($P < .001$), the expression of microglia increased ($P < .01$), autophagy was increased ($P < .05$), and conversion of microglia to the M2 type increased ($P < .05$). After 3-MA administration, the level of autophagy decreased ($P < .05$), the damage to the hippocampal region was reduced, neuronal apoptosis decreased ($P < .01$), and the activity of the microglia decreased ($P < .01$).

Conclusion: Radiation can activate the Microglia. 3-MA inhibits autophagy and excessive activity in microglia, and promotes the conversion of microglia from the M1 to the M2 type, thereby promoting the recovery of brain tissue following radiation exposure.

Keywords

radiation-induced brain injury, autophagy, microglia, 3-methyladenine, apoptosis

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Introduction

At present, there are not many treatment options for brain tumors. Radiotherapy is a common treatment that is applied to the affected location. However, radiotherapy also has significant side effects, especially for the nervous system, in which cognitive dysfunction may occur. Studies show that direct and indirect damage to the hippocampus by radiation can affect cognitive function. The dose of radiation has noticeable effects on the appearance of these complications. There is often an incubation period and continuous damage to the brain that occurs over time after radiotherapy, so cognitive impairment is a severe complication that can destroy a patient's quality of life.^{1,2} Radiation may have a direct or indirect influence on blood vessels, glial cells, and surrounding tissues, resulting in persistent, and irreversible damage to the hippocampus, which ultimately causes memory disorders and cognitive dysfunction. The early effects of radiation exposure occur within a few days of exposure and typically include damage to the body's hematopoiesis. However, its delayed response generally involves damage to the lung, skin, heart, brain, and kidney, and may occur over months or years. With respect to brain injury, radiation can damage neurons, neuroglia, and blood vessels of the brain, whereas small colloidal cells are found throughout the brain tissue, which are innate immune cells of the nervous system that are involved in brain tissue damage and repair.³⁻⁵

Autophagy is a metabolic pathway that is widespread in eukaryotic cells involving more than 40 proteins.⁶ This unique process functions in eukaryotic cells⁷ to eliminate intracellular metabolic waste through the lysosomal pathway and stabilize the homeostasis of the intracellular environment. However, excessive activation of autophagy can accelerate apoptosis in nerve cells.⁸ Thus, the appropriate inhibition of autophagy may delay the progression of some diseases. Some research shows that irradiation increased autophagy flux in GL261 glioma cells as evidenced by increased levels of LC3-II but decreased levels of p62 (*SQSTM1*). Indicating that autophagy plays a cytoprotective role in glioma cell survival.⁹ According to this study, changes in autophagy are closely associated with the onset and development of Alzheimer disease (AD), Parkinson's disease (PD), and other diseases. Regulating autophagy can effectively remove abnormally accumulated proteins in the nervous system and alleviate disease progression at the cellular level. Radiotherapy produces an inflammatory response in the brain following acute injury, with microglia and autophagy involved. Type 2 A lysosomal associated membrane protein (LAMP2A) is a marker of molecular chaperone-mediated autophagy (CMA). During the repair of brain injury, LAMP2A is significantly increased in the microglia, indicating that CMA is associated with microglial activation.¹⁰ This results in the production of diversified factors, including IL-6 and IL-1 β , which promote inflammation and inhibit the replenishment of hippocampal neurons. Moreover, it inhibits the synthesis of neurons,

regulates the expression of the Arc gene in the hippocampal region, and mediates the emergence of cognitive dysfunction.¹¹ Some scholars promote the transformation of M1 type to M2 type by interfering with the activity of microglia, which can promote the recovery of inflammation and promote the recovery of brain tissue.¹²

Currently, studies on microglia autophagy outnumber those on the acute phase of radiation brain injury, and its underlying mechanism is unclear. Accordingly, we established a rat model of radiation-induced brain injury using medium dose X-ray exposure. Rats were injected with 600 nmol of 3-MA into the lateral ventricle. A Morris water maze experiment was used to test the effects of 3-MA on cognitive dysfunction, such as spatial learning and memory in RBI rats. H&E staining was used to observe the pathological changes in the ipsilateral hippocampus. The morphological and quantitative changes of positive neuronal nuclear (NeuN) neurons and Iba-1 microglia in the ipsilateral hippocampus were evaluated by immunohistochemistry. Immunoblot analysis was used to observe the changes of NeuN, Iba-1, Arg-1, and the apoptosis-related proteins, Bcl-2 and Bax. The changes in autophagy of microglia were evaluated by double fluorescence staining with Iba-1 and LC3. The inflammatory response was evaluated in the hippocampus by measuring the levels of TNF- α and IL-6 by Enzyme-Linked Immunosorbent Assay (ELISA). Apoptosis of the hippocampal neurons was detected by TUNEL. Overall, this study was aimed to explore the effect of 3-MA on RBI damage and its potential mechanism.

Aim: To determine the effect of the autophagy inhibitor, 3-methyladenine (3-MA), on cognitive function changes, microglia activity, neuronal apoptosis, and inflammation in rats following radiation-induced brain injury.

Methods

Animals: Thirty-six healthy male SD rats weighing 180–200 g were purchased from the Beijing Huafukang Biotechnology Co., Ltd (LX201871). The rats were fed in the Medical Experimental Animal Center of North China University of Technology in a room temperature controlled at (22 \pm 2) $^{\circ}$ C environment with natural light for 12 hours every day, and they had free access to food and water.

Materials: Anti-LC3 protein antibody was purchased from MBL. Anti-NeuN antibody, anti-ionized calcium-binding adapter molecule 1 (Iba-1), anti-Bax, anti-Bcl-2, anti-Arg-1, and anti- β -actin antibodies were purchased from Abcam. The autophagy inhibitor, 3-MA, was purchased from Cayman. The IL-6 and TNF- α ELISA kits were purchased from the Wuhan ELISA LAB Co., Ltd, China.

The rats were adaptively fed for 1 week before the experiment and were randomly divided into three groups: a control group, model group, and 3-methyladenine (3-MA) group, with 12 animals in each group. The RBI model established by administering single whole brain irradiation of 20 Gy. All rats were irradiated after fasting for 12 hours and

were anesthetized with 5% chloral hydrate at 6 mL/kg and placed on the radiotherapy table for less than 5 min. The rats in the Sham group were fasted and given anesthesia, but no irradiation. Following the completion of irradiation, the rats were immediately fixed on a stereotaxic instrument, their scalps were cut open, skulls exposed, the surface tissue was digested with H₂O₂, and the coordinates of the lateral ventricle of the rats were located with the aid of stereotaxic instrument. In the model group, 5 μ L of .9% NaCl was injected with a microinjector. For the 3-MA group, 600 nmol of 3-MA was injected (.9% NaCl diluted to 5 μ L in volume), the scalps of rats were cut open. No drugs were injected into the rats of the control group. We continued feeding the rats for 8 weeks.

Analysis of learning and memory ability were tested using a Morris water maze.¹³ The temperature in the laboratory was 22°C \pm 2°C, the water temperature was 22°C, and the roof and walls were smooth and tidy. The maze was covered with a black shading cloth to avoid light, and the lighting equipment was located directly above the water maze and consisted of soft lighting. Rats in all groups were trained for 4 days prior to irradiation. (1) Positioning navigation experiment: The researcher placed the rats facing the pool wall in the middle of the 3 quadrants outside the platform and recorded the movement trajectory of the rats and the time they required to climb onto the platform within 120 s (escape latency). After climbing the platform, the rats were on their feet for 30 s. Then, the rats were returned to the cage for 60 s. If the rats did not find the platform within 120 s, they were towed to it where they remained for 30 s. The escape latency was recorded as 120 s and the rats were put back into the cage to rest for 60 s. The rats were trained 4 times a day in the morning and afternoon for 4 days; (2) Space exploration experiment: The next day after the positioning navigation experiment was done, the platform was removed, and the number of rats passing through the platform within 120 seconds was recorded. All rats were subjected to the water maze test again before 8 weeks of treatment. The conditions and operation were the same as above, and the learning and memory abilities of the rats were recorded.

H&E Staining: The rats were provided anesthesia (5% chloral hydrate) and their blood was drawn through the abdominal aorta, fixed with 4% paraformaldehyde, decapitated, and the brain was harvested and fixed overnight at 4°C. The rats were embedded in paraffin to obtain serial sections using a cryostat. After H&E staining, the pathological changes of the ipsilateral hippocampus were observed with three rats in each group.

Western Blot Analysis: The frozen brain tissue was removed, the hippocampus was separated, and transferred to an EP tube. The frozen brain tissue was cut into small pieces with small scissors. The brain tissue (50 mg) was mixed with 300 μ L RIPA lysate buffer and homogenized using an ultrasonic homogenizer for 30 min (all steps were performed on ice). Following high-speed centrifugation, the supernatant was collected as the protein sample to be tested. The samples were

separated on a 12% SDS-PAGE gel (50 μ g per well). After transfer and blocking, the membrane was incubated with NeuN, Iba-1, Bcl-2, and Bax antibodies. Following washing and incubation with the appropriate secondary antibody, the membrane was developed with AB luminescent solution (Beijing Applygen Company). The results were analyzed by Image J software and the ratio of the target protein to β -actin was calculated. There were three rats in each group.

Immunohistochemical Staining: The paraffin sections were fully dewaxed, soaked sequentially in xylene, an ethanol gradient, and distilled water. They were restored by high pressure treatment for 4 min in citrate using a high pressure steam cooker. The slides were cooled under running water for 15 min. After treatment with 3% H₂O₂ and blocking with 5% BSA, antibodies were added dropwise and incubated overnight at 4°C. After a 1-h rewarming period at 37°C, the secondary antibody was added and incubated (sheep anti-rabbit). The slides were developed with DAB substrate (Beijing ZSGB-BIO Co., Ltd), washed in PBS, counter-stained, and sealed.

Immunofluorescence: The primary antibodies for immunofluorescence were mouse anti-Iba-1 (1:200) and rabbit anti-rat LC3 (1:200). After blocking with 5% BSA, they were co-incubated with primary antibody overnight at 4°C. After rewarming, the secondary antibody was added. Alexa Fluor 568 goat anti-mouse antibody was used for Iba-1 and Alexa Fluor 488 goat anti-rabbit antibody was used for LC3. We double-labeled Iba-1 and LC-3 to detect the expression of autophagy protein in the microglia.

TUNEL Staining: A TUNEL kit was used to detect apoptosis. After dewaxing in xylene, the slides were washed with n-butanol and ethanol at various concentrations. After washing in PBS, 3% H₂O₂ was added, followed by Equilibration Buffer, TdT enzyme, and anti-digoxigenin conjugate at a working concentration. DAB was used for developing and the slides were incubated with .5% methyl green at room temperature. Finally, they were dehydrated with xylene, sealed with neutral gum, and observed under a microscope.

ELISA: After feeding for 8 weeks, the rats in each group were anesthetized with 10% chloral hydrate. Rapid dissection was conducted of the brain. The hippocampal specimens were separated, cut into small pieces, sucked into a pipette, and homogenized with an ultrasonic homogenizer for 8 s (all steps were done on ice). The homogenate was centrifuged at 7500 rpm for 20 min. The supernatant was used to measure TNF- α and IL-6, which was done using a commercial ELISA kit (Wuhan ELISA LAB Co., Ltd, China) following the manufacturer's instructions.

Statistical Analyses: All experiments were repeated at least three times. Statistical analysis was performed using SPSS statistical software, version 17.0. Results are presented as means \pm SEMs. A one-way analysis of variance (ANOVA) and repeated measures by ANOVA were used for multivariate data analyses. $P \leq .05$ was considered statistically significant.

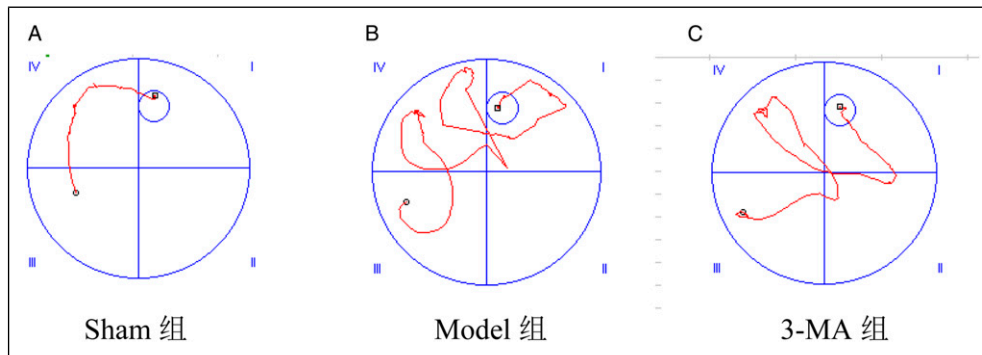


Figure 1. Eight weeks after RBI in each group, the Morris water maze positioning navigation swimming trajectory is shown. A Sham 组 B Model 组 C 3-MA 组.

Table 1. Comparison of escape latency of positioning navigation test in four days in each group.

| Groups | 1d | 2d | 3d | 4d |
|--------|---------------|--------------|-----------------------------|---------------------------|
| Sham | 67.87 ± 10.46 | 61.60 ± 9.19 | 48.62 ± 5.98 | 32.96 ± 4.55 |
| Model | 73.75 ± 7.46 | 67.82 ± 5.11 | 62.84 ± 4.62 ^b | 50.75 ± 4.30 ^b |
| 3-MA | 71.97 ± 12.50 | 64.90 ± 8.52 | 55.24 ± 8.85 ^{a,c} | 42.53 ± 4.65 ^b |
| F | 1.020 | 1.902 | 13.471 | 46.943 |
| P | .372 | .165 | .000 | .000 |

Table 1vs Sham.

^a $P < .05$.

^b $P < .01$; vs Model * $P < .05$.

^c $P < .01$.

Results

Morris Water Maze

Eight weeks after RBI, the trajectories of the rats in each group were recorded (Figure 1). Table 1 contains the data analysis instructions for the positioning navigation test for each group.

For D1 and D2, compared with the sham group, there was no statistical difference ($P > .05$), therefore, radiation did not have a relevant effect on memory function in rats. For D3, the escape latency of the rats in the model and 3-MA groups was significantly ($P < .05$) longer compared with that of the Sham group. However, in the 3-MA group, it was shorter compared with that of the Model group and there was a statistically significant difference ($P < .01$). After 8 weeks of RBI, the incubation period of the rats searching for the underwater platform was significantly prolonged, suggesting a certain learning and memory dysfunction after radiotherapy. After administration of 3-MA, the escape latency of the rats was significantly shorter compared with the Model group and the learning and memory ability was restored to some extent. The results showed that cognitive and memory impairment started on the third day.

Space Exploration Test

Table 2 shows the comparison of the frequency of the rats passing through the original platform (times of crossing).

Table 2. Comparison of the number of crossing platforms in space exploration experiments of rats in each group.

| Group | Sample(n) | Mean | SD | F | P |
|-------|-----------|------|---------------------|--------|------|
| Sham | 12 | 6.75 | 1.71 ^c | 10.355 | .000 |
| Model | 12 | 4.00 | 1.60 ^a | | |
| 3-MA | 12 | 5.25 | 1.06 ^{b,d} | | |

Table 2vs Sham.

^a $P < .05$.

^b $P < .01$ vs Model.

^c $P < .05$.

^d $P < .01$.

The rats crossing the platform had the shortest times in the Model group and the most in the Sham group. There were significant differences among the three groups ($P < .05$). Thus, the frequency of rats crossing the platform decreased significantly after RBI, suggesting some spatial memory impairment. The crossing frequency of the rats in the 3-MA group was significantly higher than that of the Model group and the spatial memory ability recovered to some extent. The results showed that the cognitive function of the rats was significantly damaged after radiation injury, and the times of crossings was significantly reduced compared with the sham group.

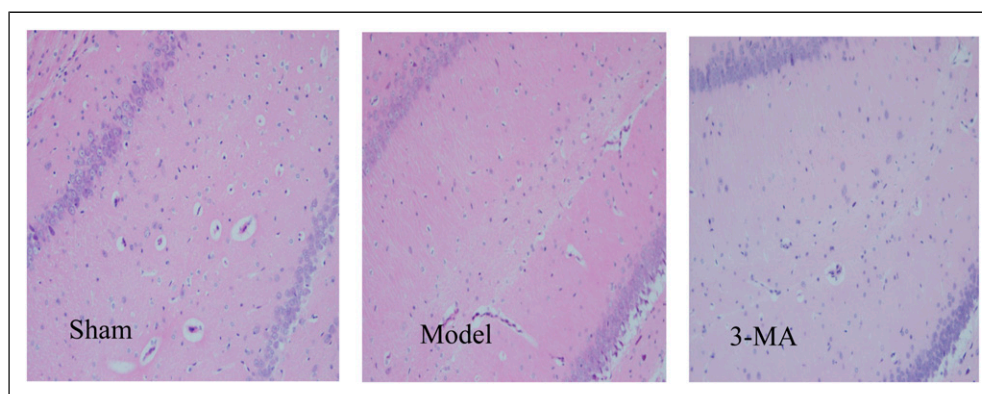


Figure 2. H & E staining results for the ipsilateral hippocampus ($\times 200$).

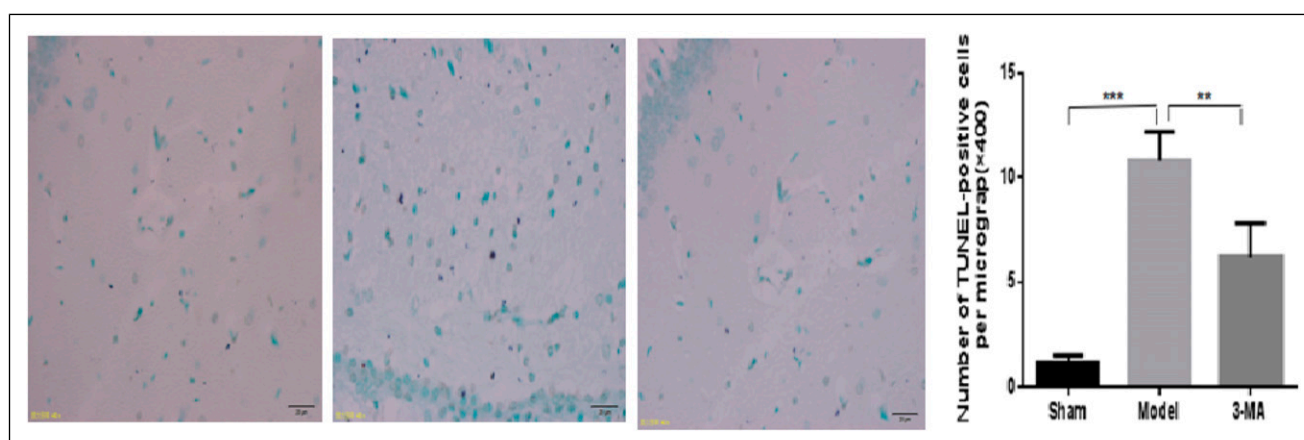


Figure 3. Terminal deoxyribonucleotidyl transferase-mediated deoxyuridine 50-triphosphate nick-end labeling (TUNEL) staining of the ipsilateral hippocampal region. Arrows indicate TUNEL-positive cells. (Magnification $\times 400$) Mean SD. $n = 3$. $***P < .001$, $**P < .01$ vs. Model group.

H & E Staining

The model group showed more obvious nuclear pyknosis and pyramidal tract disorder. The change in the 3-MA group was smaller compared with that of the model group. After the rats experienced radiation injury, there was evident damage appearing in the hippocampus and nuclear pyknosis was observed in the nerve cells, which was alleviated by 3-MA treatment (Figure 2).

TUNEL Staining

TUNEL staining was used to evaluate apoptosis in the injured hippocampus. Compared with the Sham group, there were morphological changes and a large number of positive TUNEL cells in the model group. The positive TUNEL cells of the 3-MA group were decreased significantly compared with the model group. Therefore, after suffering radiation injury, the hippocampus of the rats was clearly damaged, which caused neuronal apoptosis, and 3-MA treatment reduced the level of apoptosis (Figure 3).

Immunohistochemistry

After radiation, neurons may be damaged directly or through the activity of microglia. The level of NeuN decreased significantly, whereas the level of Iba-1 increased. After 3-MA treatment, the level of NeuN increased significantly, whereas the level of Iba-1 decreased. Therefore, radiation activated microglia and damaged neurons simultaneously, whereas 3-MA inhibited the excessive activation of microglia and promoted the recovery of neurons (Figure 4).

Immunofluorescence Staining

Some positive LC3 cells were observed in the Sham group by immunofluorescence, suggesting autophagy activity in the microglia of normal brain tissue. Eight weeks after RBI, LC3 expression in the model group was significantly up-regulated compared with that in the Sham group. The expression of LC3 in the 3-MA group was lower compared with that in the model group. RBI activated microglia autophagy, whereas 3-MA treatment significantly reduced the level of microglia

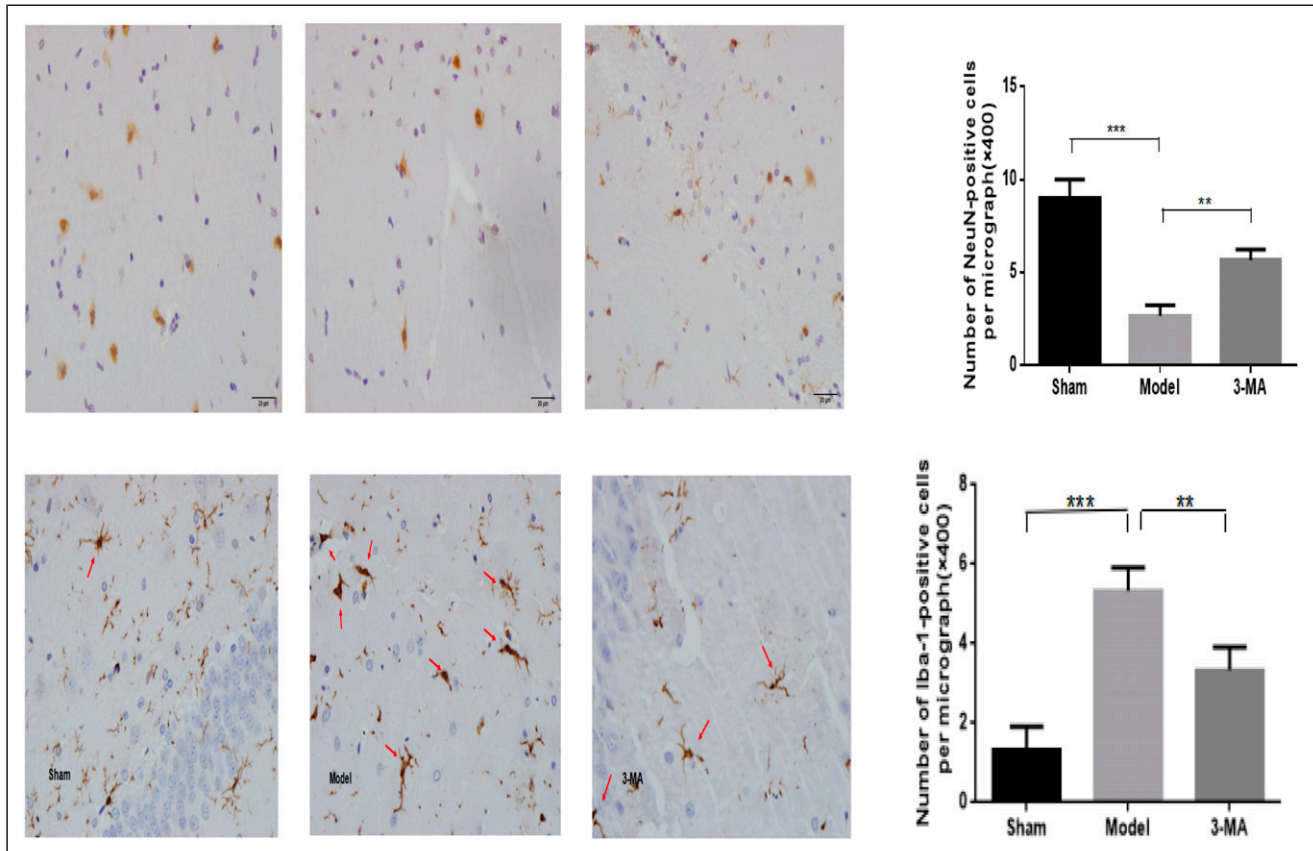


Figure 4. IHC staining of NeuN and Iba-1 8 weeks after RBI in the 3 groups ($\times 400$) $**P < .01$ $***P < .001$ (Magnification $\times 400$).

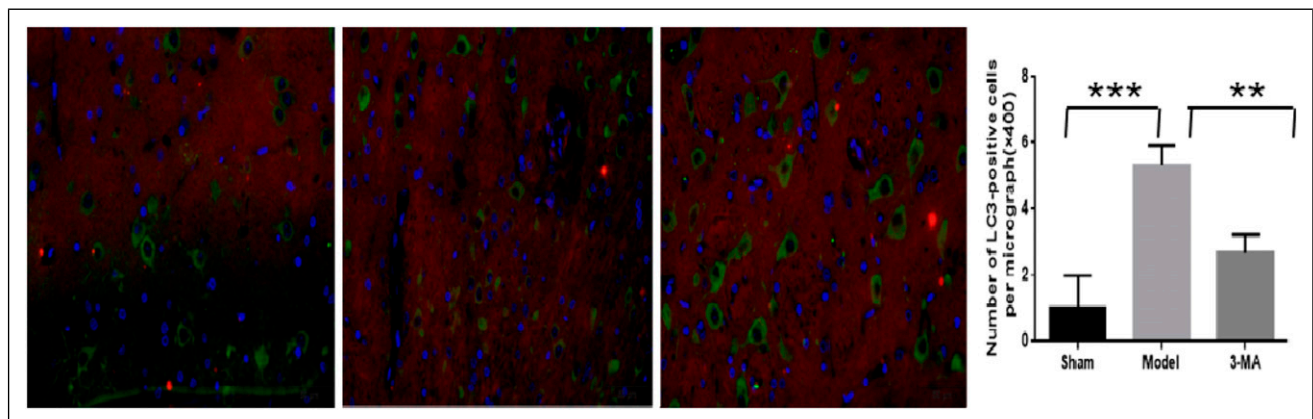


Figure 5. Immunohistochemical staining of ionized calcium-binding adapter molecule 1 (Iba-1) in the ipsilateral hippocampus. $**P < .01$; $***P < .001$ (Magnification $\times 400$).

autophagy, thus inhibiting the activity of the microglia (Figure 5).

Western Blot Analysis

We measured inflammatory factors and a marker of microglia, then evaluated the effect of 3-MA on the activity of microglia

and apoptosis in neurons using western blot analysis. Radiation can activate microglia, whereas 3-MA can promote the recovery of brain tissue (Figure 6).

Next, we determined the molecular mechanism of RT-induced cell apoptosis. We measured the classical mitochondria-associated pro- and anti-apoptotic proteins, Bax and Bcl-2, involved in RD-induced cell apoptosis. In the

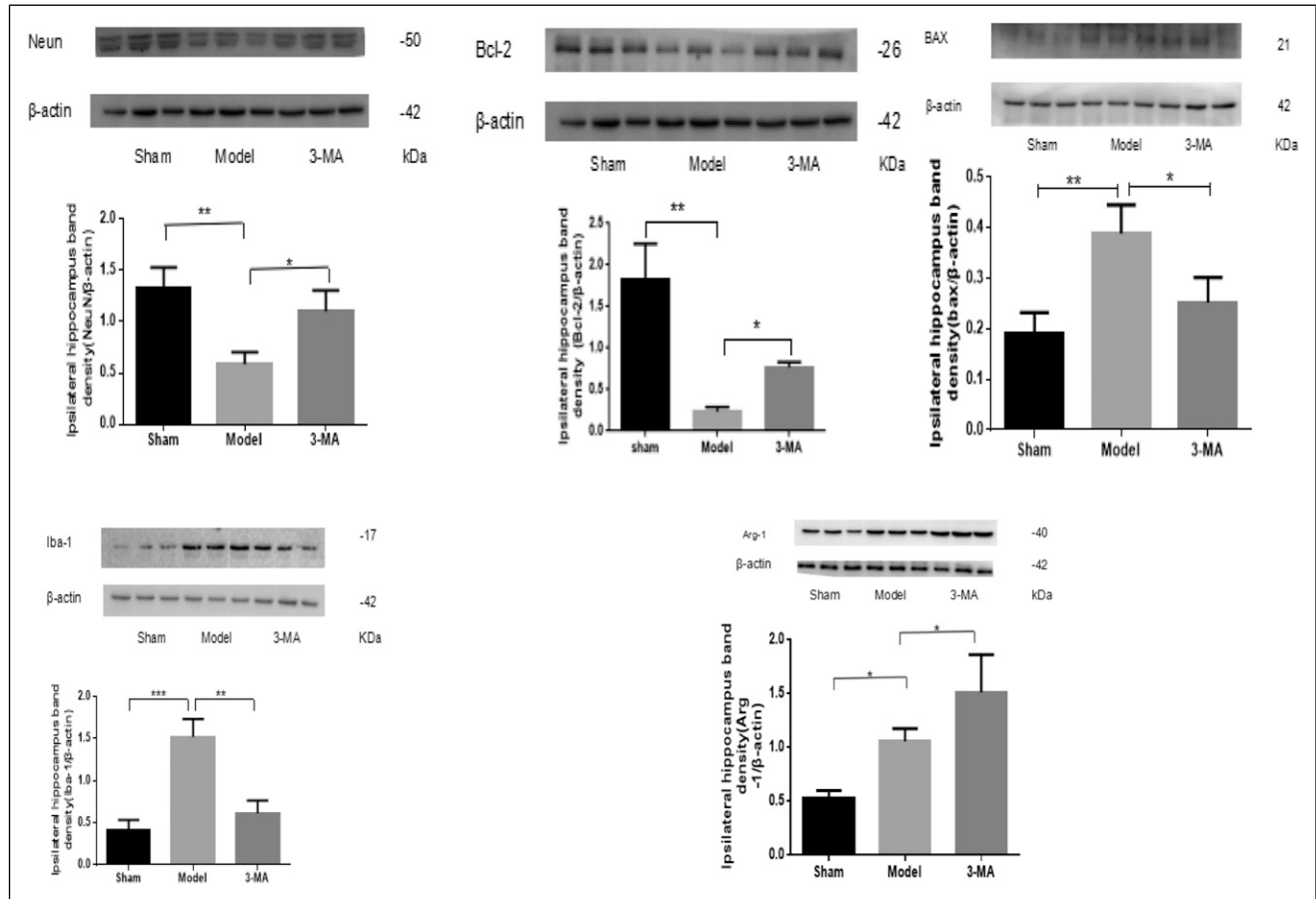


Figure 6. Western blot of NeuN, Bcl-2, Bax, Iba-1, and Arg-1 of the ipsilateral hippocampus. Data in the bar graph represent the mean \pm SE. β -Actin was used as a loading control. * $P < .05$ ** $P < .01$ *** $P < .001$.

radiation exposed group, there were higher levels of Iba-1 and Bax, whereas there were lower levels of NeuN and Bcl-2. Radiation activated the microglia and promoted inflammation. In the 3-MA group, 3-MA inhibited the expression of Bax and Iba-1, promoted the repair of neurons, and decreased the activity of microglia. These results suggest that 3-MA can protect the brain tissue after radiation by attenuating the activity of microglia (Figure 6).

Microglia may be divided into an M1 and M2 type. The M2 type can protect brain tissue. Arg-1 is a marker of M2 type microglia. Radiation can increase the expression of Arg-1, thus microglia can protect themselves in a harsh environment. The 3-MA group exhibited a higher level of Arg-1, indicating that 3-MA can promote the transformation of microglia into the M2 type (Figure 6).

ELISA

TNF- α and interleukin are important signaling molecules involved in the inflammatory response and they are significantly increased in the early stage of brain injury. To determine the changes in the inflammatory response, we measured the levels of TNF- α and IL-6. After 8 weeks of radiation, although

the expression of TNF- α and IL-6 increased, there was no significant difference ($P > .05$). Although the expression of these factors decreased in the 3-MA group, it was also not significant ($P > .05$) (Figure 7). Therefore, the inflammatory response in the brain tissue disappeared after 8 weeks.

Discussion

In this study, we established a rat brain injury model by exposure to medium dose radiation. After radiation, the microglia was activated and autophagy in the microglia increased. The 3-MA-treated group showed a significant decrease in microglia activity, promoted microglia to M2 differentiation, and inhibited neuronal apoptosis. These data indicate that the inhibition of microglia autophagy may be a new treatment strategy for brain injury caused by radiation.

Establishment of RBI Model and Selection of Radiation Dose

The establishment of various rat models of radiation-induced brain injury has been reported, and 10–30 Gy is often used at different exposures. The dose is primarily associated with the

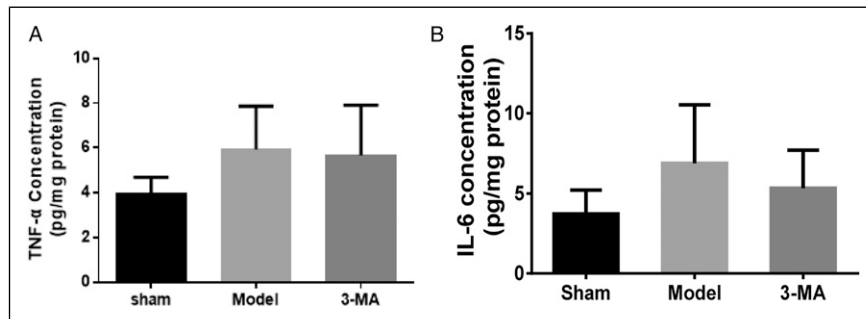


Figure 7. Enzyme-linked immunosorbent assay of (A) tumor necrosis factor- α (TNF- α) and (B) interleukin-6 (IL-6) in the ipsilateral hippocampus. $P > .05$.

variety, body weight, and research purpose of the rats. In addition, there are lots of radiation sources, such as X-rays and γ -rays. As early as 1991, Delapaz et al¹⁴ established a rat model of radiation-induced brain injury using X-rays for the first time and observed corresponding changes in the brain tissue. Based on the literature, we irradiated the whole brain with X-rays of 10 Gy, 20 Gy, and 30 Gy, the total dose was offered in 2 minutes. The rats were fasted and provided intraperitoneal anesthesia before irradiation, covering the eyes to avoid damage and blindness. After irradiation, the weight, and hair of the rats were recorded. One week later, H&E staining was performed in the hippocampus of the brain. We found that there was no obvious damage in brain tissue in the 10 Gy irradiation group. There was no significant change in all of the brain tissues. There was obvious cell edema and hippocampal pyramidal tract disorder in both the 20 Gy and 30 Gy groups, followed by weight loss, loss of appetite, vomiting, and mental malaise. The symptoms of the rats in the 30 Gy group were more serious and the number of deaths increased, which seriously affected the statistical analysis of the data. Comparatively, we determined that the appropriate experimental radiation dose was 20 Gy and the irradiation mode was single whole brain irradiation.

Autophagy and Tissue Damage

Autophagy is a widespread metabolic pathway in eukaryotic cells, which mainly degrades macromolecular substances and organelles in the cytoplasm through the lysosomal pathway. LC3 is the gold standard for detecting autophagy. Under normal circumstances, autophagy remains stable with the function of regulatory pathways. However, under some pathological conditions, autophagy is overactivated, or inhibited, which aggravates injury. Autophagy can be highly induced under different conditions, such as nutritional deprivation, oxidative stress, hypoxia, and chemotherapeutic drugs exposure.¹⁵⁻¹⁸ Mitochondrial autophagy is a type of autophagy that is critical for mitochondrial homeostasis. Some studies have indicated¹⁹⁻²¹ that the deficiency and excess of mitochondrial autophagy promotes cell damage and death. Jin et al²² found that, through the study of the changes of

autophagy after traumatic brain injury in rats, double fluorescence staining of the hippocampus was positive for the autophagy-associated proteins, LC3 and Beclin-1. The expression was significantly different from the microglial marker, Iba-1. The positive cells in the hippocampus increased significantly in the radiation group, indicating that autophagy of the microglia in the damaged brain was significantly up-regulated. Autophagy inhibitors further aggravated the original damage and its underlying mechanism may be the result of PI3K/Akt/mTOR pathway activation by radiation.²³ In a study of Parkinson's disease, Redmann et al²⁴ found that an autophagy activator increased autophagy as well as LC3-II levels, which ameliorated Parkinson's disease. This suggests that the symptoms of Parkinson's disease may be related to the reduction of autophagy and abnormal protein accumulation. New evidence suggests that autophagy plays an important role in the pathogenesis of amyotrophic lateral sclerosis (ALS).²⁵⁻²⁷ Xiaojie et al²⁸ found that the autophagy protein marker, LC3-II, was increased significantly and specifically in spinal motor neurons of ALS mice and autophagy vacuoles accumulated significantly in the dystrophic axons of spinal motor neurons of ALS mice. After the classical autophagy activator rapamycin was used to treat transgenic mice with ALS, disease progression occurred, and promoted the death of the mice. It is evident that autophagy stability is an important physiological process needed to maintain the homeostasis of the intracellular environment, and excessive activation aggravates the damage to the original tissue and cells. In contrast, inhibition prevents the excretion of damaged organelles and metabolites, thus causing chronic and persistent damage to tissues and cells.

Autophagy and Microglia Activity

Most studies on cognitive impairment and brain damage are focused on the relationship between autophagy and neurons; however, recent studies have found that autophagy can also regulate the function of glial cells, thus regulating acute and chronic brain injury.^{29,30} Some studies indicate a role for the autophagy-associated protein, BECN-1, in microglia phagocytosis of the β -amyloid protein (A β). The down-regulation of

BECN-1 decreased the phagocytosis of A β by microglia, suggesting that BECN-1 may be necessary for microglia-mediated phagocytosis.³¹ Some studies have suggested that autophagy can also regulate the inflammatory response of microglia.³² The mTOR pathway may be a bridge between autophagy and microglia activity. The activation of class I phosphatidylinositol-3-kinase leads to the activation of the Akt-mTOR signal transduction cascade and suppresses autophagy.^{33,34} In this study, the autophagy inhibitor, 3-MA, was used to specifically block the transformation of LC3-I to LC3-II^{35,36} by inhibiting phosphatidylinositol-3-kinase. Double fluorescence staining showed that the expression of LC3 in microglia decreased significantly, as did Iba-1, a specific marker of microglia. Thus, autophagy can have an effect on the activity of microglia. Whereas mTOR can significantly affect the microglia, Erlich et al³⁷ demonstrated that the inactivation of the mTOR pathway inhibited the activation of microglia, increased the number of neurons in the injured brain tissue, and improved brain injury. Finally, there are a few studies on the effect of autophagy on microglia flux; however, more data is needed to confirm the relationship between autophagy and microglia activity.

Radiation Brain Injury and Microglia

Microglia are innate immune cells of the nervous system. In a healthy adult brain, microglia constantly detect changes in the surrounding environment. Any slight change may cause the activation of microglia, including changes in morphology and the regulation of inflammatory factors.^{38,39} In studies of radiation-induced brain injury, radiation exposure was found to be associated with an immune response,^{40,41} as well as cell necrosis and apoptosis.⁴² Bálentová et al⁴³ found that when studying the changes in cognitive function following radiotherapy, microglia were significantly activated after radiotherapy; however, the activity of the microglia gradually returned to normal over time. In an experiment on rhesus monkeys after whole brain radiotherapy, Andrews et al⁴⁴ found that the white matter was damaged after radiotherapy and microglia were activated. The microglia could mediate neuroinflammatory reactions and aggravate the brain tissue injury. Similarly, using moderate doses of radiation in rats, Lia et al found that the inflammasome *NLRP3* (NLR pyrin domain containing 3) and caspase-1 in microglia were activated, but not in neurons.⁴⁵ Microglia are involved in the repair of brain tissue, but they may release inflammatory factors after over-activation to mediate brain tissue damage.

In the present study, radiation caused brain damage to rats. We found that the expression of Iba-1 was significantly increased and double fluorescence staining showed that autophagy was increased in microglia, which indicates that after 8 weeks of radiation injury, microglia are activated and the brain could not recover in a timely manner. After treating the rats with 3-MA, the expression of Iba-1 was significantly down-regulated and autophagy was decreased in the microglia. The detection of

apoptosis-related indicators and the neuron-specific marker NeuN revealed that in the group in which autophagy was inhibited, the number of necrotic neurons decreased and the expression of apoptosis-related proteins improved, which resulted in a certain degree of recovery from brain injury. However, because of time limitations, the mechanism of microglia activation (i.e., mechanism of autophagy) was not fully examined, although it warrants further investigation.

Conclusion

This study evaluated the pro-survival and anti-apoptotic effects of 3-methyladenine on radiation-induced brain injury. Through the detection of apoptosis-related proteins and microglia markers, we found that 3-MA regulates autophagy and affects the activity of microglia. This further promotes the repair of brain tissue, which may represent a potential treatment for brain injury. However, the detailed molecular mechanisms still require further study.

Declaration of Conflicting Interests

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References

1. Yeh S-A, Tang Y, Lui C-C, Huang Y-J, Huang E-Y. Treatment outcomes and late complications of 849 patients with nasopharyngeal carcinoma treated with radiotherapy alone. *Int J Radiat Oncol Biol Phys.* 2005;62(3):672-679.
2. Xiao W-W, Han F, Lu T-X, Chen C-Y, Huang Y, Zhao C. Treatment outcomes after radiotherapy alone for patients with early-stage nasopharyngeal carcinoma. *Int J Radiat Oncol Biol Phys.* 2009;74(4):1070-1076.
3. Shemer A, Erny D, Jung S, Prinz M. Microglia plasticity during health and disease: An immunological perspective. *Trends Immunol.* 2015;36(10):614-624.
4. Schafer DP, Stevens B. Microglia function in central nervous system development and plasticity. *Cold Spring Harbor Perspect Biol.* 2015;7(10):a020545.
5. Casano AM, Peri F. Microglia: Multitasking specialists of the brain. *Dev Cell.* 2015;32(4):469-477.
6. Mochida K, Oikawa Y, Kimura Y, et al. Receptor-mediated selective autophagy degrades the endoplasmic reticulum and the nucleus. *Nature.* 2015;522(7556):359-362.

7. Yorimitsu T, Klionsky DJ. Autophagy: Molecular machinery for self-eating. *Cell Death Differ.* 2005;12(S2):1542-1552.
8. Kiffin R, Bandyopadhyay U, Cuervo AM. Oxidative stress and autophagy. *Antioxidants Redox Signal.* 2006;8(1-2):152-162.
9. Cho YH, Lee SH, Lee SJ, Kim HN, Koh JY. A role of metallothionein-3 in radiation-induced autophagy in glioma cells. *Sci Rep.* 2020;10(11):2015.
10. Park Y, Liu C, Luo T, Dietrich WD, Bramlett H, Hu B. Chaperone-mediated autophagy after traumatic brain injury. *J Neurotrauma.* 2015;32(19):1449-1457.
11. EkdahiClaasen CTJH, BondeKokaia SZ, Lindvall O. Inflammation is detrimental for neurogenesis in adult brain. *Proc Natl Acad Sci USA.* 2003;100(23):13632-13637.
12. Wang J, Pan H, Lin Z, et al. Neuroprotective effect of fractalkine on radiation-induced brain injury through promoting the M2 polarization of microglia. *Mol Neurobiol.* 2021;58(3):1074-1087.
13. Vorhees CV, Williams MT. Morris water maze: Procedures for assessing spatial and related forms of learning and memory. *Nat Protoc.* 2006;1(2):848-858.
14. DelaPaz RL, Shibata D, Steinberg GK, Zarnegar R, George C. Acute cerebral ischemia in rabbits: Correlation between MR and histopathology. *AJNR. Am J neuroradiol.* 1991;12(1):89-95.
15. Chen Z, Teo AE, McCarty N. ROS-induced CXCR4 signaling regulates mantle cell lymphoma (MCL) cell survival and drug resistance in the bone marrow microenvironment via autophagy. *Clin Cancer Res.* 2016;22(1):187-199.
16. Sui X, Chen R, Wang Z, et al. Autophagy and chemotherapy resistance: A promising therapeutic target for cancer treatment. *Cell Death Dis.* 2013;4(10):e838-e838.
17. Zhang H, Chen Z, Miranda RN, Medeiros LJ, McCarty N. TG2 and NF- κ B Signaling coordinates the survival of mantle cell lymphoma cells via IL6-mediated autophagy. *Cancer Res.* 2016;76(21):6410-6423.
18. Zhang H, McCarty N. Tampering with cancer chemoresistance by targeting the TGM2-IL6-autophagy regulatory network. *Autophagy.* 2017;13(3):627-628.
19. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature.* 2008;451(7182):1069-1075.
20. Remasters JJV. Necroptosis and the mitochondrial permeability transition: Shared pathways to necrosis and apoptosis. *Am J Physiol.* 1999;276(1):122-135.
21. Levine B, Klionsky DJ. Development by self-digestion: Molecular mechanisms and biological functions of autophagy. *Dev Cell.* 2004;6(4):463-477.
22. Jin Y, Wang R, Yang S, Zhang X, Dai J. Role of microglia autophagy in microglia activation after traumatic brain injury. *World Neurosurgery.* 2017;100:351-360.
23. Tao H, Shan X, Li X, et al. Inhibition of post-ischemic adaptation on autophagy induced by focal cerebral ischemia and reperfusion in rats. *Chin J Pathophysiol.* 2017;33(10):1896-1900.
24. Redmann M, Wani WY, Volpicelli-Daley L, Darley-Usmar V, Zhang J. Trehalose does not improve neuronal survival on exposure to alpha-synuclein pre-formed fibrils. *Redox Biol.* 2017;11:429-437.
25. Li L, Zhang X, Le W. Altered macroautophagy in the spinal cord of SOD1 mutant mice. *Autophagy.* 2008;4(3):290-293.
26. Kabuta T, Suzuki Y, Wada K. Degradation of amyotrophic lateral sclerosis-linked mutant Cu,Zn-superoxide dismutase proteins by macroautophagy and the proteasome. *J Biol Chem.* 2006;281(41):30524-30533.
27. Morimoto N, Nagai M, Ohta Y, et al. Increased autophagy in transgenic mice with a G93A mutant SOD1 gene. *Brain Res.* 2007;1167:112-117.
28. Zhang X, Li L, Chen S, et al. Rapamycin treatment augments motor neuron degeneration in SOD1G93A mouse model of amyotrophic lateral sclerosis. *Autophagy.* 2011;7(4):412-425.
29. Dello Russo C, Lisi L, Feinstein DL, Navarra P. MTOR kinase, a key player in the regulation of glial functions: Relevance for the therapy of multiple sclerosis. *Glia.* 2013;61(3):301-311.
30. Yamamoto A, Yue Z. Autophagy and its normal and pathogenic states in the brain. *Annu Rev Neurosci.* 2014;37(1):55-78.
31. Lucin KM, O'Brien CE, Bieri G, et al. Microglial beclin 1 regulates retromer trafficking and phagocytosis and is impaired in Alzheimer's disease. *Neuron.* 2013;79(5):873-886.
32. Su P, Zhang J, Wang D, et al. The role of autophagy in modulation of neuroinflammation in microglia. *Neuroscience.* 2016;319:155-167.
33. Chang Y-Y, Juhász G, Goraksha-Hicks P, et al. Nutrient-dependent regulation of autophagy through the target of rapamycin pathway. *Biochem Soc Trans.* 2009;37(1):232-236.
34. Corradetti MN, Guan K-L. Upstream of the mammalian target of rapamycin: Do all roads pass through mTOR? *Oncogene.* 2006;25(48):6347-6360.
35. Heckmann BL, Yang X, Zhang X, Liu J. The autophagic inhibitor 3-methyladenine potently stimulates PKA-dependent lipolysis in adipocytes. *Br J Pharmacol.* 2013;168(1):163-171.
36. Wu Y-T, Tan H-L, Shui G, et al. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *J Biol Chem.* 2010;285(14):10850-10861.
37. Erlich S, Alexandrovich A, Shohami E, Pinkas-Kramarski R. Rapamycin is a neuroprotective treatment for traumatic brain injury. *Neurobiol Dis.* 2007;26(1):86-93.
38. Davalos D, Grutzendler J, Yang G, et al. ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci.* 2005;8(6):752-758.
39. Perry VH, Holmes C. Microglial priming in neurodegenerative disease. *Nat Rev Neurol.* 2014;10(4):217-224.
40. Hwang S-Y, Jung J-S, Kim T-H, et al. Ionizing radiation induces astrocyte gliosis through microglia activation. *Neurobiol Dis.* 2006;21(3):457-467.
41. Raju U, Gumin GJ, Tofilon PJ. Radiation-induced transcription factor activation in the rat cerebral cortex. *Int J Radiat Biol.* 2000;76(8):1045-1053.
42. Zhang Y, Cheng Z, Wang C, Ma H, Meng W, Zhao Q. Neuroprotective Effects of Kukoamine a against Radiation-

- induced Rat Brain Injury through Inhibition of Oxidative Stress and Neuronal Apoptosis. *Neurochem Res.* 2016; 41(10):2549-2558.
43. Báľentová S, Hajtmanová E, Filová B, et al. Effects of fractionated whole-brain irradiation on cellular composition and cognitive function in that brain. *Int J Radiat Biol.* 2018;94(3): 105-112.
44. Andrews RN, Metheny-Barlow LJ, Peiffer AM, et al. Cerebrovascular remodeling and neuroinflammation is a late effect of radiation-induced brain injury in non-human primates. *Radiat Res.* 2017;187(5):599-611.
45. Liao H, Wang H, Rong X, Li E, Xu R-H, Peng Y. Mesenchymal stem cells attenuate radiation-induced brain injury by inhibiting microglia pyroptosis. *BioMed Res Int.* 2017;2017:1-11.