

Original Article

The mammalian target of rapamycin (mTOR) inhibitor rapamycin improves postoperative cognitive dysfunction (POCD)

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Abstract: Background: Several lines of evidence have indicated that rapamycin acts as an inhibitor of the mammalian target of rapamycin (mTOR), and this inhibition has therapeutic benefits as a treatment for postoperative cognitive dysfunction (POCD). Thus, we hypothesized that inhibition of mTOR signaling could affect the occurrence of POCD. Methods and results: Here, 12-14-week-old C57BL/6J mice were randomly divided into four groups. Conditioned fear tests on all mice were carried out on postoperative day 1. The expression levels of mTOR and LC3B proteins in the hippocampus were evaluated by western blotting and immunofluorescence assay. The conditioned fear tests showed that rapamycin could improve the learning and memory of mice. Rapamycin reduced the level of p-mTOR (Ser2448) protein in the hippocampus ($P < 0.05$ vs Sur group) and increased the content of LC3B protein in the hippocampus ($P < 0.05$ vs Sur group). Immunofluorescence assay showed that LC3B was mainly expressed in the cytoplasm. Conclusions: The mTOR inhibitor rapamycin can significantly impede mTOR signaling, up-regulate the expression of LC3B protein in the hippocampus, and improve cognitive dysfunction caused by surgical trauma.

Keywords: Postoperative cognitive dysfunction, mammalian target of rapamycin, autophagy, rapamycin

Introduction

Postoperative cognitive dysfunction (POCD) is a central nervous system complication of anesthesia and surgery in elderly patients [1]. Its specific pathogenesis is still not clear. Age and surgery are the recognized risk factors [2, 3]. Patients with POCD often exhibit memory decline, cognitive impairment, anxiety, language comprehension skill loss, social integration ability loss, and other clinical manifestations, and patients with serious forms may further develop dementia [4].

A previous study has shown that about 25.8% of elderly patients suffer from POCD in the week after non-cardiac surgery, and the proportion was higher for cardiac surgery [5]. POCD can affect patient quality of life, lengthen patients' hospital stay, increase medical expenses, and increase patient mortality, which cause a heavy burden for individuals and families. Therefore, elucidating the mechanism

of POCD and identifying effective prevention and treatment methods are increasingly becoming popular medical science issues for society [6].

The mammalian target of rapamycin (mTOR) is the target of rapamycin in mammalian cells, and it plays an important role in the process of neurodegenerative disease [7, 8]. It is hypothesized that the pathogenesis of POCD has a similar pathway as neurodegenerative diseases [9]. It is assumed that the mechanism of POCD is that anesthesia induces abnormalities in mTOR signal activation in the brain, and then, protein synthesis becomes abnormal, ultimately damaging the functioning of the nervous system [10].

Therefore, we hoped to verify whether mTOR signaling was closely related with postoperative cognitive function changes by first establishing a POCD model. We then aimed to clarify the mechanism of mTOR signaling in POCD and to

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determine whether the role and mechanism of rapamycin in POCD involves influencing mTOR signaling, and the results indicate a theoretical basis and new approach for the prevention and treatment of POCD.

Material and methods

Experimental animals

Male C57BL/6J mice (12-14 weeks old, SPF grade, body weight 20-30 g), were provided by the animal center of Capital Medical University. The mice were randomly divided into four groups: the control group (Ctrl group, n=8), rapamycin group (provided by Shanghai Chenyi Biological Technology Co., LTD) (Ctrl + Rapa group, n=8), surgery group (Sur group, n=8), and surgery + rapamycin group (Sur + Rapa group, n=8). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of Capital Medical University.

Establishment of the animal model

Sur model (tibial fractures with open reduction and intramedullary nail fixation): The tibia was sheared at the middle position, and open reduction and internal fixation were performed. Sur + Rapa group: 0.5 mg/Kg rapamycin was intraperitoneally injected in the mice for 3 d before the operation. The dose of rapamycin was based on a previous study [11]. Ctrl + Rapa group: 0.5 mg/Kg rapamycin was intraperitoneally injected in the mice for 3 d. The mice in the control group were placed in the anesthesia induction box with an 80% oxygen concentration for 15 min.

Behavior test

The mice were placed in the training room for 2 h the day before anesthesia to allow them to become familiar with the environment. The mice were placed in the training box for 5 min at first. Then, the mice were exposed to three consecutive 2000-Hz 90-dB sound stimulations. The mice were given consecutive 2-sec 0.85-mA electrostimulations at the end of the sound stimulation. The above training sessions

were repeated five times, and the mice were removed from the training box and returned to their cage. After completion of the training, the mice underwent a conditioned fear test. The mice were returned to the original training box for 3 min to become familiar with the environment, and the total freezing time over 3 min was observed without sound stimulation or electrostimulation administration. The mice received the same condition-related fear memory test on postoperative day 1. The freezing time percentage of the mice was measured under the fear condition.

Western blot

Total protein extracts were obtained, and the protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit. Equal amounts of proteins were fractionated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies (rabbit polyclonal, 1:4000, Maxim, China) after blocking with 3% BSA in TBS. The membranes were incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody. Actin was used as a loading control.

Immunofluorescence assay

The rat sections were blocked in PBS containing normal goat serum (diluted 1:10, by vol.), 0.5% BSA, 0.2% Tween-20, and 0.05% sodium azide for 4 h at 4°C. The cells were labeled with the respective primary antibodies at a 1:100 dilution followed by incubation with fluorescein-conjugated secondary antibodies at a 1:100 dilution. The nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI).

Statistical methods

The statistical analysis was performed using SPSS for Windows 18.0 software. The measurement data are expressed as the mean \pm standard deviation. Comparisons among groups were performed using the normality test and homogeneity test of variance. The statistical analysis of the experimental data was performed using single factor analysis of variance. If the variance analysis showed that the results were statistically significant, the Newman-

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Table 1. Body weight comparison between four groups of mice (n=12)

Groups	Weight (g)
Ctrl	27.2±2.6
Ctrl + rapa	28.2±3.2
Sur	29.5±3.7
Sur + rapa	26.3±2.7

Ctrl: Control group, Ctrl + rapa: rapamycin group, Sur: surgery group, Sur + rapa: surgery + rapamycin group.

Table 2. Comparison of the vital signs monitoring results between four groups of mice (n=8)

Groups	MAP (mmHg)	HR (bpm)	SpO ₂ (%)
Ctrl	93.16±12.3	539±136	96.6±0.7
Ctrl + rapa	92.87±14.4	508±109	97.1±1.0
Sur	90.22±11.8	562±103	97.5±1.3
Sur + rapa	91.3±10.8	498±118	97.8±1.0

Ctrl: Control group, Ctrl + rapa: rapamycin group, Sur: surgery group, Sur + rapa: surgery + rapamycin group.

Table 3. Comparison of blood gas index between four groups (n=4)

Groups	pH	pCO ₂ (mmHg)	pO ₂ (mmHg)
Ctrl	7.42±0.14	33.8±1.7	137±9
Ctrl + rapa	7.38±0.23	36.0±1.8	145±8
Sur	7.37±0.13	35.5±0.6	133±7
Sur + rapa	7.40±0.18	34.8±2.2	143±6

Ctrl: Control group, Ctrl + rapa: rapamycin group, Sur: surgery group, Sur + rapa: surgery + rapamycin group.

Keuls post-hoc test was used. $P < 0.05$ indicated statistical significance.

Results

General condition of the mice

There were no significant differences in body weight between the groups in this experiment (Table 1). The vital signs of the mice in all groups were stable during the surgery. There was no obvious hypoxia, respiratory depression, hypotension, or other symptoms. The specific data are shown in Tables 2 and 3.

Cognitive function

In order to investigate whether inhibition of mTOR signaling could improve POCD induced

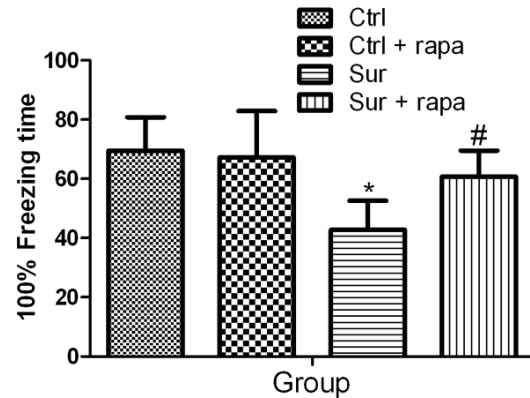


Figure 1. Effect of mTOR inhibitor rapamycin on conditioned fear memory. The mTOR inhibitor rapamycin did not affect the learning and memory function of the normal mice (Ctrl + rapa group vs Ctrl group, $P > 0.05$, $n = 8$), but could significantly improve the hippocampus dependent learning and memory induced by the surgery (Ctrl + rapa group vs Sur group, $\#P < 0.05$, $n = 8$). At the same time, the surgery could cause hippocampus dependent learning memory impairment ($*P < 0.05$ Sur group vs Ctrl group, $n = 8$). Ctrl: control group; Ctrl + rapa: rapamycin group; Sur: Surgery group; Sur + rapa: Surgery + rapamycin group.

by surgery, the effect of rapamycin on learning and memory impairment was evaluated using the conditioned fear test. The results showed that the surgical trauma could cause hippocampus-dependent learning and memory damage ($*P < 0.05$ Sur group vs Ctrl group). The mTOR inhibitor rapamycin did not affect the learning and memory function of normal mice, but it did significantly improve the learning and memory impairment in POCD mice ($60.0 \pm 8.1\%$ vs $43.4 \pm 8.0\%$, $\#P < 0.05$) (Figure 1).

mTOR signaling activation

Previous results showed that surgery could activate mTOR signaling in the hippocampus. In order to further verify the role of mTOR signaling activation in the occurrence of POCD, the mTOR inhibitor rapamycin was administered in this experiment before the surgery, and the changes in postoperative hippocampal mTOR signaling were evaluated. The expression levels of mTOR protein and phosphorylated mTOR protein in the hippocampus were detected by western blotting. The results further confirmed that the phosphorylation level of the mTOR protein was increased by the surgery ($*P < 0.05$ vs Ctrl group) but was significantly decreased in the

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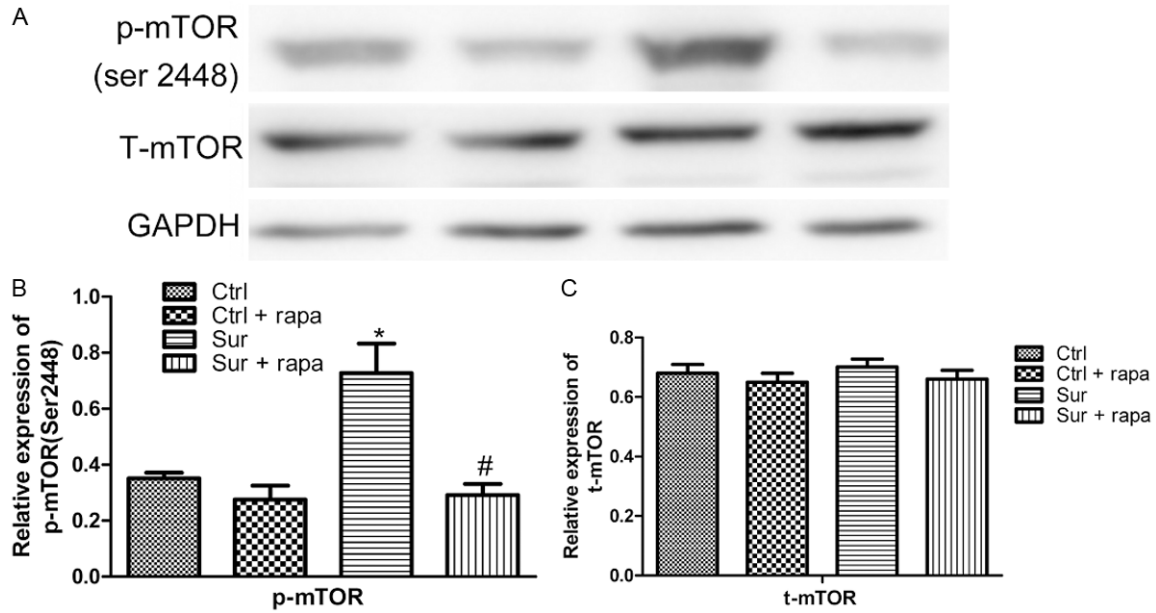


Figure 2. Effect of mTOR inhibitor rapamycin on the expressions of hippocampal total mTOR protein and phosphorylated mTOR. A. Western Blot result showed the expressions of phosphorylated mTOR and total mTOR protein in the hippocampus of mice in postoperative 1 d. B. Compared with Ctrl group, the expression of p-mTOR (Ser2448) protein in Sur group was significantly increased ($*P < 0.05$ vs Ctrl group, $n = 8$). Compared with Sur group, the expression of p-mTOR (Ser2448) protein in Sur + Rapa group was decreased in postoperative 1 d, which was statistically significant ($\#P < 0.05$ vs Sur group, $n = 8$). Compared with Ctrl group, the expression of p-mTOR (Ser2448) protein showed no statistical significance in Ctrl + rapa group ($P > 0.05$, $n = 8$). C. There was no significant difference in the expression of mTOR protein between all groups ($P > 0.05$, $n = 8$). The histogram represented mean \pm standard deviation.

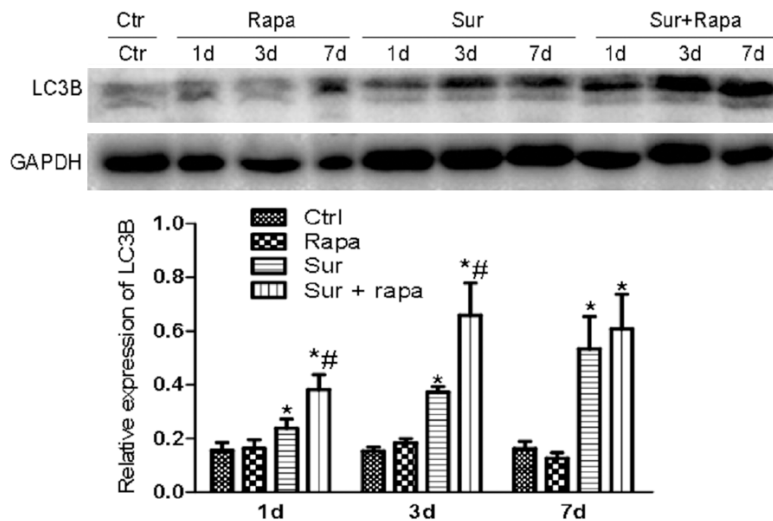


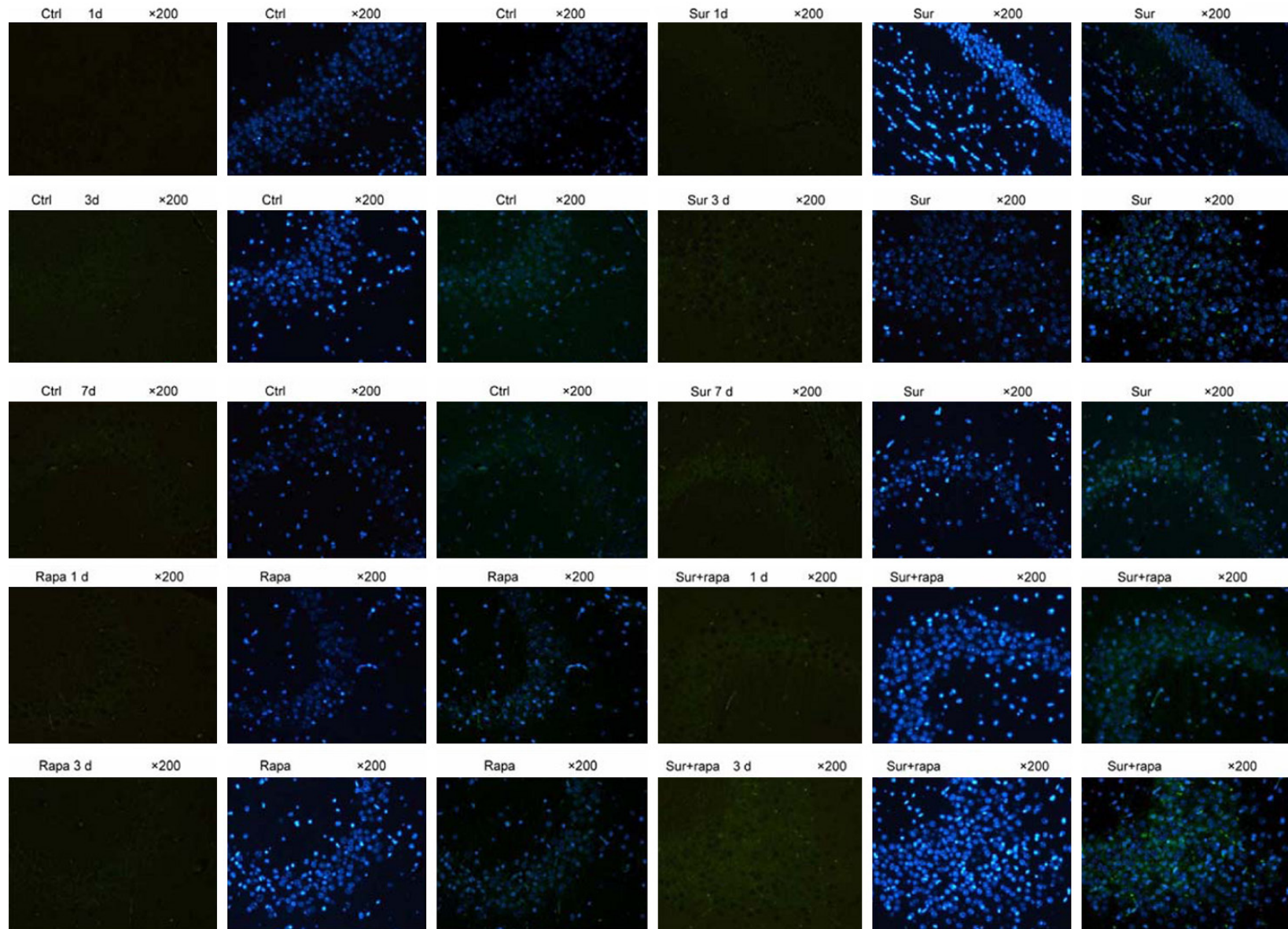
Figure 3. Effect of mTOR inhibitor rapamycin on the expression of LC3B protein in hippocampus. The expression of LC3B protein was detected by WB in postoperative 1 d, 3 d and 7 d. Postoperative 1 d, 3 d and 7 d: compared with Ctrl group, the expression of LC3B protein was significantly increased in Sur group and Sur + rapa group ($*P < 0.05$ vs Ctrl group, $n = 8$). Compared with Sur group, the expression of LC3B protein in Sur + Rapa group was significantly higher ($\#P < 0.05$ vs Sur group, $n = 8$). At the same time, there was no significant difference in protein expression between Ctrl + rapa and Ctrl groups ($P > 0.05$, $n = 8$). The histogram represented mean \pm standard deviation.

surgical group treated with rapamycin ($\#P < 0.05$ vs Sur group) (Figure 2). Rapamycin treatment did not affect the expression of total mTOR protein (Figure 2).

Content of LC3B protein

Previous studies have shown that the content of autophagy protein in the hippocampus in surgically treated animals was significantly decreased along with the activation of mTOR signaling. However, the content of autophagy protein was influenced by multiple factors. To further verify the relationship between surgical trauma, mTOR activation, and autophagy proteins, rapamycin was used to inhibit the activity of mTOR protein, and

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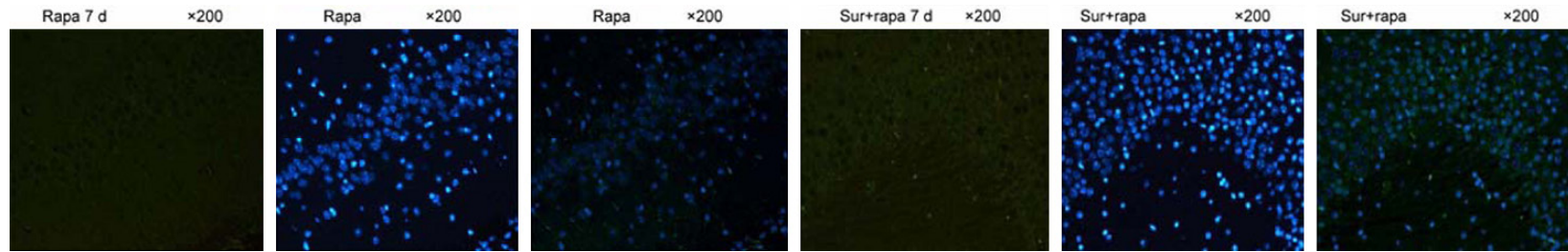


Figure 4. LC3B was mainly expressed in cytoplasm. Compared with Sur group, the expression of LC3B was stronger in Sur + Rapa group. The expression intensities showed no significant difference in 1 d, 3 d and 7 d in Ctrl + Rapa group.

the expression of LC3B protein in hippocampus was evaluated after the surgery. The results showed that the mTOR inhibitor rapamycin could increase the level of the autophagy protein LC3B ($\#P < 0.05$ vs Sur group), (**Figure 3**).

Morphological changes of hippocampal neurons and glial cells

In order to investigate the effect of the mTOR inhibitor rapamycin on autophagy, the autophagy protein LC3B activity was evaluated using the morphological changes of the hippocampal neurons and glial cells. Multiple bright green fluorescent spots were observed under fluorescence microscopy. The autophagy activity was evaluated by counting. The fluorescence detection results (**Figure 4**) showed that LC3B was mainly expressed in the cytoplasm. The expression tendency of LC3B was consistent between the Sur group and the Sur + Rapa group, and LC3B increased at 1 d, 3 d, and 7 d. Compared with the Sur group, the expression of LC3B was stronger in the Sur + Rapa group. The expression intensities showed no significant differences at 1 d, 3 d, and 7 d in the Ctrl + Rapa group.

Discussion

POCD is a common postoperative complication in elderly patients, and it has received increasing attention among medical professionals. However, to date, the specific pathogenesis of POCD is still not clear. Recently, advances in POCD research showed that POCD might have a similar or common mechanism as neurodegenerative disease, and the abnormal function of mTOR also plays an important role in some neurodegenerative diseases, such as Alzheimer's disease [12-14].

mTOR, as an atypical serine/threonine protein kinase, is involved in controlling protein synthesis, cell growth, proliferation and apoptosis, and other pathophysiological processes. Previous studies have shown that in addition to mTOR deficiency, excessively activated mTOR could also cause neural functional damage [15, 16]. Puighermanal et al. showed that the excessive activation of mTOR in the hippocampus was not beneficial for the formation of learning and memory [17], as it damaged the function of hippocampus-dependent learning and memory. Therefore, we speculated that

abnormal mTOR function might play a role in the occurrence of POCD.

In this experiment, the effects of anesthesia surgery and rapamycin on the activity of mTOR signaling in the hippocampus of mice were investigated. The results showed that the surgical trauma could not only cause cognitive dysfunction but also led to the excessive activation of mTOR signaling in hippocampus. The excessive activation of mTOR caused cognitive dysfunction due to many factors. Surgical trauma could cause an increase in A β and Tau protein expression levels as well as the up-regulation of Tau protein phosphorylation [18]. These toxic proteins excessively activate the mTOR/p70S6K pathway, induce the production of the toxic amyloid β protein (A β), and inhibit autophagy, forming a vicious cycle and resulting in cognitive dysfunction [19, 20]. Our study confirmed that rapamycin inhibited the excessive activation of mTOR signaling and improved cognitive dysfunction caused by surgical trauma.

Autophagy is another important cytobiological phenomenon that follows cell necrosis and apoptosis. Autophagy is ubiquitous in most eukaryotic cells. Autolysosomes function in cellular metabolism and in the renewal of organelles through the phagocytosis and degradation of cellular structures [21, 22]. The literature suggests that the regulatory pathway of autophagy mainly involves the classical phosphatidylinositol-3-kinase/protein kinase B/rapamycin target protein (PI3K/Akt/mTOR) signal [23, 24]. This interconnected and interdependent signal pathway inhibits the cytotoxic response to agrin in the cell and protects cells from damage and apoptosis. Caccamo et al. showed that increasing the activity of mTOR increased the Tau protein phosphorylation level and inhibited autophagy. The mTOR inhibitor rapamycin can reduce Tau protein expression and enhance its phosphorylation [20]. Rapamycin can inhibit the phosphorylation of p70S6K and decrease the activity of microglia [25]. Meanwhile, rapamycin can regulate the expression of Tau protein and its phosphorylation level, which are also involved in the mechanism of postoperative cognitive impairment [26].

The study showed that the expression of the autophagy protein LC3B in the hippocampus of mice was enhanced under the stress condition

of operative trauma, which is likely related to the intense stress caused by trauma. In addition, the expression of the autophagy protein LC3B was stronger when rapamycin was administered before the operation. Meanwhile, rapamycin could inhibit the activation of mTOR signaling and enhance the expression of the autophagy protein LC3B. Autophagy had a clear anti-inflammatory effect and removed toxic proteins. CCI-779 (a rapamycin analogue) can inhibit the activity of mTOR in an HD mouse model, promote autophagy, reduce the aggregation of mtHTT, and inhibit cell apoptosis [27]. Immunofluorescence assays also confirmed that the level of the autophagy protein LC3B in the hippocampus increased following surgical stress. The expression of the LC3B protein in the hippocampus increased significantly when rapamycin was administered before the surgery, and autophagy was enhanced, and POCD was alleviated.

In summary, the mTOR inhibitor rapamycin reversed the activation of mTOR signaling and as a result improved POCD, suggesting that rapamycin inhibited the neuro-inflammatory response, reduced the abnormal protein accumulation seen in neurodegenerative disease, and alleviated POCD through inducing and promoting autophagy. Rapamycin may be a potential drug for use in preventing and treating POCD, and it is also expected to be used to prevent and treat neurodegenerative diseases.

Disclosure of conflict of interest

None.

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