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Neuroprotective and anti-inflammatory effects of Yingqiao San on epileptogenesis are mediated by inhibiting HMGB1 translocation and regulating the HMGB1/TLR4 signaling pathway

Hanjiang Chen
First Teaching Hospital of Tianjin University of Traditional Chinese Medicine
Xilian Zhang
First Teaching Hospital of Tianjin University of Traditional Chinese Medicine
Ying Song
Tianjin University of Traditional Chinese Medicine
Zhaoyuan Chen
Tianjin University of Traditional Chinese Medicine
Yanli Lu
First Teaching Hospital of Tianjin University of Traditional Chinese Medicine
Ping Rong
First Teaching Hospital of Tianjin University of Traditional Chinese Medicine
Rong Ma (antiepilepsy2021@outlook.com)
First Teaching Hospital of Tianjin University of Traditional Chinese Medicine

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Abstract

Background: Epileptogenesis was the latency period of epilepsy. Neuronal damage and subsequent inflammatory response were the basic mechanisms of epileptogenesis. From now on, there was still no effective drugs to prevent the process of epileptogenesis. Yingqiao San, a famous classic prescription over 400 years in China, was widely used in sorts of infectious disease. In preclinical study of childhood epilepsy complicated with upper respiratory tract infections, we found that YQS can not only reduce the frequency of respiratory tract infections, but also decrease the frequency and duration time of seizures, and can alleviate sterile inflammation to some extent. HMGB1, an important late mediator in sterile inflammation, has been seem as a potential target of epileptogenesis. So we hypothesized that YQS had effect of antiepileptogenic, may be related to inhibit the translocation of HMGB1 and modulate neuroinflammation.

Methods: The pilocarpine-induced status epileptic model was adopted to induce brain injury model in mice. Observation and sampling were performed at 7 days, 14 days and 28 days. The number of seizures, duration time of seizures, mortality rate and the appearance of brain were employed to determine the anti-seizure effects of YQS. The Nissl staining, FJB staining were employed to determine the neuroprotective of YQS. The double immunofluorescent staining were employed to observe inflammatory response and the location of HMGB1 in glia cells. The Western blot and immnuohistochemical staining were used to calculate the quantitative and qualitative of HMGB1 to detect the translocation of HMGB1 and the expression of HMGB1/TLR4 signaling pathway.

Results: It was found that YQS can effectively decrease the frequency of seizures, shorten duration time of seizures, reduce the mortality of mice and alleviate brain edema. YQS can significantly attenuate neuronal damage and inhibit the activation and proliferation of microglia and astrocytes with time-dependent. Moreover, YQS can modulate the subsequent inflammatory response by inhibiting HMGB1 translocation and regulating the expression of HMGB1/TLR4 signaling pathway.

Conclusions: YQS can prevent the process of epileptogensis. Its neuroprotective and anti-inflammatory effects on epileptogenesis may mediate by inhibiting HMGB1 translocation and modulating neuroinflammation. It can still be used as an adjunctive therapy for treatment of epilepsy.

Background

It was a gradual process of the initial seizure to chronic epilepsy, this progressive process was called epileptogenesis. The concept of epileptogenesis is the process by which the previous normal brain is functionally altered and biased towards the generation of the abnormal electrical activity that subserves chronic seizures [1]. In general, it was divided into three phases: the initial brain injury stage, the epileptic stage and the chronic epilepsy stage [2]. This process of spontaneous seizures can progress over several weeks or months in animal models and for years in human beings. Thus, epileptogenesis was the latent stage and window period of epilepsy, which provided broad window time for anti-epileptogenic

therapeutic interventions [3]. However, the current antiepileptic drugs (AEDs) merely provide symptomatic control of seizures, and wholly ineffective in preventing the process of epileptogenesis [4]. Therefore, it was an urgent unmet clinical need to explore some novel therapeutic agents against epileptogenesis.

Mechanisms of epileptogensis included widespread alteration in both neuronal and non-neuronal cells at several levels in the brain, including epigenetic and genetic alteration and molecular and structural changes that results in the dysfunction of neuronal circuit [5]. These dynamic changes can include neurogenesis, gliosis, axonal damage or sprouting, dendritic plasticity, blood-brain-barrier damage, inflammation and reorganization of neuronal cells and tissues [6]. Neuronal damage and subsequent inflammatory response are the basic characteristics of epileptogensis. Neuroinflammation involved the whole process of epileptogenesis. This procedure leads to increased capillary permeability, blood-brain-barrier dysfunction, brain edema, which directly caused the establishment of abnormal neural circuity and abnormal of neuronal discharges [7]. Neurons, immune cells and inflammatory cytokines played a central role in this pathophysiological procedure, especially for the inflammatory cytokines. AS the classic early inflammatory mediators, TNF-α and IL-1β had been proved the effect of pro-convulsion for a long time [8]. In recent years, High mobility group protein B1(HMGB1), a late mediator of inflammatory, had been found as a potential target on the process of epileptogensis [9]. Depending on its location of intracellular and extracellular, HMGB1 has two different functions: (1) Intracellular function: as a nuclear structure protein and DNA coupled protein, HGMB1 can unspecific combine with DNA and unwind the DNA to decorate, curve and change the structure of DNA, and take part in nucleic acid-regulated processes including DNA replication, recombination, damage repair and gene transcription[10]. (2) Extracellular function: as an important late inflammatory mediator, HMGB1 can be released passively by necrotic cells or actively secreted by innate immune cells. Thus, HMGB1 via a dedicated secretion pathway: HMGB1 relocated from the nucleus to the cytoplasm and then to secretory lysosomes or directly to the extracellular space, extracellular HMGB1 (either released or secreted) trigger inflammation and adaptive immunological responses by switching among multiple oxidation states [11]. Extracellular HMGB1 can act as a damage-associated molecular pattern (DAMP) molecular and activate innate immune response. Researchers found that the HMGB1/TL4 signaling pathway take part in the process of epileptogenesis [12]. HMGB1 acts on TLR4 and facilitated inflammatory cytokines expressing, affect the expression of NMDAR2B, further promoted releasing of glutamine and influx of Ca^{2+}, and increased seizure susceptibility at the end. Researchers had demonstrated that inhibited HMGB1-TLR4 signal transduction could reduce epileptic rats seizure severity and shorten seizure duration time [13].

Yingqiao San (YQS, another names were Yingqiao Powder, Shufeng Zhijing), a classical prescription was created by Wu Jutong, an outstanding infectious scientists of Qing Dynasty in China over 400 years ago, was widely used in sorts of infectious diseases, especially used in acute upper respiratory tract infection. Modern pharmacological studies and clinical practices have demonstrated its effects on anti-inflammatory, immune-regulation and antiviral [14.15]. In fact, infection was one of most common triggers for seizures and the recurrence of epilepsy [16]. Meanwhile, children were more susceptible to respiratory tract diseases and trend to febrile seizure. In addition, parts of febrile seizures are phenotype

of related epilepsy syndrome or epileptic encephalopathies [17]. In the preclinical study of childhood epilepsy complicated with upper respiratory tract infections, we found that YQS can not only reduce the frequency of respiratory tract infections, but also decrease the frequency and severity of seizures to some extent, and improve its long-term prognosis[18]. Furthermore, we found that it still could inhibit sterile inflammation and alleviate multi-drug resistance of refractory epilepsy [19]. Furthermore, the core Traditional Chinese Medicine (TCM) monomers of YQS, such as gastrodins [20], α-asarone [21], curcumin [22], had been proved had the effect of anti-seizure. Thus, we hypothesized that YQS had dual effects of anti-convulsion and antiepileptogenic. The dual effects may be associated with inhibiting neuroinflammation and neural protection. In order to verify above hypothesis, we designed the following experiment and try to find a potent anti-epileptogenic agent in the future.

Materials And Methods

Animals

All animal experiments were approved by the first teaching hospital of Tianjin traditional Chinese Medicine Experimental Animal Ethic Committee (Tianjin, China) and were performed under strict supervision. Young male Sprague-Dawley mice, ranging between 45 and 55 g were purchased from the Beijing Vital River Laboratory Animal Technology Co, Ltd, and housed in a temperature(23±2℃) and light (12 hours light/dark cycle)-controlled room with ad libitum access to food and water.

Drugs

YQS mainly consists of sixteen Chinese herbs (Lonicerae japonicae flos, Forsythiae fructus,Schizonepetae spica, Menthae haplocalycis herba, Arctii fructus, Platycodonis radix, Aurantii fructus, Bupleuri radix, Scutellariae radix, Pinelliae rhizome, Arisaema cum bile, Acori tatarinowii rhizome, Curcumae radix, Gastrodiae rhizome,Bombyx batryticatus,Scorpio); Levetiracetam tablets (Capland, B14202068231),o.25g/tablet; β-Actin (abcam, ab79823, dilution 5000 fold); Anti-HMGB1(abcam, ab217274, dilution 5000 fold); Anti-TLR4 (abcam, ab93610, dilution 5000 fold); Goat Anti-Rabbit IgG (ZB-2301, dilution 5000 fold); Goat Anti-MOUSE IgG (ZB-2305, dilution 5000 fold); Pilocarpine Hydrochloride (S31556-100mg, LOT:B 12J 10b77879) ; NeuN (E4M5P) Mouse-mAb (CST,94403S,dilution 100 fold); GFAP (GA5) Mouse-mAb (CST,3670S, dilution 300 fold); Mouse Monoclonal CD68/SR-D1 Antibody (NOVUS, NB600-985AF405, dilution 25 fold)

Experimental model and drug administration

All rats were randomly assigned into Control group, Model group, YQS group, LEV group (n=12 each). These groups were further divided into three time points: 7 days, 14 days and 28 days (n=4, each time point). pilocarpine status epileptic model induced as pretest dosage(intraperitoneally injected with 127mg/kg lithium chloride, and 18 hours later, intraperitoneally injected with 200mg/kg pliocarpine) to observe the frequency and duration time of seizures. When seizures reached the level of Ⅳ or over, lasted for more than 30 minutes seem as successful model. Intraperitoneal injection of diazepam (10mg/kg) to

terminate convulsions when convulsions lasting more than 1 hours. All procedures were the same for each group except the control group.

Drug intervention began 30 minutes after the end of convulsions in each group. A total of 146 g crude drug of YQS was concentrated 100 ml, total 250 mg of Levetiracetam was dissolve in 0.9% Nacl to 125 ml. After daily weight measurements, the mice were given average at 1ml/100 g once per day. The Control group and Model group was administrated 0.9% Nacl 1ml/100g by gavage once per day, the YQS group was administrated YQS 1ml/100g by gavage once per day, the LEV group was administrated LEV 1ml/100 g by gavage once per day. Each group at different points were administrated the corresponding drugs or saline interventions for 7 days, 14 days and 28 days respective.

Then brain tissue samples were initiated collection after each time points. The mice were anesthetized by diazepam. Mice were deeply anesthetized and perfused with ice-cold saline, and brain were removed from following decapitation, gently detached and removed the fascia layer and blood of brain tissues. Separated cortical and exposed hippocampus, then were dissected the hippocampus into two parts along the sagittal midline. The left part was flash-frozen in liquid nitrogen, and restored at -70 ℃,which were used for the Western blot. The right part was fixed by 4% paraformaldehyde. Then paraffin sections were prepared by dehydration, transparency, wax dipping and embedding, each brain tissues from continuous coronal sections were sliced for 15 sections with 5 *μm* thickness, and 50 μm interval between two successive sections, which were used for the Nissl staining, FJB staining, immunoflurosence staining and immunohistochemical staining.

Nissl staining and Fluoro-jade B staining

Paraffin sections were dewaxed routinely and hydrated with gradient ethanol, 1% thionine staining 1for 15 min, 95% ethanol 1to color separation, 100% ethanol 2for 1 min to hydration, 100% xylene 2for 5 min to transparent. After hydration of gradient ethanol and sealing slices with neutral gum, slices were observed under a microscope. Then Fluoro-jade B staining: Paraffin sections were dewaxed routinely and hydrated with gradient ethanol. 1% NaOH/80% ethanol 2for 5 min, 70% ethanol 1for 2 min, 0.06% potassium permanganate solution 1for 10 min, 0.0004% FJB(room temperature and avoid light) 1for 20 min, distilled water washed 3 times, baked at 50-60°Cuntil the slices were completely dry. 100% xylene 1for 2 min to transparent and sealing slices with neutral gum. The fluorescence microscope (excitation wavelength 450-490 nm) was used to observe and collect images.

Western blot analysis

Protein were extracted with RIPA lysis buffer (Beyotime Biotechnology, P0013B), and 50μg of total protein was loaded on a gel and separated by sodium dodecyl sulfate polyacrelamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes and probed with primary antibodies against HGMB1, TLR4, β-actin. Followed by incubation with appropriate Goat Anti-Rabbit IgG (ZB-2301, dilution 5000 fold) secondary antibodies. Immunoblots were visualized using the Western blot detection system. Expression levels were normalized against β-Actin (abcam, ab79823, dilution 5000 fold).

Immnunohistochemical and Immunofluorescent staining

Paraffin sections were dewaxed routinely and hydrated with gradient ethanol; Antigen repairing: the sections were immersed in citrate buffer(0.01 M, PH 6.0) and then microwaved for 15 min and cooled to room temperature; rinsed by PBS for 3 times; incubated in 3% hydrogen peroxide at room temperature for 10 min; blocked with 10% goat serum for 30 min; discarded extra serum, added primary antibodies (HMGB1 1:500; TLR4 1:100) and incubated overnight at 4℃;the secondary Goat Anti-Rabbit IgG (ZB-2301, dilution 5000 fold) was added dropwise, incubated at 37℃ for 30 min and then washed with PBS three times for 5 min each time; after washing, streptavidin-HRP were added and incubated at 37℃ for 30 min; DAB substrate kit were added to visualize positive staining and counterstaining with hematoxylin; washed with PBS and differentiated in 1% acid alcohol for 5-10 sec; then dehydrated in gradient ethanol, transparently treated with xylene, sealed with neutral gum, acquisited images with microscope.

Paraffin sections were dewaxed routinely and hydrated with gradient ethanol; Antigen repairing: the sections were immersed in citrate buffer(0.01 M, PH 6.0) and then microwaved for 15 min and cooled to room temperature; rinsed by PBS for 3 times; incubated in 3% hydrogen peroxide at room temperature for 10 min; blocked with 10% goat serum for 30 min; discarded extra serum, added primary antibodies (anti-HMGB1 1:500; mouse anti-GFAP 1:300; mouse anti-NeuN 1:100;mouse anti-CD86 1:25), incubated overnight at 4℃; the Goat Anti-MOUSE IgG (ZB-2305, dilution 5000 fold) incubated at 37℃for 45min, protected from light; nuclear staining with DAB incubated at 37℃for5min; sealed with anti-fluorescence quenching sealing tablets and observed under fluorescence microscope.

Statistical analysis

All statistical analyses were performed using SPSS.19 statistical analysis. The results were expressed by mean±standard deviation, statistical differences among the groups were assessed by one-way ANOVA, and post hoc multiple comparisons were performed using Student-Newman-Keuls tests. Values P□0.05 seem as statistically significant.

Results

1. YQS had the effect on anti-seizures that could reduce the duration time and frequency of seizures, decrease the mortality rate of epileptic mice

In order to better simulated the process of epileptogensis, we adapted the classic pilocarpine-induced status epileptics model, which manifested with recurrence seizures and characteristics with three periods: acute period (several hours to 4 days), latent period (1 week to 4 weeks) and chronic period (4 weeks to several months) (Fig. 1.a).This pathology procedure was greatly similar as epileptogensis process of human beings and was an ideal epileptic animal model. Actually, it had two mature methods to replicate this animal model. The first model is one-time model, intraperitoneally pilocarpine one time (rang from 180-350mg/kg was reported in literatures). It was easily to replicate but with high mortality rate. While, the other method was intraperitoneally pilocarpine 30mg/kg 2–3 times to induce epileptics status, over 3

times seemed as unsuccessful. This method had lower mortality rate but usually unsuccessful replicated or with minor brain injury. In this study, we adapted the first method and processed several dosages (300mg/kg、275mg/kg、250mg/kg and 200mg/kg) in pretest. Finally the 200mg/kg was chosen as the experimental dosage (Fig. 1.b) because the mortality rate of other dosages groups were too high or brain injury was too serious. Letting us to give up.

In order to observe the frequency and duration time of seizures, each group was monitored by electronic monitoring 24 hours. The results showed that the seizures of each groups were obviously in the first week, gradually decreased in the 2–3 weeks, then recurrence seizures occurred in the 21 days or latter, generally consistent with the description of related literatures. A large number of mice died on 1–4 days, gradually stabilized from 4–7 days, and almost no death after 7 days. The results showed the YQS group had lower frequency of seizures than the Model group in the first week ($n = 4$, $P < 0.05$)(Fig. 1.c). Duration times are another index for seizures. Compared with the Model group, the duration time of the YQS group was significantly shorten at each time points ($n = 4$, $P < 0.01$) (Fig. 1.d), indicating YQS was more meaningful to shorten duration time. Meanwhile, the mortality rate was improved significant, YQS 25%, LEV 41.2%, Control 40%,(Fig. 1.e). Above results showed that YQS had effects on anti-seizures. This maybe be associated with alleviating cerebral edema, preventing brain damage. So we further observed the brain appearance of each groups, and found that, compared with the YQS group, the brain appearance of the Model group manifested with pale and swollen in obvious, cerebral vessels congested and convoluted. The average of wet brain weight more heavier, the brain tissue was fragile during sampling, and this cerebral edema could continue to 4 weeks (Fig. 1.g), indicating the brain injury will persist if without any intervention. However, although the weight of mice was steady growth, but the average weight of the YQS group was lagged behind and lighter that of the Model group in temporal (Fig. 1.f), which was mismatched their morality rates. It maybe related to the compensatory growth for the body through intake more food produced surplus energy to repair the tissue injury after convulsion.

2. YQS had the effect on neuroprotective that could attenuate hippocampal neurnal damage and apoptosis

In order to observe the neuroprotective effect of YQS, we performed double staining, Nissl staining and Fluoro-jade B (FJB) staining, to observe neuronal morphology and quantity from different perspectives. Nissl staining was conducted to observe the normal neurons in hippocampus. FJB staining had high affinity for degenerating neurons and can selectively labeled death or injured neurons. Nissl staining showed that the pyramidal cell layer and granule cell layer of the Control group distributed in bands. The volume of neurons in CA1 and DG regions slightly small and arranged neatly, neurons in CA3 were large and arranged sparsely. The structure of neurons was intact, with a clear borderline, with a clear cytoplasm, uniform and clear nuclei, hyperchromatic cytoplasm with abundant Nissl bodies. With the extension of time, the volume of hippocampus gradually enlarged and extracellular space of neurons became more widen, while with high degree of contiguity and completeness. The Model group showed that hippocampus neurons injured obviously, manifested as pyramidal cells layer broke off, neurons

liquefaction and hydrolysis, residual cells varied in size, swollen, faint staining, cytoplasm disappeared and Nissl bodies were significantly reduced or even disappeared. The damage of CA1 region was more obvious than the CA3 and DG region and more notable with time extension(Fig. 2.C). Nissl bodies were used as a morphological indicator of neuronal survival. The amounts of Nissl bodies of CA1, CA3, DG showed that the YQS group was obviously superior to the other groups at each time points. The CA3 regions were more prominent（n = 4,P＜0.01）（Fig. 2.C）. The FJB staining results broadly were line with the Nissl staining. Compared with the YQS group and LEV group, a large number of fragmented or damaged neuronal with yellow fluorescence were observed in the Model groups（Fig. 2.b）. In order to observe the location between Neurons and HMGB1, we further adapted the double immunofluorescence staining to reveal the co-localization of HMGB1/NeuN in neurons. The results showed that the neurons with blue fluorescence in the YQS group had completely morphology, neatly arrangement and little damage. The HMGB1 was uniformly distributed and most of them was overlapped with cytoplasm, which indicated that HMGB1 was located in the intracellular without translocation. But neurons in the Model group were damaged seriously, without completely morphology, thin fluorescence and most of HMGB1 located in the extracellular space. This phenomenon lasted from 7 days to 28 days, indicating the neuronal damage and neuro-inflammation were persistent (Fig. 3, Additional file.Fig.S1.S2).

3. YQS had the effect on anti-inflammatory that could significantly inhibit the activation and proliferation of microglia and astrocytes

Subsequently immune response was closely related to the severity, progression and prognosis of epilepsy and run through the whole process of epileptogenesis. Astrocytes and microglia were the major innate immune cells in the brain. As the macrophage cells in the brain, microglia can quickly activated and timely engulf the injury neuronal cells at early stage of inflammation. Astrocytes, acted as structural and metabolic support cells, played an important role in maintaining integrity of brain-blood-barrier(BBB), and regulating brain homeostasis. So we adapted the double immunofluorescence staining to reveal co-localization of HMGB1/CD68, HMGB1/GFAP in microglia and astrocytes to observe the reaction of glial cells. The results showed that in the Model group, a large number of activated microglia cells with amoeboid-like change accumulated around the fragmented pyramidal cells in the CA1 and DG areas, recruited a large number of yellow HMGB1 at the extracellular space, this phenomenon lasted from day 7 to day 28. In contrast, the YQS group almost with no or few activated microglia cells, even fewer than the LEV group and equivalent to the Control group (Fig. 4, Additional file.Fig.S3.S4). These indicated YQS significantly inhibit the activation of microglia and block the early stage of inflammation. In the double immunofluorescence staining of HMGB1/GFAP, numerous of abnormal proliferated astrocytes in the Model group dishevelled distributed in the CA1 and DG areas where neurons damaged seriously, exhibited round-shaped cell bodies, hypertrophy and numerous fibrillary process. Compared with the Model group, the number of activated astrocytes in the YQS group was relatively small, large in size but regular in shape (Fig. 5, Additional file. Fig. S5.S6). The amounts of HMGB1 at each group showed increased in the beginning and then gradually decreased, either intracellular or extracellular. These phenomenon generally consistent with the results of Western blot and immunohistochemical staining, indicating that HMGB1

not only original from damaged neurons, but also from glia cells, it may be related with the passively secretion of astrocytes and the dysfunction of brain-blood-barrier.

4. YQS can inhibit the translocation of HMGB1,regulate the HMGB1/TLR4 signaling pathway to modulate neuroinflammation

Above results have demonstrated that YQS can significantly prevent neuronal damage, and inhibit subsequently immune response with time independent. Thus, we hypothesized that YQS can inhibit the translocation of HMGB1 and regulate the HMGB1/TLR4 signaling pathway to prevent the process of epileptogenesis. The Western blot results showed that the total HMGB1 of the YQS group were significantly higher than the Control group in day 7 and day 14 in respective($n = 4$, $p < 0.05$), but with no difference between the YQS group and the Model group from day 7 to day 28($n = 4$,$p > 0.05$) (Fig. 6a.b). Does that mean YQS can upregulate the expression of HMGB1? Therefore, we further adapted the immunohistochemical staining, which could better localize and detect the expression level of intracellular HMGB1. The immnuohistochemical staining results showed that HMGB1 mainly located in cytoplasmic and nuclear, represented in deep blue staining. The OD score of intracellular HMGB1 in the YQS group significantly higher than the Control group at three time pionts ($n = 4$,CA3.$P < 0.05$;CA1.$P < 0.01$) (Fig. 6c, Fig. 7, Additional file. Fig.S7). Although the comparison between each groups at CA1 region and CA3 region had slighter differences, but the general trend was the same. This results indicated than YQS can effectively prevent neuronal damaging and inhibit the translocation of HMGB1, even could promote the synthesis of intracelluar HMGB1. Compared with the Control group, the expression of TLR4 of the YQS group represented with high level at 7 days($n = 4$,$P < 0.05$), and gradually decreased at 14 days and 28 days ($n = 4$,$P < 0.05$)(Fig. 6d, Fig. 7), generally consistent with the expression of extracellular HMGB1.The high expression level of TLR4 at 7 days may be not only associated with the secretion of HMGB1, but also elevated with other inflammatory mediators.

Discussion

Epileptogenesis was the latency period and early stage of epilepsy [23]. Neural injury and secondary immune injury are the basic characteristics of epileptics brain injury and are two main directions of medication intervention, the later one is even more important [24]. From now on, there had no ideal drugs with effects of either anti-seizure or anti-epileptogenetic. Levetiracetam (LEV) was supposed to have parts of function on anti-epileptogenetic [25], thus being selected as an antagonist group in this study. YQS was a classic prescription consisting of sixteen herbal medicines. It has been used in China for more than 400 years and widely used in clinical practices, especially on respiratory tract infectious diseases. Modern pharmacology had demonstrated that it had multiple effects on anti-inflammatory, immune-regulation and antiviral [14.15]. In fact, infection was also one of most common triggers for convulsions. Children were more susceptible to respiratory tract diseases and trend to febrile seizure. In the preclinical study of children epilepsy complicated with upper respiratory tract infection, we found that YQS can not only reduced the frequency of respiratory tract infections, but also can reduce the frequency and severity

of seizures, improve long-term prognosis. Furthermore, we found that it still could reduce the seizures without infectious trigger and had effective on the adjunctive therapy for pharmacoresistant epilepsy, may be related to mediate the pathological consequences of sterile inflammation. What's more, part of monomers of the YQS, such as gastrodins, α-asarone, curcumin, had been proved had effects on anti-seizure and anti-inflammatory. So, we proposed that YQS had effect on preventing the process of epileptogensis. But the detail mechanism was no clear, maybe related to inhibit the translocation of HMGB1 and regulate the expression of HMGB1/TLR4 signaling pathway to protect neuronal damage and alleviate subsequent immune response.

PAMP(pathogen-associated molecular pattern) and DAMP(damage-associated molecular pattern) are two basic molecular patterns in immune response [26]. Compared with direct brain injury induced by infection, sterile inflammation were more common in the brain. In this study, we adapted the pilocarpine-SE model as the convulsive brain injury model, and selected three time-points with representative significant: 7 days, 14 days and 28 days, to observe the neural protection and anti-inflammation efficacy of YQS. The results showed that YQS can significant reduce the frequency and duration time of seizures, alleviate brain edema, decrease the morality of rates. These indicated that YQS had efficacy of anti-seizure. The Nissl staining, FJB staining and double immunofluorescence staining showed that neurons in the YQS group had intact structure, arranged regularly and damaged lighter with time dependent, specifically in the CA3 and DG region. It means that YQS can effectively prevent neuronal damage. In fact, most of seizures and deaths of mice occurred at the first week, but the neuronal damage continued through the whole process, indicating secondary immune injury was more important in the later stage. As the innate macrophage cell in the central nervous system, microglia were seem as the marker of the acute inflammatory response [27]. The double immunofluorescence staining showed that the number and morphology of microglia in the YQS group were significantly lower than the Model group and the LEV group, even no difference with the Control group, indicating that YQS can significantly inhibit the activation of microglia and the release of pro-inflammatory in early stage. Meanwhile, astrocytes as the key element of blood-brain-barrier (BBB), can nourish, support neurons and maintain brain homeostasis [28]. Some articles had reported that the pathological slices of epileptic brain tissue in human beings can observe amounts of proliferation astrocytes and released cytokines, epileptic discharges often were ignited beside the anomaly site due to neural injury and gliosis [29]. In addition, researches confirmed that astrocytes also were the original of HMGB1 [30]. The results of this article also supported this phenomenon. In the CA1 and DG region of the Model group, activated astrocytes replaced the necrosis neuronal cells, coexistent with a large of yellow HMGB1, extracellular more than intracellular, especially obviously on the 7 days and continued to 28 days. The number of activated astrocyte in the YQS group is relatively small and the extracellular of HMGB1 gradually decreased or even disappeared at 28 days. It means that YQS can effectively inhibited the activation and proliferation of astrocytes no matter directly or indirectly.

Extracellular HMGB1 was considered as the central component of late inflammatory response, a potentially mediator of DAMP and gradually been much account for the epilepsy research [31]. The translocation and secretion of HMGB1 were key step in the HMGB1-induced inflammation. Extracellular

HMGB1 specificity binds to TLR4 and activated the NF-kB signaling pathway, ultimately leading to neuroinflammation in the central nerves system [32]. In this article, we mainly discussed the translocation of HMGB1.The western blot results showed the total HMGB1 of the YQS groups represented high expression level at day 7 and day 14. These results been repeated twice and were still the same. Does that mean YQS can upregulate the expression of HMGB1? By searching a large number of literatures, we found that some articles also had no difference or higher expression in the total HMGB1, but had difference in the cytoplasmic HMGB1. It speculated that the total HMGB1 were easily affected by peripheral blood and passively secretion by glia cells in possible [9, 33]. Regrettably, we did not detect the cytoplasmic HMGB1 due to design negligence. Fortunately, the immunohistochemical staining remedied the design flaws. The immunohistochemical staining results showed that the intracelluar HMGB1 in the YQS group higher than the Model group in each time points. However, the results of Western blot between two groups had no significant difference. Theses means that the overexpression of HMGB1 was mainly located on the intracellular. Meanwhile, the double Immunofluorescence staining was used to observe the location of HMGB1 in the GFAP-positive astrocytes and CD68-positive microglia. These figures showed that yellow HMGB1 in the Model group scattered around the damaged neurons, with activated and proliferated microglia and astrocytes. While in the YQS group and LEV group, much yellow HMGB1 located in the cytoplasmic of microglia and astrocytes. Thus we speculated that elevated serum HMGB1 not only originated from damaged neurons, but also originated from astrocytes and microglia through autocrine and paracrine mechanisms. In addition, it also possible been affected by the dysfunction of brain-blood-barrier at early stage, the HMGB1 in peripheral blood penetrated into the central nerves system through brain-blood-barrier leakage, but the detail mechanism was not clear and need to further study. The expression of TLR4 generally consistent with the trends of HMGB1, manifested high expression level at 7 days and gradually decreased in the day 14 and day 28. Why did TLR4 with high expression level at 7 days? we believed that HMGB1 not the only mediator binding the TLR4,and TLR4 also not the only receptor in the transcriptional activation.

This study provided a beneficial exploration for epileptogensis from the perspective of neuroinflammation. Above results demonstrated that YQS had dual functions of anti-seizures and anti-inflammation, may inhibit the translocation of HMGB1 and modulate the HMGB1/TLR4 signaling pathway to prevent the process of epileptogensis. But this study still had some disadvantages or defects need to further refinement. First, single therapeutic dosage. Because the limitation of funds and restriction of experimental conditions, the YQS group was only arranged single dose group and did not set up multi-dose groups that would affect the rigor of this research. In fact, this therapy dose was generally based on the preliminary clinical practice from human beings, which had been proved effectively and safely for a long time. Second, the complex compositions of YQS restricted further researches. Indeed, the characteristic of multi-targets and multi-components of Traditional Chinese Medicine limited itself development. However, as a classic prescription, YQS had been widely used in clinical practice for more than 400 years in China, its efficacy and safety had been well proven. At the same time, the core monomers of YQS, such as asarone, baicailin and saikosaide, had been proved to be efficacy of anti-seizure and anti-inflammation in experiments. So the next step in the further was to clarify

the key components and targets of YQS by using network pharmacology, mass spectrometry, snRNA-Sequence and so on. Third, one flaw of this experiment was that Western blot only detected the total HMGB1 and neglected the cytoplasm HMGB1, but the immunohistochemical staining remedied this flaw from another perspective. Last but not least, the priority of this research was studied the translocation of HMGB1 in epileptogenisis, on account of the restriction of fund, not involve the acute and chronic stage of epilepsy, not detect the upstream and downstream signaling pathways of HMGB1/TLR4, not detect the parallel signaling pathway, such as HMGB1/RAGE, which would be the priority and direction of our next research.

Conclusions

To sum up, these results showed that YQS had effects on neuroprotective and anti-inflammatory to prevent the process of epileptogenisis, it may be mediated by inhibiting the translocation of HMGB1 and regulate the HMGB1/TLR4 signaling pathway. It can still be used as an adjunctive therapy for treatment of epilepsy.

Abbreviations

HMGB1
High-mobility group box 1
YQS
Yingqiao san, another names were Yingqiao Powder, Shufeng Zhijing
LEV
Levetiracetam
DAMPs
Damage-associated molecular patterns
PAMPs
Pathogen-associated molecular patterns
TLR4
Toll-like receptor4
SE
Status epilepticus
TCM
Traditional Chinese Medicine

Declarations

Acknowledgements

Not applicable

Author's contributions

Hanjiang Chen and Xilian Zhang wrote the manuscript, Rong Ma and Ping Rong designed and supervised this experiment, Hanjiang Chen, Ying Song and Zhaoyuan Chen performed this experiment, Yanli Lu performed the statistical analysis of this article. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets supporting the conclusions of this article are available from the corresponding author, on reasonable request.

Ethic approval and consent to participate

All animal use and care protocol conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and was approved by the first teaching hospital of Tianjin traditional Chinese Medicine Experimental Animal Ethic Committee (Tianjin, China).

Consent for publication

No applicable

Competing interests

The authors have no conflicts of interests to disclose.

Author details

1.Department of Pediatrics, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, National Clinical Research Center for Chinese Medicine Acupuncture and Moxibustion;

2.Graduate College of Tianjin University of Traditional Chinese Medicine, Tianjin, China

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Figures

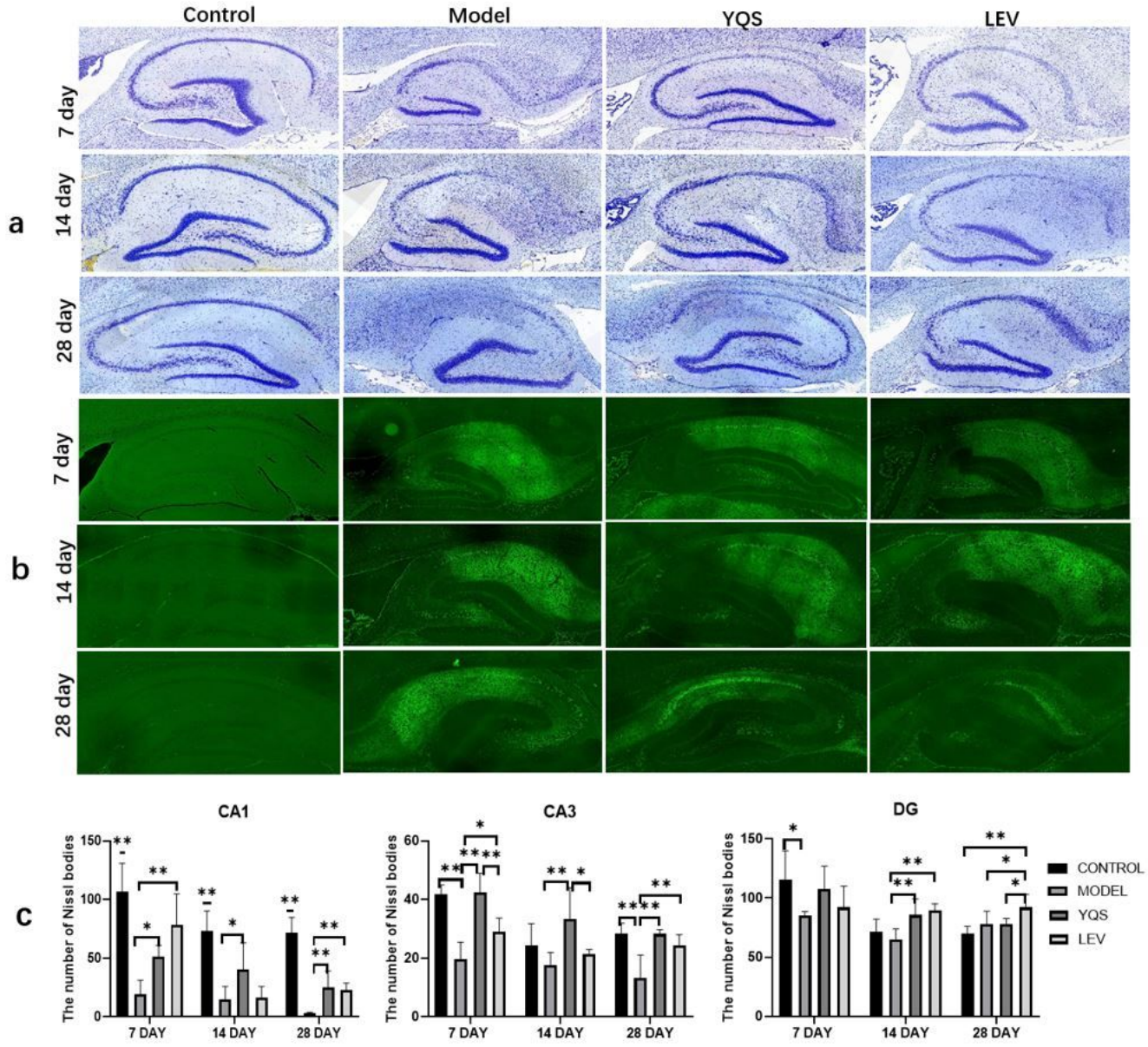

Figure 2

The Nissl staining and Fluoro-jade B (FJB) staining of pyramidal cells in hippocampus. **a**. the Nissl staining results showed that the vertebral cells in CA1 and CA3 of the Model group were loosely arranged and damaged seriously, the YQS group generally intact, regularly arranged and compacted, the difference was more evident with the extension of time. **b.** the FJB staining results were generally consistent with the Nissl results, a large number of fragmented neuronal with yellow fluorescence were observed in the Model group, the CA1 region was more serious than the CA3 and DG region. **C**. the counts of Nissl bodies showed that the CA1 region was damaged more seriously than the CA3 and DG regions, the YQS group

can significantly inhibit the injury and necrosis of neural cells than the Model group at CA1 region (n=4,P<0.05) and CA3 Region(n=4,P<0.05), the injury relatively slighter at DG region.

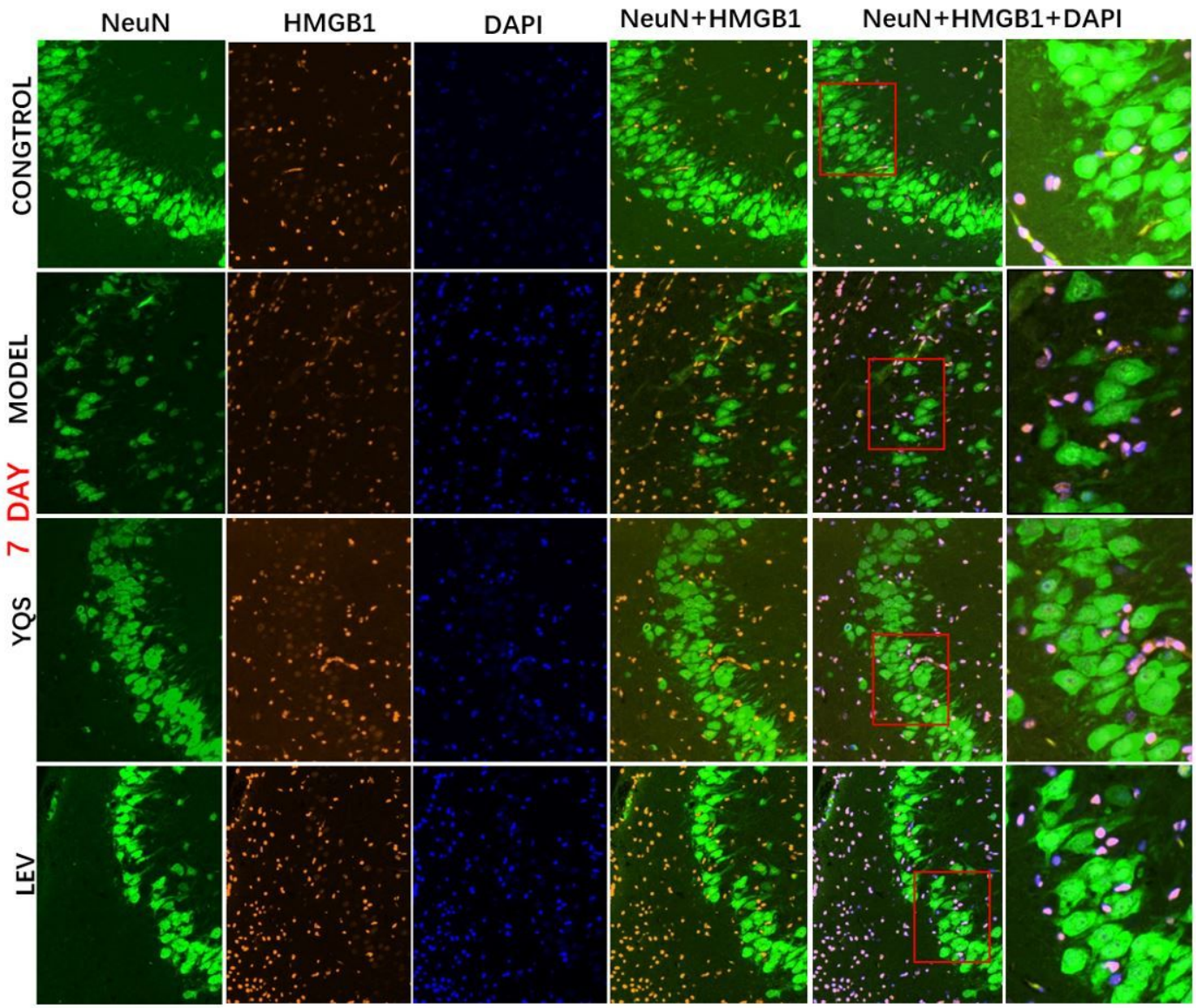

Figure 3

Double Immunofluorescence staining for HMGB1/NeuN in the CA3 region of hippocampus(7 days). The figures of 14 days and 28 days at supplementary file.Fig.S1.S2.This images(20×, 50×, magnification) were obtained by double immunofluorescence label for NeuN (green), HMGB1(yellow) and nuclear counter staining with DAPI(blue). The results showed that a large number of neurons in the Model group were loosely arranged, necrotic and dissolved, and surrounded with large amounts of extracellular HMGB1.The neural structures of the YQS group generally intact and arranged closely, most of HMGB1 located in the cytoplasm. This phenomenon was more obvious with time extension. This results indicated YQS can effectively protect neurons and inhibit the translocation of HMGB1 with time independent.

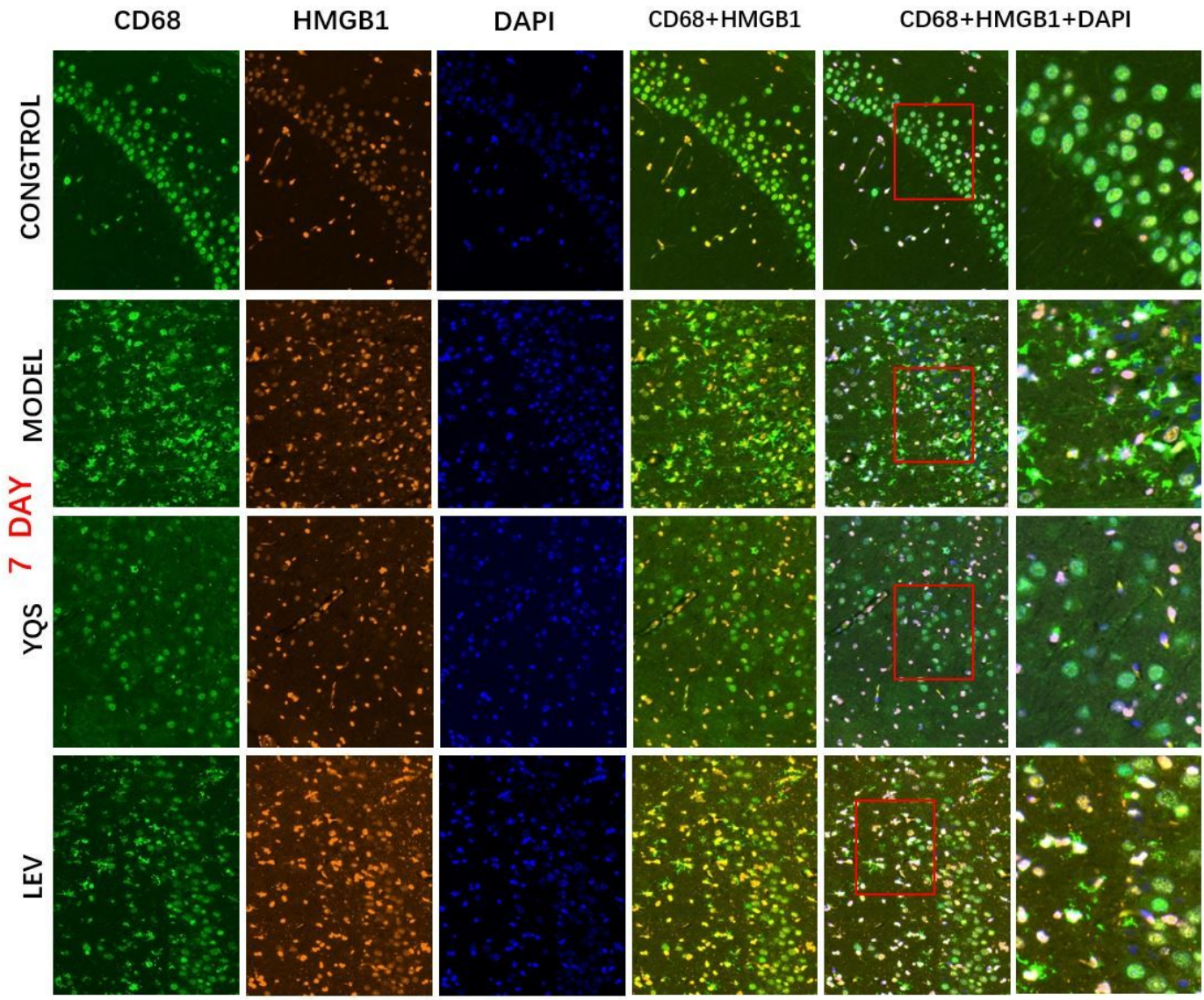

Figure 4

Double immunofluorescence staining for HMGB1/CD68 in the CA1 region of hippocampus(7 days). The figures of 14 days and 28 days at supplementary file.Fig.S3.S4. This images (20×, 50× magnification) were obtained by the double immunofluorescence label for CD68-positive cells (green), HMGB1 (yellow) and nuclear counter staining with DAPI (blue). In the Model group, a large number of activated microglia with amoeboid-like changes accumulated around necrotic neurons and recruited large amounts of HMGB1. In the YQS group, almost no or very few activated microglia cells. This phenomenon lasted for the whole process, indicating that YQS can significantly inhibit the activation of microglia and acute inflammatory response.

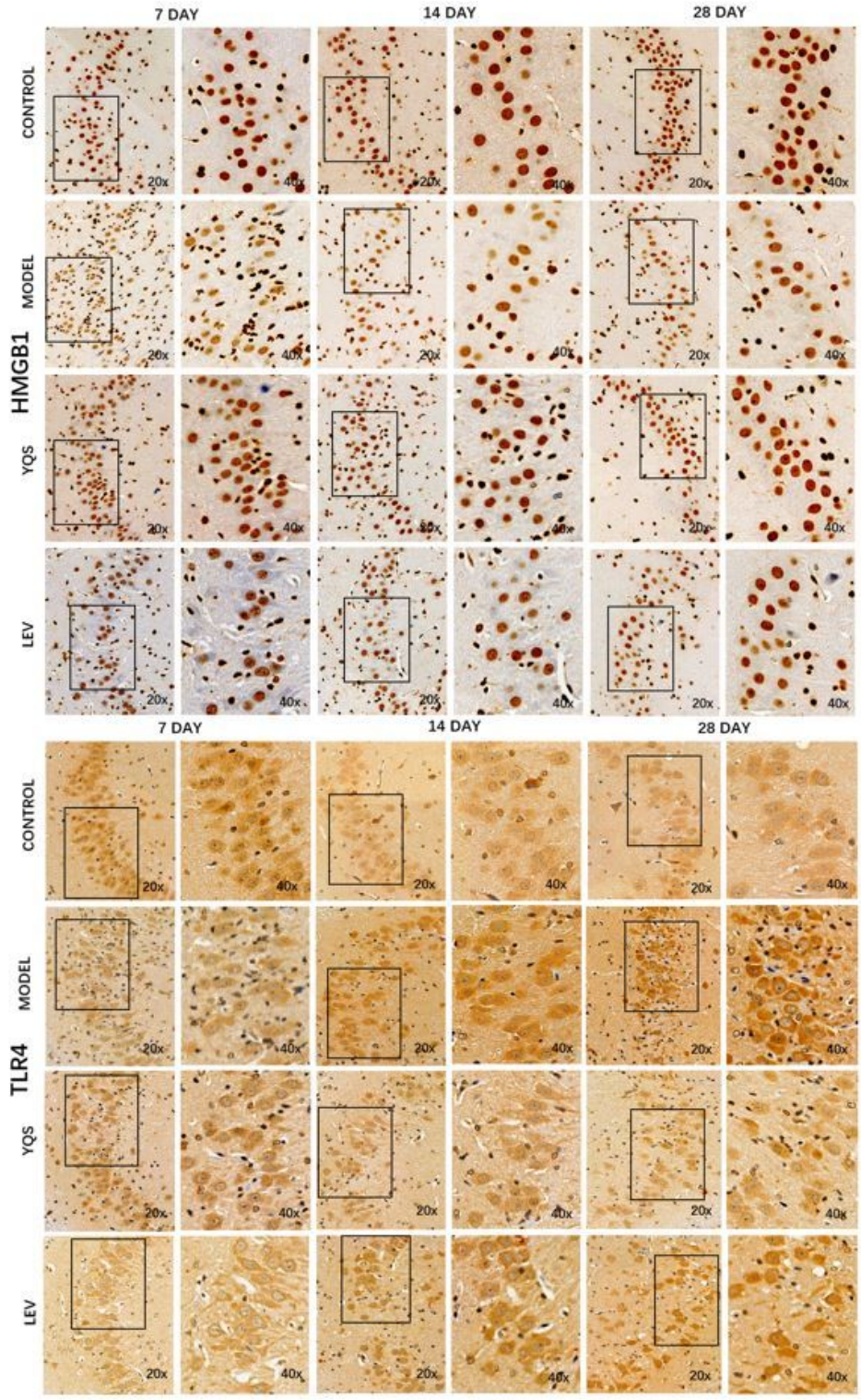

Figure 7

The Immunohistochemical staining of HMGB1 and TLR4 of CA3 region in three time points. The figure of CA1 region at supplementary file.Fig.S7. In the Control group, the Immunohistochemical staining of HMGB1 mainly located in the cytoplasmic of hippocampal pyramidal cells, with complete structure and arranged neatly; the pyramidal cells of the Model group were loosely arranged and damaged seriously, with massive and generalized necrotic cells and loss of intracellular HMGB1, which means that HMGB1

had translocated from cytoplasmic and released into extracellular. Compared to the Model group, the YQS group and LEV group damaged relatively slighter. As a membrane receptor, the expression level of TLR4 generally consistent with host cells and inflammatory stimuli, compared to the Control group and Model group, the YQS group and LEV group represented with high expression in day 7 and gradually low expression in day 14 and day 28.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS3.tif
- FigureS7.tif
- HMGB11.jpg
- actin2.jpg
- actin4.jpg
- HMGB13.jpg
- actin1.jpg
- TLR42.jpg
- TLR43.jpg
- TLR44.jpg