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Quercetin can improve anesthesia induced neuroinflammation and cognitive dysfunction by regulating miR-138-5p/ LCN2

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Abstract

Background Anesthesia can lead to functional cognitive impairment, which can seriously affect postoperative recovery. To investigate the effect and mechanism of quercetin (Que) in anesthetized rats, the study provided a new therapeutic idea for the prevention of cognitive dysfunction caused by anesthesia.

Methods Cognitively impaired rats were constructed using Isoflurane (ISO) anesthesia and treated with Que. The capacity of the rats to learn and remember was tested using the Morris water maze test. Rat hippocampal tissues were collected and analyzed for inflammatory factor concentration and miR-138-5p expression using ELISA and qRT-PCR, respectively, and the targeting link between miR-138-5p and LCN2 was verified by dual luciferase reporter.

Results Que treatment was found to improve ISO-induced cognitive dysfunction and inhibit the level of hippocampal inflammatory factors in rats. miR-138-5p was down-regulated in rats with cognitive dysfunction, while Que treatment increased miR-138-5p expression. The study found that knockdown miR-138-5p can reverse the positive effects of Que therapy, aggravate cognitive dysfunction, and promote the secretion of TNF-α, IL-1β, and IL-6 in the hippocampus. In addition, LCN2, a target gene of miR-138-5p, was significantly up-regulated in the hippocampus after ISO induction.

Conclusion Que may inhibit ISO-induced hippocampal neuroinflammation and ameliorate functional cognitive deficits in rats by modulating miR-138-5p/ LCN2.

Keywords Isoflurane anesthesia, Cognitive dysfunction, Quercetin, Neuroinflammation, miR-138-5p, LCN2

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Background

Isoflurane (ISO) is widely used in clinical anesthesia as an inhaled anesthetic [1, 2]. In recent years, the adverse effects of ISO on the nervous system have received increasing attention. It has been pointed out that ISO can cause patients' learning and memory decline, induce neuronal apoptosis, and lead to cognitive dysfunction [3, 4]. Cognitive dysfunction is a postoperative complication that occurs in psychologically normal patients before surgery, and is commonly seen in elderly surgical patients, manifesting as memory changes, disorientation, and changes in social adaptation [5]. Cognitive dysfunction causes great pain and economic burden to patients' families. Therefore, it is necessary to explore an effective therapeutic regimen for ISO-induced cognitive dysfunction.

Quercetin (Que) is a flavonoid compound extracted from the roots, flowers and fruits of plants with a broad spectrum of biological activities like anti-inflammatory, antioxidant, neuroprotective, cardioprotective, and anticancer [6]. It is extensively employed in the therapy of cardiovascular and cerebrovascular diseases, malignant tumors and inflammatory diseases [7]. It was demonstrated that Que exerts neuroprotective effects in cerebral ischemia-reperfusion injury by inhibiting NF-κB phosphorylation [8]. Although there is ample support for a protective function of Que in many pathways associated with neurological disorders, the effect of Que on ISO-induced cognitive dysfunction and its mechanisms remains to be further explored.

MicroRNAs (miRNAs) are highly conserved noncoding ribonucleic acids that regulate the expression of target genes through degradation or translation. An increasing variety of miRNAs have been shown to have a critical part to play in the amelioration of anesthesia-induced cognitive dysfunction. It has been shown that miR-96 promotes sevoflurane anesthesia-induced apoptosis in hippocampal neurons and exacerbates the effects of sevoflurane on hippocampal neurons and cognitive function [9]. The lncRNA Riken can inhibit sevoflurane-induced neurotoxic effects by targeting miR-101a and potentially contributes to amelioration of cognitive dysfunction [10]. One study screened for miRNAs that appeared to be aberrantly expressed in the hippocampus of neonatal rats after sevoflurane exposure, in which miR-138-5p was dramatically downregulated [11]. miR-138-5p was involved in the regulation of a variety of diseases, and it can affect macrophage polarization and regulate tumor progression by targeting KDM6B [12]. It has also been found to act on the progression of osteoporosis and is a key target for osteogenic differentiation in old age [13]. Furthermore, it was recently shown that miR-138-5p expression was significantly correlated with neuronal survival [14] and can participate in the regulation of neuroinflammation by affecting the NLRP3 inflammasome [15]. It can be inferred that miR-138-5p has an important effect on the regulation of neural function. However, the functional molecular mechanism of miR-138-5p in the induction of ISO anesthesia and how to improve cognitive dysfunction remain unclear.

Based on the above background, this study established a rat model of cognitive impairment using ISO anesthesia and investigated the influence of Que on rats and its specific mechanisms.

Methods

Animal treatment and ethics statement

Thirty-five healthy male Sprague-Dawley (SD) rats, body weight 570 ~ 680 g, 20 months old [16, 17], were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., LTD. They were fed normally in SPF animal houses with relative humidity of 40% ~ 60%, temperature of 22 ~ 28 ℃, light time of 12 h a day and night cycle. The rats were randomly assigned to the following groups: control check group (CK), ISO anesthesia group (ISO, RWD, China), dimethyl sulfoxide treatment group (3% DMSO, Thermo, USA), Que treatment group (3% Que, 5 mg/kg, Solarbio, China), Que treatment + miR-138-5p negative control group (Que + miR-NC), and Que treatment + miR-138-5p inhibitor group (Que + miR-inhibitor). The required sample size was preliminarily determined using G*Power software (version 3.1; original German version). The calculations were based on an α error probability of 0.05, an effect size f of 0.8, and a target power (1 - β error probability) of 0.8. Considering the health status of the rats during breeding, healthy rats were selected and evenly divided into each group, with five rats per group. Inhalation of 3% ISO was performed in all groups except the CK group. miR-NC and miR-inhibitor vectors (Lipofectamine2000 transfection reagent, Thermo, USA) were injected via the lateral ventricle 1 d before ISO anesthesia, respectively. Rats were monitored for oxygen saturation and pulse rate during anesthesia and were returned to the cage for rearing at the end. It was obtained from the Shengli Oilfield Central Hospital Ethics Committee and complied with the National Institutes of Health "Guidelines for the Protection and Use of Laboratory Animals".

Morris water maze test

The Morris water maze test [18, 19] was performed 48 h after the establishment of the ISO anesthetized rat model. The rats were placed in the water from the four quadrants of the water maze with their backs facing the underwater platform, and the time spent by the rats to search for the underwater platform and the distance they swam to reach the platform were recorded within 120 seconds. If the rats did not find the underwater platform within 120 second, the rats were guided to the underwater

platform and stayed there for 10 seconds, and the evasion latency was recorded to be 120 seconds. The rats underwent four training sessions daily, with an inter-session interval exceeding 4 min, for a total of 5 consecutive days. The final measure of avoidance latency was determined as the average evasion latency across these 5 days. On day 6 of the experiment, the platform was removed. The rats were reintroduced into the water from the other side, and the number of times the rats crossed the original platform, and the residence time were recorded.

qRT-PCR

At the end of the water maze experiment, the rats were executed, and the hippocampal tissues were rapidly isolated and preserved in liquid nitrogen. Frozen hippocampal tissue was taken, and total RNA was extracted by adding TRIzol reagent (Invitrogen, USA). A reverse transcription system was used to reverse transcribe the total RNA into cDNA, which was then used as a template for PCR amplification using the SYBR PrimeScript™ RT-PCR kit (TaKaRa, Japan). U6 and GAPDH were employed for miR-138-5p and LCN2 as internal controls, respectively, and the relative expression was determined by the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Take frozen hippocampus tissue, add appropriate amount of protein extraction buffer, grind it into homogenate, centrifuge it at 4 ℃ and 3 500 r/min for 10 min. The supernatant was preserved at -80 °C in a refrigerator. The frozen supernatant was taken during the test and the ELISA kit was strictly followed. After the reaction was terminated, the absorbance was detected by an enzyme-labeled instrument, and the levels of IL-1β, IL-6 and TNF-α were calculated.

Cell culture and dual luciferase reporter

Rat brain neuronal cells PC12 (ATCC, USA) were purchased and frozen at -80 ℃. When used, it was thawed first, and then incubated in culture flasks containing DMEM medium with fetal bovine serum (10%), penicillin (1%) and streptomycin (1%). The culture flasks were cultured in a 5% CO_2 incubator at 37 ℃, and when the cell density reached 80% of the flasks, 1:3 passaging culture was performed. The miR-138-5p predicted target sequence or mutation sequence 3'-UTR of LCN2was constructed into the pmirGLO vector. The mutation sequence was modeled on the 3' -UTR sequence of normal LCN2, and point mutation was performed at the predicted site bound to miR-138-5p. Using Lipofectamine2000 reagent, miR-NC, or miR-138-5p inhibitors were co-transfected with the constructed vectors into the PC12 cells, respectively, and placed in the incubator for 48 h. Luciferase activity was determined with a dual luciferase reporter assay kit and standardized with renin luciferase activity.

Statistical analyses

Statistical analyses were conducted using SPSS 11 and GraphPad Prism 5.0. For pairwise comparisons, the independent sample t-test was employed. For multiple group comparisons, One-Way ANOVA was initially applied. If ANOVA indicated significant differences ($P<0.05$), a post hoc analysis (Tukey's Honestly Significant Difference Test) was subsequently performed to identify specific group differences. Data are presented as mean ± SD (standard deviation). Statistical significance was set at $P<0.05$.

Result

Effect of que on anesthetized mice

It was found that the escape latency and distance traveled by ISO-anesthetized rats were significantly higher than those of the CK group (Fig. 1a and b), and the residence time in the target quadrant and the number of times the ISO-treated rats traversed the original platform were significantly lower than those of the CK group (Fig. 1c and d). Que treatment significantly improved the learning and memory abilities of ISO rats. In addition, the levels of TNF-α, IL-1β and IL-6 were significantly increased in the hippocampus of ISO anesthetized rats, whereas treatment with Que reduced the secretion of inflammatory factors to some extent (Fig. 2a-c).

Mir-138-5p mediates que in the treatment of anesthetized rats

To investigate the mechanism of action of Que affecting cognitive dysfunction in ISO rats, qRT-PCR was performed, and miR-138-5p was notably reduced in the hippocampus of ISO anesthetized rats compared with the CK group (Fig. 3a). Further exploration revealed that Que promoted miR-138-5p expression (Fig. 3b). It was found that miR-138-5p down-regulation significantly reversed the therapeutic effects of Que on the learning memory ability of ISO anesthetized rats, increasing the time to find the platform and the distance travelled in the test (Fig. 4a-b), and decreasing the dwell time in the target quadrant and the number of times the ISO anesthetized rats passed the original platform (Fig. 4c-d). miR-138-5p down-regulation significantly reversed inhibitory effects of Que on the inflammatory factors in ISO anesthetized rats (Fig. 5a-c).

Effect of que on mir-138-5p downstream target genes

A database search revealed that LCN2 is the downstream target gene of miR-138-5p, to verify the targeting relationship between the two, a dual luciferase gene reporter assay was carried out, and the designed sequences are

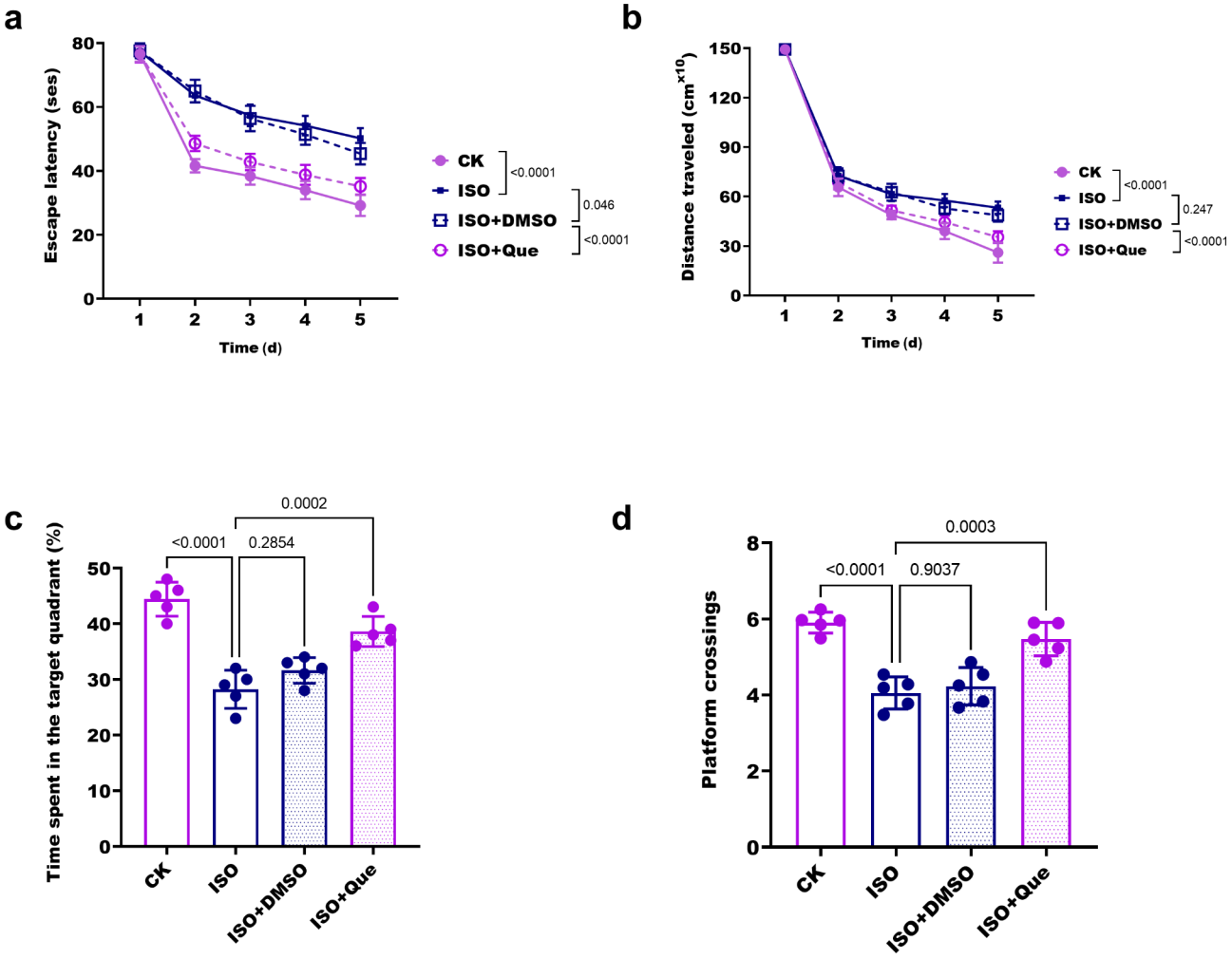

Fig. 1 Effect of Que on functional cognitive impairment in ISO-anesthetized rats. Que decreased the escape latency (**a**) and traveling distance of anesthetized rats (**b**); Que increased the residence time in the target quadrant (**c**) crossing time on the original platform (**d**) of anesthetized rats

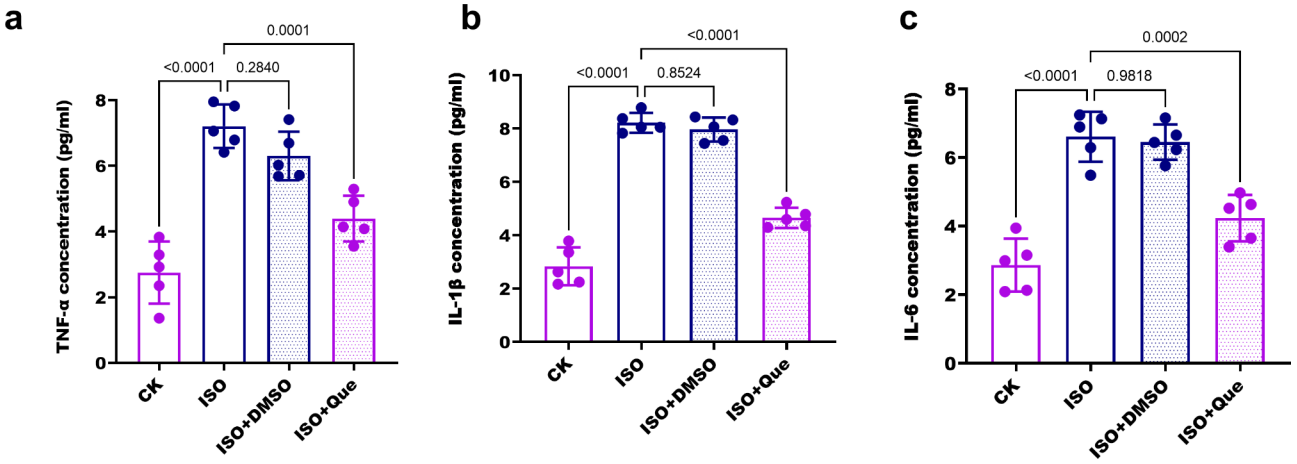

Fig. 2 Effect of Que on neuroinflammation in the rat hippocampus. Que can reduce the level of TNF-α (**a**), IL-1β (**b**) and IL-6 levels (**c**) in the hippocampus of anesthetized rats

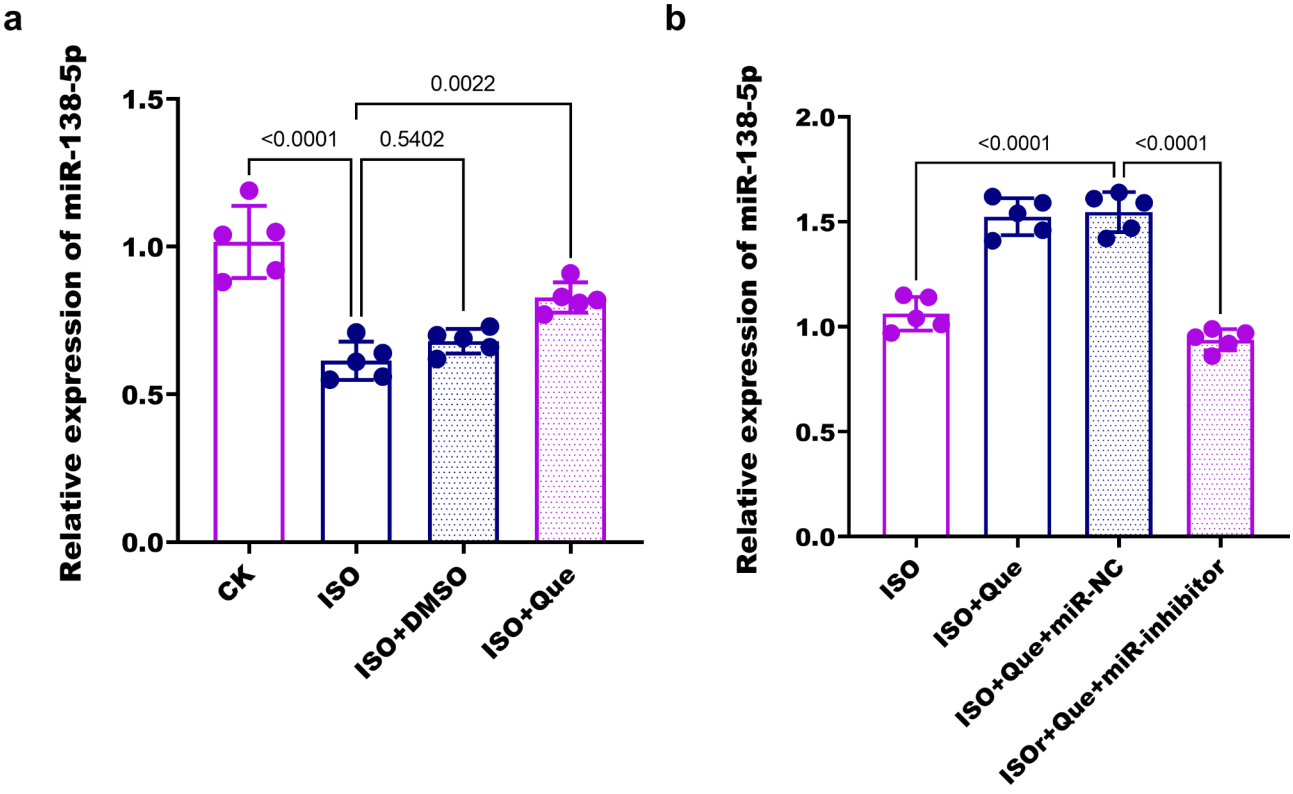

Fig. 3 miR-138-5p expression in anesthetized rats. miR-138-5p was significantly down-regulated in anesthesia (**a**); Que treatment significantly increased miR-138-5p expression (**b**)

shown in Fig. 6a. The results revealed that there is a targeting relationship between miR-138-5p and LCN2 (Fig. 6b). Que treatment significantly promoted LCN2 expression, which was reversed by knockdown of miR-138-5p (Fig. 6c). LCN2 was found to be significantly up regulated in ISO anesthetized rats, while Que significantly reduced LCN2 expression (Fig. 6d).

Discussion

It has been found that anesthetized patients with ISO application are prone to cognitive dysfunction, which hinders the recovery of normal cognition [3, 4]. Similarly, the present study found a significant decrease in spatial memory capacity in rats after ISO exposure compared to normal rats. In addition, it has been shown that repeated exposure of the central nervous system to ISO can cause extensive neuroinflammation, resulting in neurological decline and leading to cognitive dysfunction in patients. In the present study, we found that the levels of proinflammatory factors in the hippocampus of ISO-exposed rats were significantly elevated. Kong et al. similarly found that the mechanism of anesthesia-induced cognitive dysfunction was closely related to neuroinflammation [20]. This suggests that ISO-induced neuroinflammation in the hippocampal region of the brain is a key factor in cognitive dysfunction.

It was found that Que could exert anti-inflammatory effects in vitro and in vivo by reducing the expression of inflammatory factors [21]. It can attenuate neutrophil infiltration in inflammatory tissues, inhibit the secretion of cellular pro-inflammatory factors, and inhibit TNF-α-induced inflammatory response and apoptosis by blocking the transcription of NF-κB and AP-1 signaling molecules in human umbilical vein endothelial cells [22]. To find an effective treatment plan to prevent cognitive dysfunction, this study treated rats with Que, and the results showed that Que could significantly reduce the release of inflammatory factors TNF-α, IL-6 and IL-1β in rat hippocampus and alleviate ISO-induced cognitive dysfunction in rats. Wang et al. also demonstrated that Que can reduce the expression of inflammatory factors such as TNF-α, IL-6 and IL-1β in RAW264.7 cells, thereby alleviating LPS-induced inflammatory damage [23]. It can be preliminarily concluded that Que can reduce ISO-induced cognitive dysfunction by inhibiting the inflammatory response of rat hippocampus. However, the mechanism by which Que suppresses the hippocampal inflammatory response is unknown.

Numerous studies have demonstrated that miRNAs play an important part in regulating neuroinflammation and improving cognitive dysfunction. Some studies have indicated that miR-138-5p suppresses the activation

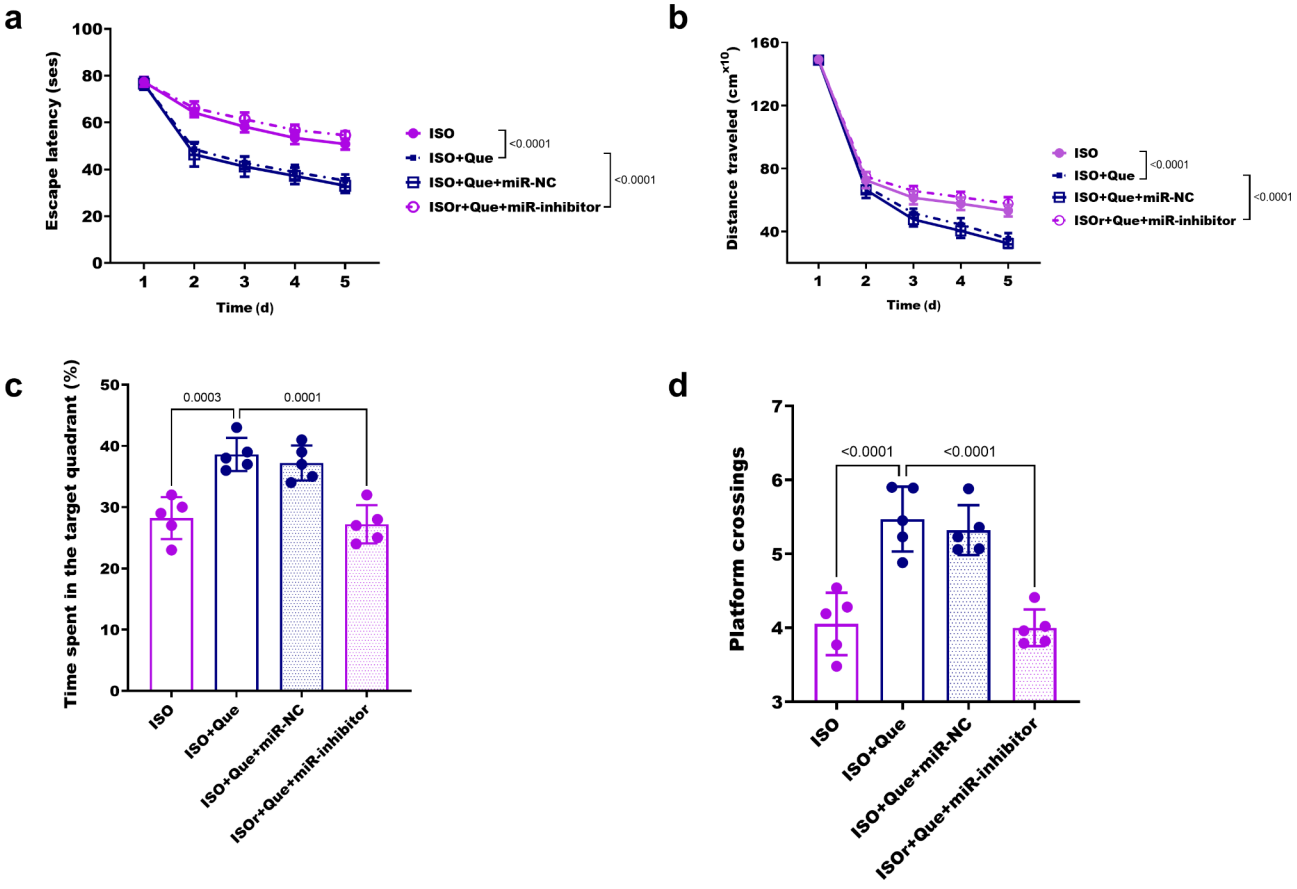

Fig. 4 Effect of miR-138-5p expression on anesthetized rats. miR-138-5p down-regulation increased the escape latency (**a**) and traveling distance of anesthetized rats (**b**); miR-138-5p down-regulation decreased the residence time in the target quadrant (**c**) crossing time on the original platform (**d**) of anesthetized rats

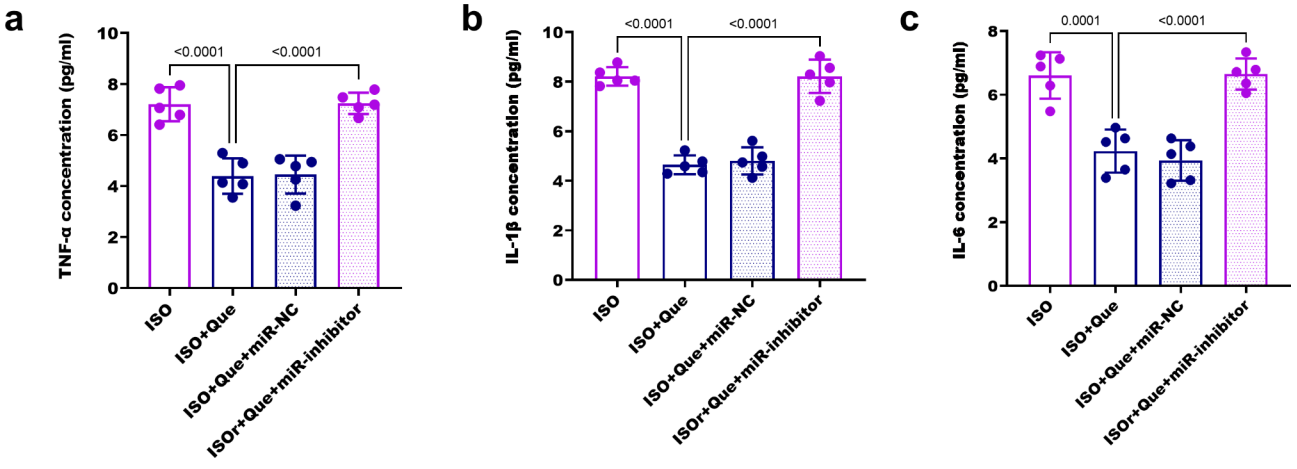

Fig. 5 Effect of miR-138-5p expression on neuroinflammation in rat hippocampus. miR-138-5p down-regulation promotes TNF-α (**a**), IL-1β (**b**) and IL-6 levels (**c**)

of NLRP3 inflammatory vesicles, thereby depressing the release of inflammation-associated factors [15, 24]. This study found that miR-138-5p expression was notably down-regulated in the ISO-anesthetized cognitive dysfunction rat model, and down-regulated the expression of miR-138-5p could aggravate the degree of cognitive dysfunction in ISO rats and increase the secretion of inflammatory factors in the hippocampus. miR-138-5p was found to be significantly down-regulated in LPS-induced cellular inflammation models [25].

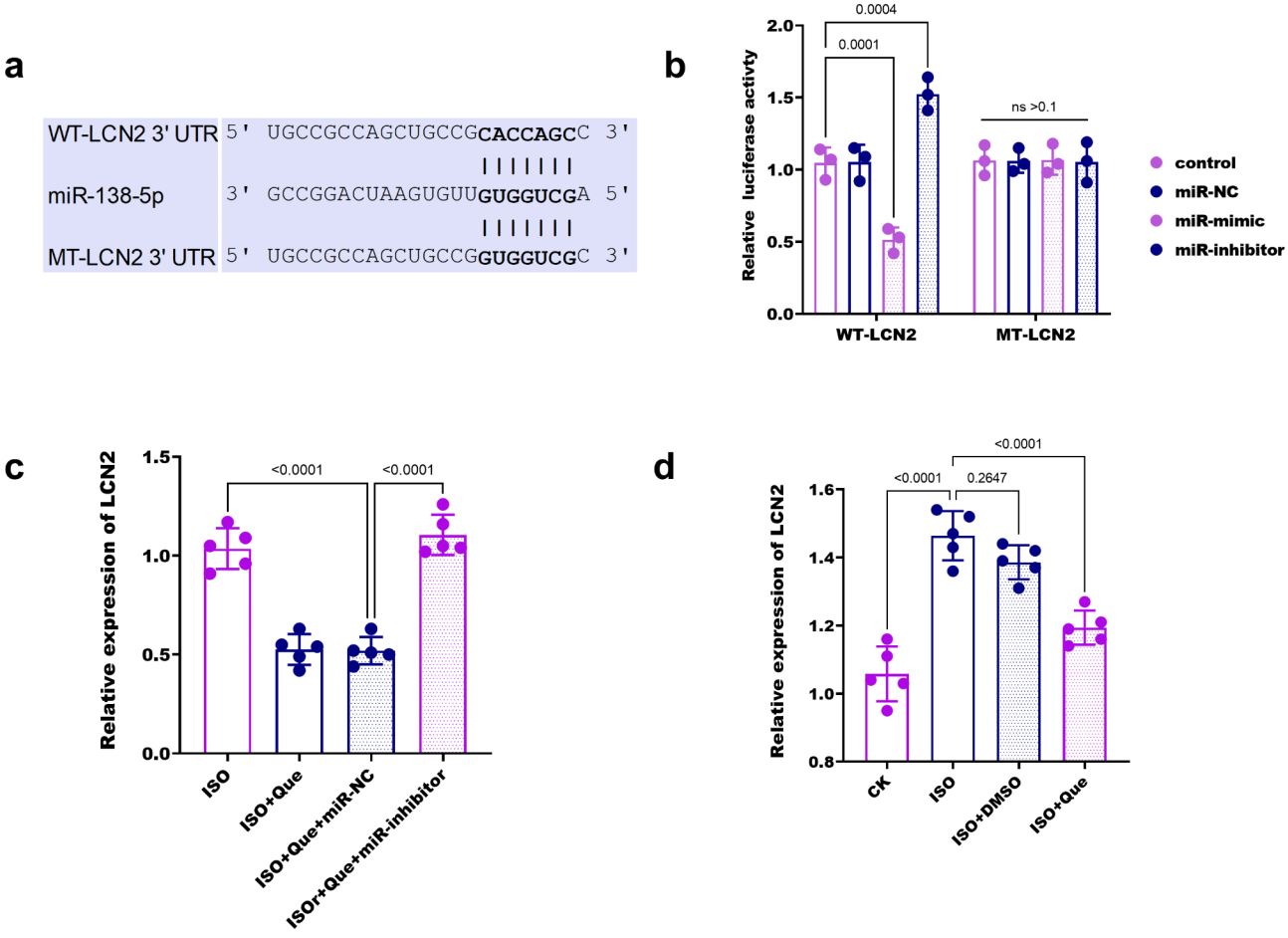

Fig. 6 Relationship between miR-138-5p and LCN2. The binding sites between miR-138-5p and LCN2 (**a**); miR-138-5p negatively regulates LCN2 luciferase activity (**b**); Que significantly inhibited LCN2 expression (**c**). LCN2 was overexpressed in anesthetized rats (**d**)

Knocking down lncRNA H19 could negatively regulate the expression of miR-138-5p and inhibit the inflammatory response of hypoxic cells [26]. These results suggest that miR-138-5p may act as a suppressor of hippocampal inflammation, alleviating cognitive dysfunction induced by ISO anesthesia. Further analyses revealed a significant upward trend of miR-138-5p in Que-intervened rats, and in addition Que could reverse the negative effects of miR-138-5p downregulation on the ISO rat model. It has been suggested that Que can promote miR-369-3p expression and alleviate the inflammatory response in chronic inflammation [27]. Wang et al. found that Que induced upregulation of miR-135b, which ameliorated atrial fibrillation by negatively regulating the TGF-β/Smads pathway [28]. The above evidence suggests that Que may improve the hippocampal inflammatory response and cognitive dysfunction in ISO mice by targeting miR-138-5p.

To further determine the mechanism of miR-138-5p in ISO-induced cognitive dysfunction, this study demonstrated that miR-138-5p can bind to the 3′-UTR of LCN2 and negatively regulate its expression. In other diseases, the regulatory relationship between miR-138-5p and LCN2 is consistent with the results of this study. For example, miR-138-5p can negatively regulate LCN2 expression and thus inhibit hypoxia-induced apoptosis in cardiomyocytes [29]. LCN2 has been found to be involved in a variety of cellular processes, such as innate immunity and apoptosis [30, 31]. Li et al. found that inhibition of LCN2 expression significantly suppressed the secretion of proinflammatory factors in stroke and improved cognitive function in mice [32]. In addition, a study showed that LCN2 was significantly up regulated in the brain after LPS stimulation, and that LCN2 overexpression induced a delayed process in brain neurons, which reduced spatial memory capacity in rats [33]. The above evidence suggests that Que may have played a role in inhibiting the inflammatory response and ameliorating cognitive dysfunction by inducing miR-138-5p expression to reduce LCN2 expression.

LCN2 has been reported to play a powerful therapeutic role in cerebral ischemia by inhibiting neuroinflammatory damage through the JAK2/STAT3 pathway [34]. It has shown that JAK2/STAT3 pathway is related to cognitive function, for example, dexmedetomidine alleviates

ISO-induced cognitive impairment through JAK2/STAT3 pathway [35]. Therefore, it is speculated that Que plays a role in LCN2/JAK2/STAT3 pathway by targeting miR-138-5p. Unfortunately, due to the cost and time of the experiment, it has not been studied deeply, and it will be further discussed in the follow-up experiment.

Conclusions

In general, this study found that Que may inhibit the inflammatory response in the hippocampus of ISO-anesthetized rats and improve cognitive dysfunction by upregulating miR-138-5p. In addition, Que may initially affect the LCN2 pathway by targeting miR-138-5p, but it has not been further verified through in vitro experiments. Subsequent studies will continue to explore this, to provide sufficient experimental basis for the application of Que and miR-138-5p in anesthesia.

Abbreviations

CK	Control Check
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ISO	Isoflurane
miRNAs	MicroRNAs
NC	Negative control
Que	Quercetin
SD	Sprague-Dawley

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Not Applicable.

Author contributions

LJ L, WN Y and HN Z designed the research study. YY J, DL W, L C, MQ D and S W performed the research. Y C, Q L and S W analyzed the data. LJ L and WN Y wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate

It was obtained from the Shengli Oilfield Central Hospital Ethics Committee and complied with the National Institutes of Health "Guidelines for the Protection and Use of Laboratory Animals".

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

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