Brief Report

Protective effect of baicalin against cognitive memory dysfunction after splenectomy in aged rats and its underlying mechanism

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Postoperative cognitive dysfunction is a common neurological complication, characterized by impaired learning and memory, that occurs after anesthesia and surgery, especially in elderly patients. The traditional Chinese medicine baicalin is known to have neuroprotective effects. Therefore, we have investigated whether baicalin can improve postoperative cognitive impairment in aged rats after splenectomy. A total of 60 Sprague Dawley rats were randomly divided, equally, into the splenectomy, sham operation (Sham), low-dose baicalin (Baicalin A), medium-dose baicalin (Baicalin B), and high-dose baicalin (Baicalin C) groups. Splenectomy was performed under anesthesia in all groups except for the Sham group, in which an appropriate concentration of saline was administered. The effects of baicalin on learning and memory were examined by the Y-maze behavioral experiments. Although splenectomy had a negative effect on cognitive function in the acute phase, all the rats spontaneously recovered on a postoperative day seven. Nonetheless, in the acute phase, the medium and high doses of baicalin slightly alleviated these effects of the procedure. The protein expression of the inflammatory cytokines tumor necrosis factor-α, Interleukin-6, and Interleukin-1β was assessed using enzyme-linked immunosorbent assay. Their levels were elevated in the acute phase but were returned to normal with the medium and high dose of baicalin. Real-time PCR analysis of the mRNA expression of the N-methyl-D-aspartic acid receptor TNF-α, which is known to be involved in long-term potentiation, revealed that baicalin promoted its transcription. Thus, the findings indicate that baicalin may improve postoperative cognitive memory dysfunction in postoperative cognitive dysfunction in rats via anti-inflammatory mechanisms and pathways that involve N-methyl-D-aspartate receptor 2B subunit.

Keywords

Baicalin; postoperative cognitive dysfunction; inflammatory factor; quality of life; Y maze

1. Introduction

Postoperative cognitive dysfunction (POCD) is a postoperative central nervous system (CNS) complication. POCD is characterized by difficulty with cognitive, learning, memory, and thinking activities after surgery in patients who otherwise have a normal cognitive function. However, some patients experience long-term cognitive dysfunction as a result of several months or years of decline in social activity, social ability, and cognitive ability. It is generally believed that POCD is a degenerative disease that involves the CNS, and is caused by the induction or aggravation of neurodegenerative diseases and neuronal apoptosis by various factors, such as surgery, anesthesia, drugs, and diet (Laalou et al., 2008).

The hippocampus is essential for learning, memory, and cognition, and the NMDA receptor subtype NR2B plays an essential role in these functions of the hippocampus. NR2B is widely expressed in the brain and affects cognitive function by regulating membrane transport and the synaptic transmission processes of NMDA receptors and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. The hippocampus is particularly sensitive to damage as a result of epilepsy, hypoxia, ischemia, and encephalitis (Knierim, 2015). Surgical trauma stimulates an inflammatory response in the CNS as a result of which inflammatory cytokines are released, and the function of the CNS is regulated through the neuro-immune-endocrine network. The resulting changes that occur in hippocampal function are classified as POCD. Typically, the expression levels of pro-inflammatory cytokines (IL-1β, IL-2, IL-6, and TNFα) and anti-inflammatory cytokines (Interleukin-4 (IL-4) and Interleukin-10 (IL-10)) tend to be in a dynamic equilibrium, and the imbalance between the two affects the physiological function of the body (Fidalgo et al., 2011; Wan et al., 2007).

POCD seriously affects the quality of life of patients and is also associated with a high risk of disability and death (Evered et al., 2016; Steinmetz et al., 2009). The increase in the aging population in recent times has made POCD a severe problem in society. In the CNS, microglia and astrocytes mimic the function of peripheral lymphocytes and macrophages in producing inflammatory cytokines, which act as neuromodulators that regulate neurobiochemical, neuroendocrine, and behavioral changes. For ex-
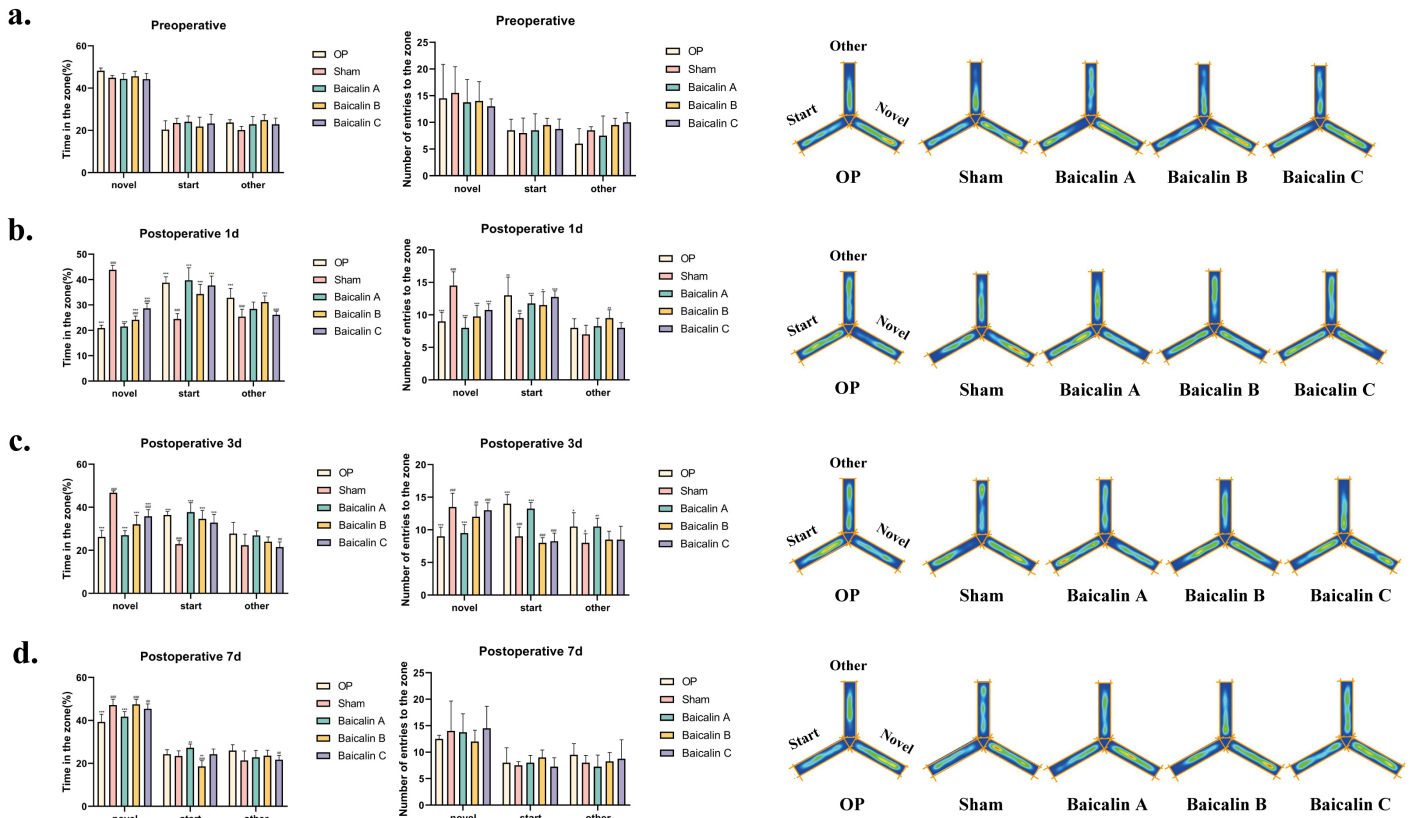
Fig. 1. Findings from the Y-maze test. (a) The percentage of time spent in the three arms, the number of entries, and the heat map of the rats' trajectories in each group during the preoperative period; (b) The percentage of time spent in the three arms, the number of entries, and the heat map of the rats' trajectories in each group on postoperative day 1; (c) The percentage of time spent in the three arms, the number of entries, and the heat map of the rats' trajectories in each group on postoperative day 3; (d) The percentage of time spent in the three arms, the number of entries, and the heat map of the rats' trajectories in each group on postoperative day 7.

[*]vs. The Sham group, [#]vs. the OP group

$0.01 < {}^*P < 0.05$, $0.001 < {}^{**}P < 0.01$, ${}^{***}P < 0.00.1$

ample, central and peripheral administration of IL-1β can disrupt spatial and non-spatial learning memory. Rosczyk et al. (2008) found that IL-1β mRNA levels in the hippocampus were significantly increased in aged rats and that their cognitive function was affected in maze experiments. Further, Cibelli et al. (2010) found that the population of microglia and the level of IL-1β expression in the hippocampus of rats were significantly increased after the operation. In contrast, rats with IL-1β receptor deficiency had a lower degree of neuroinflammatory reaction and cognitive impairment (Cibelli et al., 2010).

The elderly population has been on the increase, and the improvements in anesthesia and surgical techniques have led to an increase in the number of elderly patients undergoing surgery, and accordingly, an increase in the incidence of POCD (Kulason et al., 2018). Since the pathogenesis of POCD is not yet clear, there is no specific strategy for its treatment. As a potential strategy, controlling and inhibiting over-activated inflammatory responses in the CNS may be beneficial for the prevention and treatment of POCD. Neuropsychological tests are commonly used to assess cognitive function, such as Mini-Mental State Examination (MMSE), Mon-treal Cognitive Assessment (MoCA) and Wechsler Memory Scale (WMS) (Czyz-Szypenbejl et al., 2019).

Baicalin is a flavonoid extracted from the dried rhizome of *Astragalus membranaceus* and is the primary pharmacological component of astragalus. It has a variety of functions, such as antiviral, anti-inflammatory, anti-apoptotic, and anti-oxidative functions (Tu et al., 2011; Xu et al., 2006; Xue et al., 2010; Zhang et al., 2009). It can penetrate the blood-brain barrier and exert neuroprotective effects through anti-inflammatory and anti-apoptotic pathways, thus, providing effective protection against cerebral ischemia and other injuries. Baicalin has also been shown to protect against neuronal damage caused by amyloid β-peptide (Aβ) in an *in vitro* model.

In several studies on POCD, splenectomy on rodents is a standardized surgical procedure that causes neuroinflammation and cognitive dysfunction (Kamer et al., 2012; Lu et al., 2015; Newman et al., 2007). Y-maze takes advantage of the natural habits of rodents' exploration of new environments and is often used to test the spatial recognition and memory abilities emphasized by the hippocampus (Kraeuter et al., 2019).
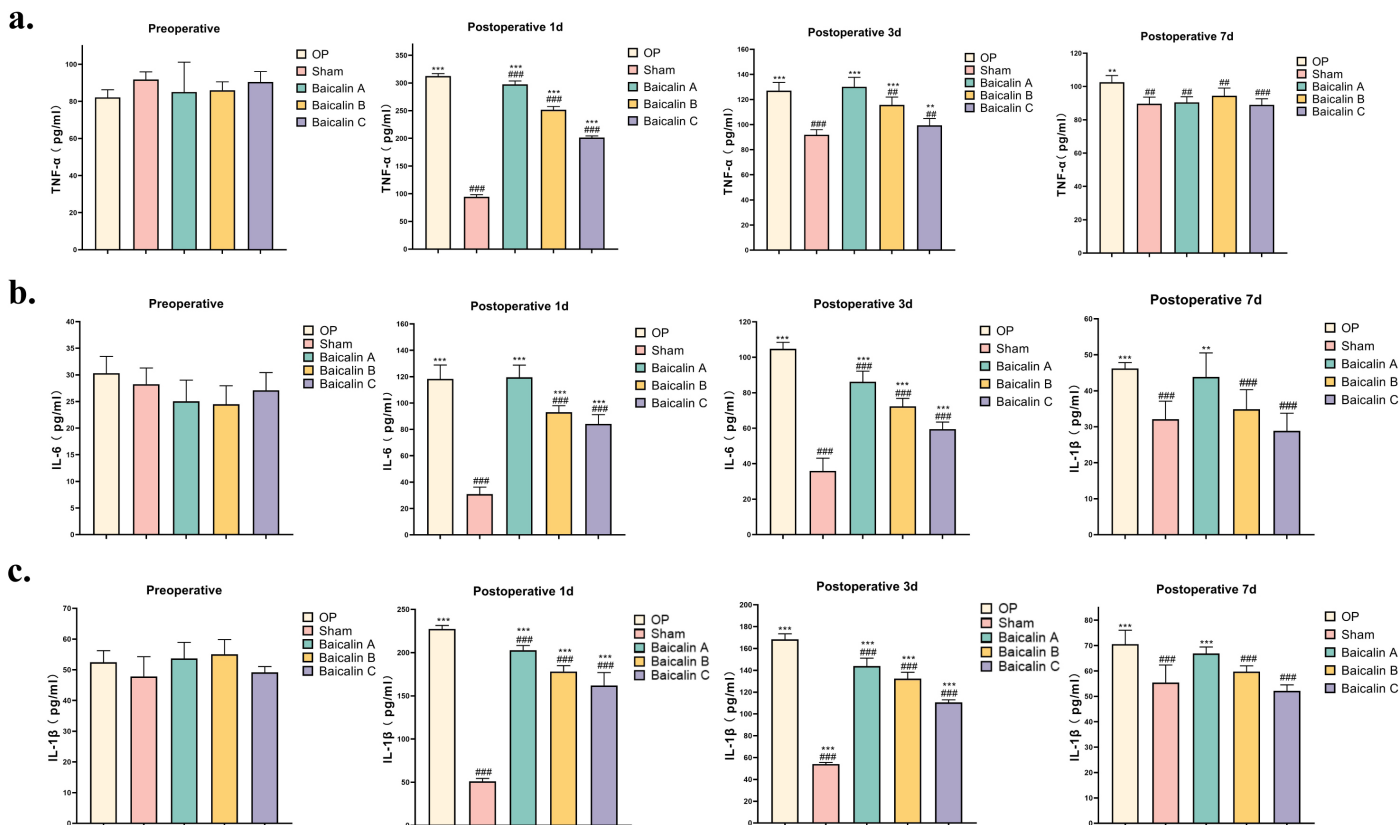
Fig. 2. ELISA findings for the protein expression of the inflammatory factor. (a) ELISA findings showing changes in serum TNF-α cytokine levels in each group before surgery, and 3 and 7 days after surgery; (b) ELISA findings showing changes in the serum IL-6 levels in each group before surgery, and 3 and 7 days after surgery; (c) ELISA findings showing changes in the serum IL-1β levels in each group before surgery, and 3 and 7 days after surgery. *vs. The Sham group, #vs. the OP group

$0.01 < {}^*P < 0.05$, $0.001 < {}^{**}P < 0.01$, ${}^{***}P < 0.001$

Based on the currently available research data, we used the Y-maze test to explored the role of baicalin in cognitive function and neuroprotection by constructing a POCD aged rat model through splenectomy. We elucidated the potential molecular mechanism underlying its neuroprotective effect, to provide a basis for the use of baicalin in the prevention and treatment of cognitive memory dysfunction after splenectomy.

2. Materials and methods

2.1 Laboratory animals and grouping

Sixty male Sprague Dawley rats aged 12 months were provided by Shanghai Slack Animal Center. The rats were randomly divided into 5 groups of 12 rats each: the splenectomy group (OP), the sham operation group (Sham), the low-dose baicalin group (Baicalin A), the medium-dose baicalin group (Baicalin B), and the high-dose baicalin group (Baicalin C). The rats were raised under specific pathogen-free (SPF) conditions, and all experiments were performed according to the ethical principles for medical research outlined. This work was approved by the ethics committee of Wuxi TCM Hospital Affiliated to the Nanjing University of Chinese Medicine.

2.2 Drug treatment and establishment of the splenectomy model

In aged rats of the Baicalin A, Baicalin B, and Baicalin C groups, 50 mg·kg^{-1}·d^{-1}, 100 mg·kg^{-1}·d^{-1}, and 200 mg·kg^{-1}·d^{-1} of baicalin, respectively, were injected intraperitoneally one day before surgery. In the aged rats of the OP and Sham groups, an equal amount of physiological saline was intraperitoneally injected on the same day. On the second day (that is, the day of the surgery), rats in the Sham group were only administered abdominal anesthesia. They did not undergo the procedure, as they served as the control group without surgery. The other four groups of rats underwent splenectomy: First, sedation was induced with 7% chloral hydrate (0.3 ml/100 g) anesthesia and 0.2% lidocaine cement analgesia, and the rats were placed on the surgical table. Under anesthesia[1], a mid-abdominal incision, a double ligature, and the spleen were removed. After hemostasis was confirmed, the abdomen was sutured.

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[1] In China, the purchase of pentobarbital sodium and isoflurane is strictly controlled, and chloral hydrate is easier to obtain. We strongly recommend that other researchers anesthetize rats with isoflurane inhalation if conditions permit.
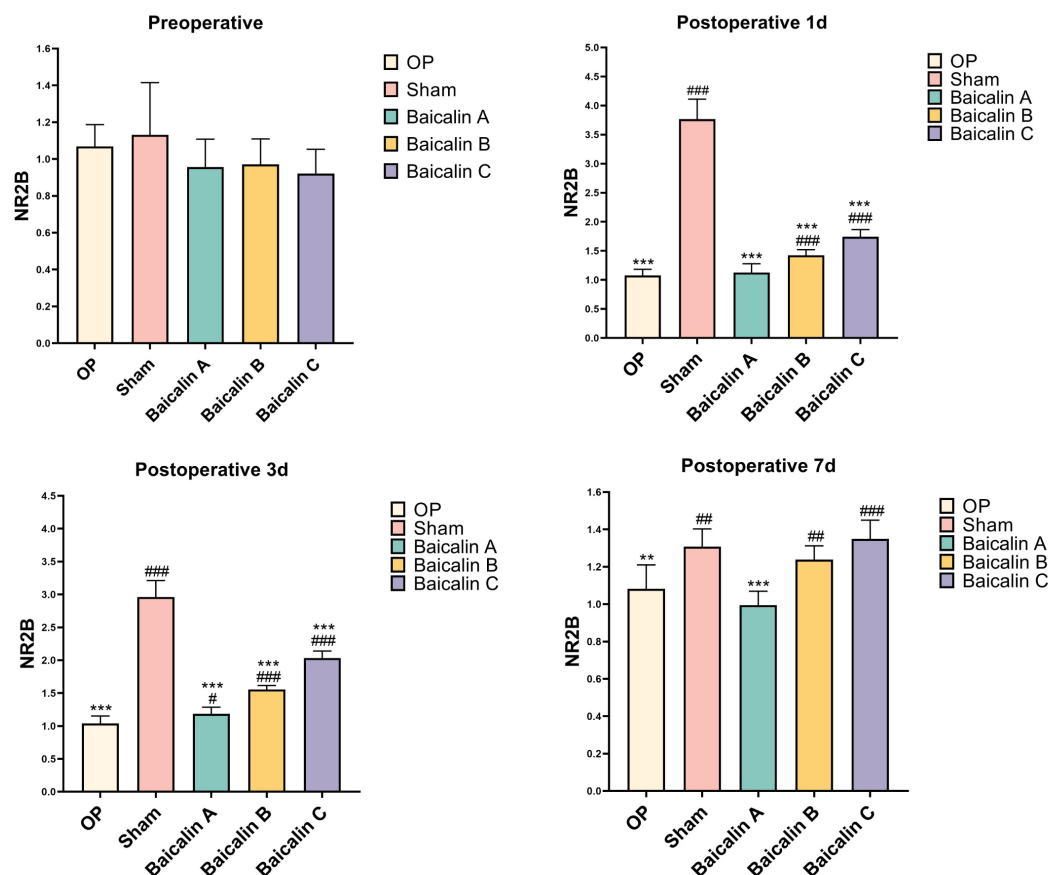
Fig. 3. Real-time PCR detection of NR2B mRNA levels. Real-time PCR detection of NR2B mRNA levels in the hippocampus of the rats in each group at 1, 3, and 7 days after surgery.
*vs. The Sham group, $^{\#}$vs. the OP group
$0.01 < {}^{*}P < 0.05$, $0.001 < {}^{**}P < 0.01$, ${}^{***}P < 0.001$

2.3 Y-maze test for examination of cognitive function

The novel arm experiment was performed in each group of rats before surgery, and 1, 3, and 7 days after surgery to assess spatial exploration and memory ability. The Y-maze has three arms with an angle of 120 ° between each of them, and the size of each arm is 50 cm × 11 cm × 20 cm (length × width × height). The three arms were named the start arm, the novel arm, and the other arm. A camera was installed at the top of the center of the maze to record the exploration behavior of the rats. During the training run, the novel arm was blocked, and the rat was placed in the start arm. It was allowed to freely explore the start arm and the other arm for 15 min. At the end of the test, the three arms were wiped down with 10% alcohol and allowed to dry out to eliminate any animal odor that could interfere with subsequent experiments.

According to the protocol of Dellu et al. (2000), the test run was initiated after 2 min. For the test run, the baffle was opened, and the rat was placed in the start arm. The rat was allowed to freely explore the three arms for 5 min. Its activity in both the trial and test runs was recorded and analyzed using the Anymaze 5.1.1 software. The recording was started after the rats entered the central area in both runs. The main parameter that was measured

as the amount of time the rats spent in each arm; was calculated as a percentage of the total exploration time. Another parameter was the number of entries into each arm, which was also calculated as a percentage of the total number of entries. These two parameters were used as indicators of the exploration behavior of animals.

2.4 Detection of serum cytokine levels by ELISA

Before surgery and 1, 3, and 7 days after surgery, 1- to 2-ml blood samples from the five groups of aged rats were collected via the eyeball. The rats were anesthetized before the blood samples were obtained from the eyeball. The levels of TNF-α, IL-1β, and IL-6 cytokines in serum were detected by enzyme-linked immunosorbent assay (ELISA).

2.5 RNA extraction and RT-qPCR

After the Y-maze behavioral experiment and blood sample collection, the animals were sacrificed immediately by intraperitoneal injection of 7% of 400 mg/kg chloral hydrate. To ensure that the euthanasia was successful, the mice were monitored to ensure that they had were not breathing for 3 min, and there were no blink reflexes. Fresh brain tissue was resected, and the hippocampus was dissected and stored at -80 °C for RT-PCR detection of NR2B.

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Total cellular RNA was extracted by the Trizol column centrifugation method, and the quality of the extracted RNA was evaluated using an Agilent 2100 Bioanalyzer (the RNA integrity values should be higher than 8). RT-qPCR was performed after synthesizing cDNA using the TaKaRa reverse-transcription kit. The amplification curves and dissolution curves were quantitatively analyzed using the Rotor-Gene Real-Time Analysis Software 6.1 and $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). The $2^{-\Delta\Delta CT}$ method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments. The threshold cycle (CT) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold.

3. Results

3.1 Effect of baicalin on spatial exploration and cognitive memory function in aged rats after splenectomy

According to the results, the spatial exploration and cognitive memory function of the aged rats were ELISZAnormal before splenectomy. Further, the percentage of time spent in and the number of entries into the novel arm were significantly higher than those for the other arm and start arm. Thus, the animals showed a better ability to explore the new arm. By tracking the trajectory of the rat, a trajectory heatmap was created within 5 min. Analysis of the map data (Fig. 1) revealed that there was no significant difference in the number of entries into and the percentage of time spent in each arm between the rats in each group.

The post-splenectomy findings showed that the spatial exploration and cognitive memory function in the aged rats had declined. On day 1 after surgery, the percentage of time spent in and the number of entries into the new arm were significantly lower in the OP group than in the sham group. The visual trajectory heatmap data confirmed this finding. Further, compared with the OP group, there were no significant differences in the number of entries, the percentage of time spent in each arm, and the trajectories in the three groups of rats treated with different concentrations of baicalin. Notably, treatment with 200 mg·kg^{-1}·d^{-1} baicalin showed a slight improvement in novel arm recognition and cognitive memory function in the aged rats.

On day 3 after surgery, the percentage of time spent, the number of entries, and the trajectories in the novel arm were significantly lower in the OP group than in the Sham group. However, when compared with the OP group, there was no significant difference in the 100 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1} baicalin groups.

On day 7, after surgery, the difference between the OP group and the Sham group was small. Further, the findings in the groups treated with 100 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1} baicalin were consistent with those in the Sham group.

3.2 Changes in serum TNF-α, IL-6, and IL-1β levels in each group

As shown in Fig. 2, the expression of the cytokines TNF-α, IL-6, and IL-1β were not significantly different between the five groups before splenectomy. On postoperative day 1, the levels of the three cytokines increased sharply, but their levels were reduced in the acute postoperative phase in the 100 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1} baicalin groups.

Compared with postoperative day 1, the TNF-α levels decreased sharply on a postoperative day 3. Treatment with 200 mg·kg^{-1}·d^{-1} returned the levels to normal (that is, the levels were comparable to those in the Sham group). Although the levels of IL-6 and IL-1β showed a decline after splenectomy, the decline was not as obvious as that of TNF-α. Nonetheless, both 100 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1} of baicalin were found to significantly reduce the levels of the three cytokines after surgery.

At day 7 after surgery, the serum levels of the three cytokines in the 100 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1} baicalin groups were restored to the normal range observed in the Sham group.

3.3 Detection of NR2B mRNA levels in the rat hippocampus by real-time PCR

As shown in Fig. 3, before splenectomy, the mRNA levels of NR2B in each group were consistent. In the acute phase on day 1 after surgery, the NR2B mRNA level of the OP group was significantly lower than that in the Sham group, and baicalin treatment was found to have a certain degree of therapeutic effect in the three baicalin-treated groups.

In the acute phase, compared with the Sham group, the NR2B mRNA level in the OP group was still low, while treatment with 100 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1} of baicalin resulted in a dose-dependent increase in the NR2B mRNA level on a postoperative day 3.

On postoperative day 7, the NR2B mRNA level in the OP group was significantly higher than that in acute phases 1 and 3 after surgery. In contrast, the NR2B level returned to normal in the aged rats treated with 100 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1} baicalin.

4. Discussion

We established an elderly POCD rat model. Behavioral and molecular experiments were used to verify whether baicalin, a traditional Chinese medicine, can exert neuroprotective functions through its anti-inflammatory and anti-oxidative effects, and thereby, improve cognitive memory impairment in the POCD model rats.

We used the Y-maze test for assessing behavior. The findings showed that in the preoperative period, the rats had normal memory and cognitive function, and the administration of baicalin did not negatively affect the neurological function of rats. The findings for postoperative days 1 and 3 showed that the procedure had hampered the ability of the old rats to explore the novel arm and their memory; however, on postoperative day 1, their function was slightly improved by treatment with 200 mg·kg^{-1}·d^{-1} baicalin or the high dose of baicalin. However, the aged rats in all the splenectomy groups seemed to spontaneously recover on postoperative day 7. Nonetheless, the findings do indicate that medium and high doses of baicalin (100 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1}) can improve exploration ability and memory in the acute phase following splenectomy in aged rats.

In the present study, cytokine analysis showed that on day 1 after surgery, the levels of TNF-α, IL-6, and IL-1β increased sharply; this may be related to the secretion of pro-inflammatory factors by inflammatory macrophages in the acute phase after spleen removal. On day 3 after surgery, the level of TNF-α decreased sharply, and the high dose of baicalin (200 mg·kg^{-1}·d^{-1}) could stabilize the TNF-α level to within the normal range. This is probably because TNF-α has a pronounced but short-lived ef-

fect as an inflammatory cytokine. The levels of IL-1β and IL-6 also decreased, but the decline was not as obvious as that of TNF-α. This is probably because the effects of these two cytokines are more prolonged. On postoperative day 3 and 7, both 100 mg·kg^{-1}·d^{-1} and 200 mg·kg^{-1}·d^{-1} of baicalin significantly reduced the levels of these three cytokines, and on day 7, the cytokine levels returned to normal. These results indicate that medium and high concentrations of baicalin can reduce the release of inflammatory factors to a certain extent. Thus, this might be one of the anti-inflammatory mechanisms by which baicalin enhances the spatial exploration and memory ability in aged rats.

Neuroscientists generally believe that long-term potentiation (LTP) is one of the crucial mechanisms of learning and memory. Electrophysiological experiments have demonstrated that AMPA receptors can mediate LTP and regulate synaptic plasticity (Borroni et al., 2000; Wiera et al., 2017). NR2B is a regulatory subunit of the NMDA receptor; therefore, we performed RT-PCR analysis of the mRNA level of NR2B in the hippocampal tissue of the five groups. The mRNA levels were consistent before the procedure. Seven days after the spleen removal procedure, the aged rats showed obvious self-recovery. Importantly, the levels of NR2B in the aged rats treated with high-dose baicalin returned to normal. These findings indicate that baicalin exerts its neuroprotective effect by increasing the level of NR2B transcription.

5. Conclusions

In summary, the present findings indicate that spontaneous recovery occurs in aged rats after splenectomy, despite a decrease in their cognitive ability in the acute phase following the procedure. Nonetheless, medium and high doses of baicalin seem to improve postoperative cognitive memory dysfunction in POCD rats in the acute phase by reducing the expression of postoperative inflammatory factors, such as TNF-α, IL-6, IL-1β, and increasing the level of NR2B transcription. Thus, the application of baicalin could be explored further for the prevention and treatment of postoperative cognitive memory dysfunction, improving the quality of life of postoperative patients, as well as reducing the social and economic burden of the patients and their families.

Abbreviations

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; Aβ: Amyloid β-peptide; Baicalin A: low-dose baicalin; Baicalin B: medium-dose baicalin; Baicalin C: high-dose baicalin; CNS: central nervous system; CT: threshold cycle; ELISA: enzyme-linked immunosorbent assay; IL-1β: Interleukin-1β; IL-4: Interleukin-4; IL-6: Interleukin-6; IL-10: Interleukin-10; LTP: long-term potentiation; MMSE: Mini-Mental State Examination; MoCA: Montreal Cognitive Assessment; NMDA: N-methyl-D-aspartic acid; NR2B: N-methyl-D-aspartate receptor 2B subunit; OP: splenectomy group; POCD: Postoperative cognitive dysfunction; RT-PCR: Real-time PCR; Sham: sham operation; SPF: specific pathogen-free; TNF-α: tumor necrosis factor-α; WMS: Wechsler Memory Scale.

Author contributions

Jian-Nan Zhang and Liang-Yu Cai are responsible for the guarantor of integrity of the entire study, study concepts & design, definition of intellectual content, literature research, clinical studies, experimental studies, data analysis & collection, statistical analysis; Hong-Mei Zhou is responsible for the study design, literature research, data acquisition, manuscript preparation; Chen-Hao Jiang and Jiao Liu is responsible for the guarantor of integrity of the entire study, study concepts, definition of intellectual content, clinical studies, experimental studies, data analysis, statistical analysis, manuscript preparation & editing & review. All authors are approved in this manuscript.

Ethics approval and consent to participate

This work was approved by the ethics committee of Wuxi TCM Hospital Affiliated to the Nanjing University of Chinese Medicine (2018011722).

Conflict of Interest

The authors declare no conflict of interest.

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