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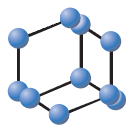
RESEARCH ARTICLE



Curcumin Reduces Neuronal Loss and Inhibits the NLRP3 Inflammasome Activation in an Epileptic Rat Model

Qianchao He[1#], Lingfei Jiang[1,2#], Shanshan Man[1], Lin Wu[1,3,4], Yueqiang Hu[1,4] and Wei Chen[1,4*]

[1]*First Cerebropathy Unit, First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning, Guangxi, P.R. China;* [2]*Department of Postgraduate School, Guangxi University of Chinese Medicine, Nanning 530200, Guangxi, P.R. China;* [3]*Scientific Laboratorial Centre, Guangxi University of Chinese Medicine, Nanning 530200, Guangxi, P.R. China;* [4]*Key Laboratory of Guangxi Basic Chinese Medicine, Nanning 530200, Guangxi, P.R. China*

ARTICLE HISTORY

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Abstract: ***Background***: Epilepsy is a chronic neurological disorder affecting an estimated 50 million people worldwide. Emerging evidences have accumulated over the past decades supporting the role of inflammation in the pathogenesis of epilepsy. Curcumin is a nature-derived active molecule demonstrating anti-inflammation efficacy. However, its effects on epilepsy and corresponding mechanisms remain elusive.

Objective: To investigate the effects of curcumin on epilepsy and its underlying mechanism.

Method: Forty Sprague Dawley rats were divided into four groups: (1) control group; (2) Kainic Acid (KA)-induced epilepsy group; (3) curcumin group; and (4) curcumin pretreatment before KA stimulation group. Morris water maze was utilized to assess the effect of curcumin on KA-induced epilepsy. The hippocampi were obtained from rats and subjected to western blot. Immunohistochemistry was conducted to investigate the underlying mechanisms.

Results: Rats received curcumin demonstrated improvement of recognition deficiency and epilepsy syndromes induced by KA. Western blot showed that KA stimulation increased the expression of IL-1β and NLRP3, which were reduced by curcumin treatment. Further investigations revealed that curcumin inhibited the activation of NLPR3/inflammasome in epilepsy and reduced neuronal loss in hippocampus.

Conclusion: Curcumin inhibits KA-induced epileptic syndromes *via* suppression of NLRP3 inflammasome activation; therefore, offers a potential therapy for epilepsy.

Keywords: Curcumin, recognition deficits, kainic acid induced epilepsy, NLRP3 inflammasomes, neuronal activity, inflammation.

1. INTRODUCTION

Epilepsy is an electrical disturbance in the brain featured with excessive or synchronous neuronal activity and enduring predisposition to produce seizures [1-3]. It affects approximately 70 million people worldwide, accounting for about 1% of the whole population [4]. Currently, there is an urgent need for the development of new drugs for patients with drug-resistant epilepsy [5]. Recently, emerging clinical and experimental evidences support the role of inflammation in the pathophysiology of epilepsy. Different inflammatory molecules and pathways have been revealed to contribute to the pathogenesis of seizure and progression in experimental epilepsy models [6-10]. Bauer *et al.,* observed consistent elevation of inflammatory cytokine IL-1β in the hippocampus of experimental epileptic animals. It is reported that the activation of IL-1R1 signaling pathway by its endogenous ligand IL-1β is pivotal in the generation of neuroinflammatory responses [11]. In the study of Vezzani *et al*., mice exposed to intra-amygdala kainate-induced epilepsy were given a combination of VX-765 (IL-1β biosynthesis inhibitor) and cyanobacterial LPS (antagonist of toll-like receptor 4) [12]. The EEG recording results demonstrated a reduction in the frequency of spontaneous seizures by 70-90% in the chronic epilepsy phase compared to the vehicle-treated kainate exposed animals [12].

The mature and functional IL-1β is processed and released from cells by inflammasomes in a caspase-1 dependent manner [13]. Inflammasomes are large intracellular multiprotein complexes that play central roles in innate immunity [14]. They response to a Pathogen-Associated Molecular Patterns (PAMPs) and damage-associated molecular patterns

*Address correspondence to this author at the First Cerebropathy Unit, First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning 530023, Guangxi, P.R. China; Tel: +86-771-584-8605; Fax: +86-771-586-2294; E-mail: chenweigxcd@163.com

#These authors contritruted equally to this work.

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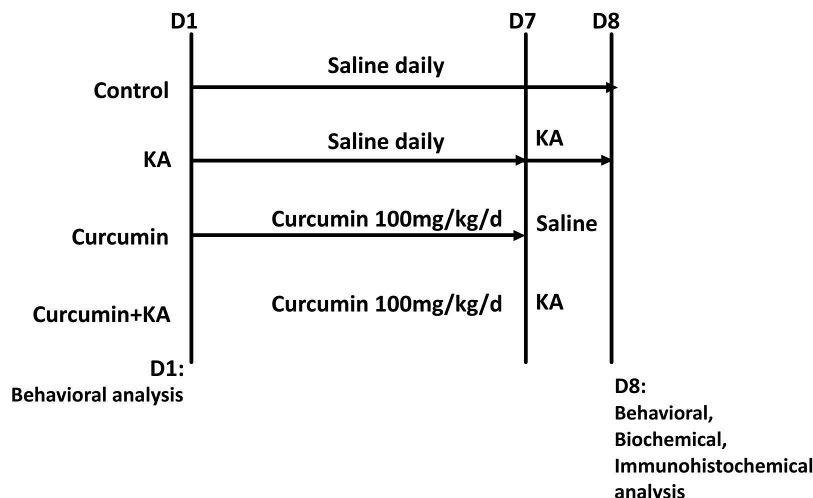

Fig. (1). Schematic of treatment paradigm.

(DAMPs) [15]. Nowadays, several inflammasomes have been reported, including NLRP1, NLRP2, NLRP3, NLRC4 and double-stranded DNA sensors Absent In Melanoma 2 (AIM2) [16, 17]. Amongst them, the best characterized molecule is the NLRP3 inflammasome. Once PAMPs or DAMPs are recognized by TLRs (toll-like receptors), NF-κB signaling is activated, leading to the upregulated transcription of inflammasome-related components, such as NLRP3, pro-IL-1β and pro-IL-18. The inflammasome-adaptor protein ASC is recruited to NLRP3, and interacts with caspase-1, leading to its activation [18, 19]. Activated caspase-1 catalyzes the maturation of pro-inflammatory cytokines IL-1β and IL-18.

Curcumin is the active ingredient of turmeric and widely used as both spice throughout Asia and western world [20]. Curcumin has a long history of medicinal applications, including anti-inflammation, anti-tumor and other conditions. In the past decades, the mechanism for its anti-inflammatory effects has been extensively studied and numerous molecular targets have been identified. The anti-inflammatory targets of curcumin including NF-κB, COX2 and pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α [21-23]. It is reported to be used in rheumatoid arthritis, psoriasis, post-operative inflammation, chronic anterior uveitis and orbital inflammatory pseudo-tumours [20]. Kong *et. al* demonstrated that curcumin could dramatically reduce NLRP3 inflammasome activation through suppression of NF-κB and P2X purinoceptor 7 (P2X7R) signaling in PMA-induced macrophages [24]. Liu and his colleagues investigated curcumin in spinal astrocytes and observed reduced NALP1 inflammasome aggregation and STAT3 signaling after curcumin treatment [25]. These findings suggest that inflammasome might be another target of curcumin. Moreover, Bertoncello *et al.*, reported that curcumin has anticonvalsant activity and can prevent cognitive deficiency in kindled animals [26]. However, the efficacy of curcumin on epilepsy remains elusive. In the present study, we investigated the effect of curcumin in Kainic Acid (KA)-induced experimental epilepsy model and revealed the underlying mechanisms. Our study demonstrated that curcumin improved recognition deficits in KA induced epilepsy rats by suppressing NLRP3 inflammasomes.

2. MATERIALS AND METHODS

2.1. Animals

A total of 40 adult Sprague Dawley rats were used for this study. All animals were housed in a pathogen-free environment with a 12-hrs light/dark cycle with a temperature of 22±1˚C and a humidity of 40-60%, fed with a standard laboratory chow and water ad libitum for the duration of the experiment. All animal care and experimental procedures were approved by the Animal Care Ethics and Use Committee of First Affiliated Hospital of Guangxi University of Chinese Medicine and performed in accordance with the guidelines of this Committee. Animals were randomly segregated into the following four groups with minimum eight animals in each group.

2.2. Kainic Acid-induced Epilepsy Model and Animal Treatment

Rats were randomly divided into four groups, namely (A). control, (B). Kainic Acid (KA) group, (C). curcumin group and (D). KA+curcumin group, with minimum 8 animals in each group. Treatment was illustrated in Fig. (**1**). KA monohydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterile saline to make 10mg/mL working solution. In (B). KA group, rats were injected with an initial dose of 5mg/kg KA intraperitoneally (i.p.) that was repeated hourly until Status Epilepticus (SE) was observed. The behavioral seizure stages were rated according to the modified Racine's scale [27]. Stage I, facial clonus; stage II, nodding and wet dog shaking; stage III, unilateral forelimb clonus with lordotic posture; stage IV, lateral forelimb clonus with rearing; and stage V, bilateral forelimb clonus with rearing, jumping, and falling. Only mice exposing the entire behavioral changes (stage 3–5) were included in the study. If that did not occur, rats were received a subsequent half dose of 2.5mg/kg until they developed stage 5 seizures or exhibited convulsive (stage 3-5) seizures for over 3h. Rats in (A). the control group were administrated with a same

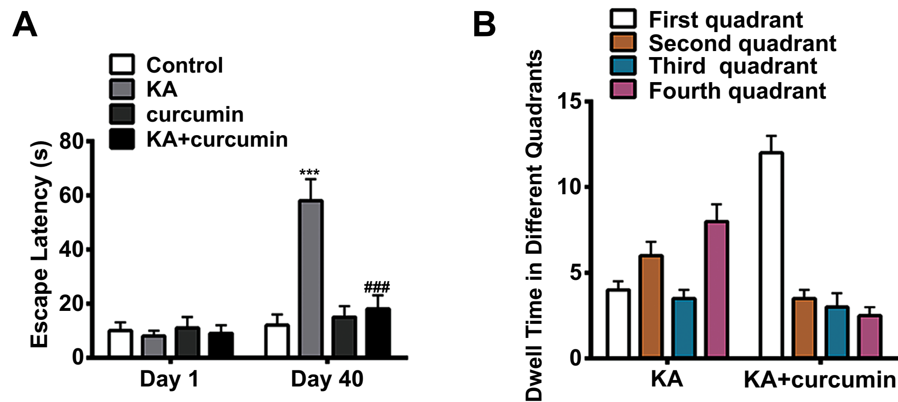

Fig. (2). Effect of curcumin treatment on cognitive functions. (**A**), Escape latency assessed using Morris water maze test in KA treated animals. (**B**), Dwell time in different quadrant. Values are expressed as mean ± SEM; *Significantly different from control group ($p< 0.05$), #Significantly different from KA treated group ($p< 0.05$).

volume of sterile saline. Animals in (C). curcumin group received a daily oral gavage of curcumin (suspended in 1% carboxymethylcellulose, a hydrophilic polymer for improving the solubility and stability of curcumin) at a dose of 100mg/kg for 7 days, then animals were injected with a similar volume of sterile saline. In (D). KA+curcumin group, animals were received daily oral administration of curcumin with the dose of 100mg/kg, starting 1 week before the KA administration.

2.3. Morris Water Maze

Animals were applied to Morris water maze test for the evaluation of spatial learning and memory. The water maze, a pool with a diameter of 140 cm and a height of 60 cm, was divided into four quadrants of equal area. The plexiglass platform was placed in the middle of one quadrant (target quadrant), 1 cm below the water surface. Several distal visual cues, serving as the navigational references for animals to locate the platform, were marked around the maze apparatus. Each animal performed 4 trials per day for 5 days. In a trial an animal was placed individually into the tank facing the inner wall. Animals were allowed to swim freely for 90s until they reached the platform where they were allowed to rest for 15s. If the animal had not reached the platform in the allotted time its latency was noted as 90s and it was gently guided to the platform and allowed to remain for 15s. Moving path and latency to reach platform was recorded and quantified using automated behavioral software (Topscan, Clever Sys, Inc., Reston, VA) over trials and days as an indicator of spatial learning.

2.4. Western Blot

Cells were lysed in RIPA solution (Sigma-Aldrich, St. Louis, MO, USA). Samples were run on SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% milk at room temperature and incubated at 4°C overnight with NLRP3, IL-1β, GAPDH (Cell Signaling Technology, Boston, MA, USA) (1:1000 dilution in TBST). Blots were then incubated with peroxidase-conjugated secondary antibodies and visualized with ECL procedure (Bio-Rad, USA). The results were analyzed using Quantity One (Bio-Rad) software.

2.5. Immunohistochemistry

Immunohistochemistry was performed for the analysis of astrocyte and microglial activation. Animals from each group were anesthetized with ether and perfused with 0.1 M phosphate buffered saline (PBS, pH 7.4) followed by 4% (w/v) paraformaldehyde in 0.1M PBS (pH 7.4). The brains were removed, fixed and immersed in 30% (w/v) sucrose for cryoprotection. Then 30mm thick frozen sections of tissues were prepared. The sections were washed with PBS for three times and later treated with 1% (v/v) Triton X-100 in PBS for 30 min. After washing with PBS, slides were blocked with 1% H_2O_2 (w/v) for 20 min at Room Temperature (RT), rinsed with PBS, blocked with normal goat serum in a humid chamber for 1 h. After rinsing off serum, slides were incubated with primary antibodies for 1 h at RT. Primary antibodies were as following: rabbit polyclonal antibody of anti-Iba-1 (1:800, Millipore), rabbit polyclonal antibody of anti-GFAP (1:2000, Cell signaling technology). The sections were then rinsed and incubated with anti-rabbit biotin labelled secondary antibodies (1:100, Millipore) for 1 h at RT. Rinsed for three times, sections were then incubated with streptavidin biotinylated horseradish peroxidase complex (1:200 Millipore) for 30 min at RT. Slides were washed once with water and counterstained with hematoxylin . Then sections were mounted and were visualized and photographed using a light microscope (Leica-DFC 295, Wetzlar, Germany).

2.6. Statistical Analysis

Data are showed as mean ± Standard Error Mean (SEM). The statistical significance between two groups was analyzed by Student's t-test using Graphpad Prism software. Ap value< 0.05 was considered as statistically significant.

3. RESULTS

3.1. Curcumin Attenuated Cognition Deficits in KA Induced Epilepsy Model

Before treatment, the spatial learning and memory of animals in four groups were evaluated using Morris water maze task. No significant difference in latency time was observed amongst four groups (Fig. **2A**). To evaluate the effect of curcumin on KA-induced epilepsy, rats were given cur-

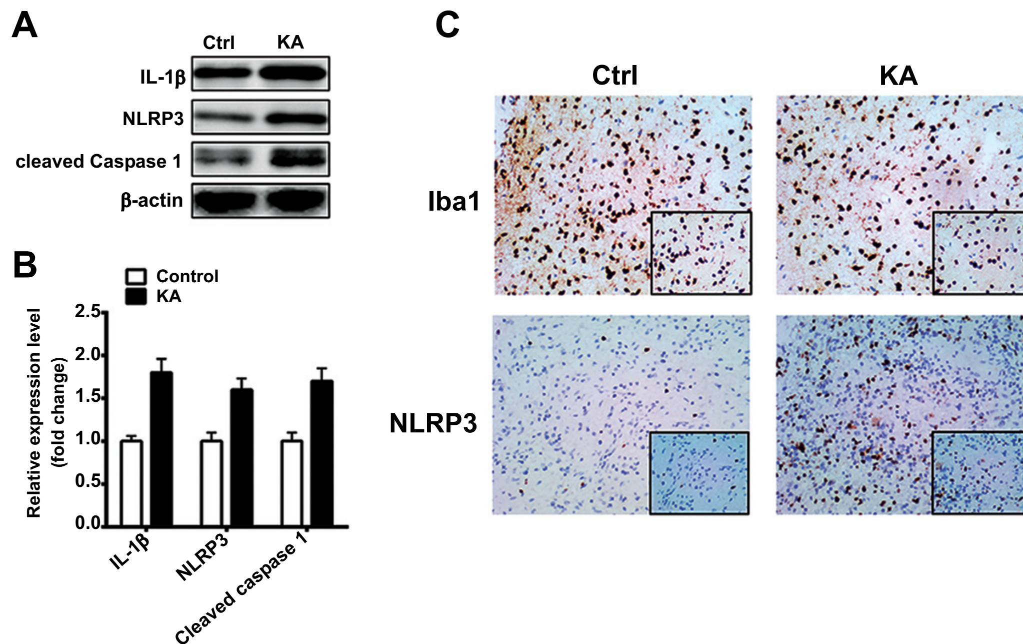

Fig. (3). Inflammasome was activated in KA induced epilepsy rats. (**A**), Representative Western blot analysis of IL-1β, NLRP3 and the cleavage of caspase-1 and protein expression in hippocampi from control group or animals after and KA induced epilepsy. (**B**), Quantification of the level of NLRP3 and cleavage of caspase-1 and IL-1β. (**C**), NLRP3 and Iba1 expression in hippocampus was detected with immunochemistry.The results represent the mean ± SEM for three experiments. *P*< 0.05 *vs*. control group.

cumin orally 7 days before KA induction. At the end of treatment, animals were subjected to another water maze task. Rats with KA-induced epilepsy spent much more time in escape, which was significantly reduced by curcumin pretreatment. As visual confirmation of the difference in two groups, swimming paths were recorded and analyzed (Fig. **2B**). Examination of the total path length indicated a significant reduction in curcumin+KA group compared to KA group (data not shown). Further analysis demonstrated that rats received curcumin prior to KA stimulation spent most of the exploration time in the dedicated quantum where target platform placed. Meanwhile, rats in KA group spent exploration time in four quantum without difference (Fig. **2B**). These results suggested that rat pre-treated with curcumin could ameliorate KA-induced deficient in spatial learning and memory.

3.2. Inflammasome was Activated in KA Induced Epilepsy Rats

Rats received KA administration and exhibiting convulsive seizures were sacrificed after behavioral test. The whole protein was extracted from hippocampus tissue and subjected to western blot. We observed that IL-1β, NLRP3 and active caspase-1 in KA group were up-regulated significantly (Fig. **3A** and **B**). Microglial activation was reported to play an active role in the induction of neuroinflammation [28-31]. Compared to the control group, KA administration resulted in a dramatic increase in the cells stained positive to ionized calcium-binding adapter molecule 1 (Iba-1), a biomarker for active microglia (Fig. **3C**). Immunohistochemical data further demonstrated that Iba-1^+ NLRP3^+ cell count increased compared to control. Taken together, these data indicatedan increased activity of inflammasome in hippocampus of KA-induced epilepsy rats.

3.3. Curcumin Attenuated Inflammation and Neuronal Death *via* Modulation of NLPR3/Inflammasome Activation in Epilepsy

Next, we investigated the effect of curcumin on inflammasome in epilepsy rats. Western blot analysis revealed that curcumin suppressed protein expression of IL-1β, NLRP3 and cleaved caspase-1 in hippocampus significantly compared to KA group (Fig. **4A** and **B**). Hematoxylin/eosin staining demonstrated that KA injection result in neuronal injury with acidophilic cytoplasm and pyknotic, shrunken nuclei in the hippocampus (Fig. **4C**). Compared with control group, dramatic neuronal loss was observed after KA treatment, with resultant narrowing and sparse staining of CA1 region and a breach of staining continuity in the CA3 region. In contract, we did not detect obvious neuronal loss in rats received curcumin treatment before KA injection. Only one of the eight rats in KA+curcumin group displayed evidence of mild neuronal injury of hippocampus, which was restricted to the CA3b subfield. Together, these results indicated that intervention of curcumin suppressed KA-induced neuronal death by inhibition.

4. DISCUSSION

In the present study, we observed a significant neuronal loss with elevated IL-1β production in KA induced experimental epilepsy in rats. After binding with IL-R1, the receptor mediating the biological responses to the IL-1β, the downstream NF-κB signaling is activated. The transcription factor, NF-κB, regulates the expression of genes involved in neurogenesis, cell death and survival. In addition to NF-κB activation, the binding of IL-1β and its receptor also leads to the rapid activation of pathways involving Src and Mitogen-Activated Protein Kinase (MAPK), pathways that directly

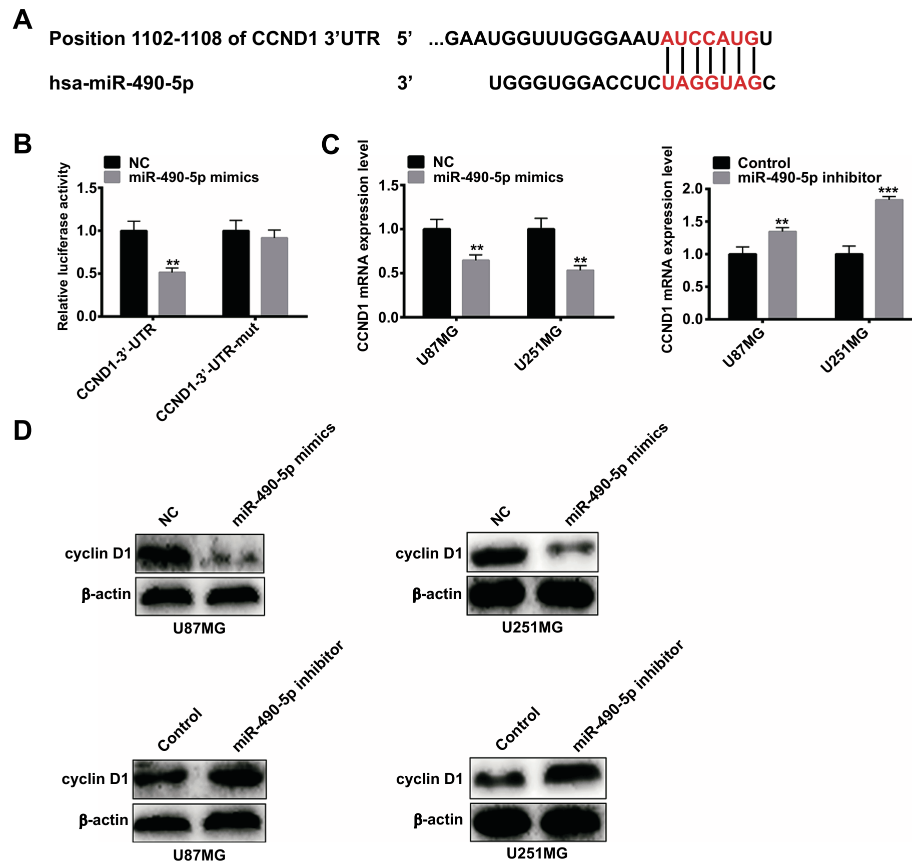

Fig. (4). Curcumin attenuated inflammation and neuronal death *via* modulating the activation of NLPR3/inflammasome in epilepsy. (**A**), Representative Western blot analysis of IL-1β, NLRP3 and the cleavage of caspase-1 and protein expression in hippocampi from rats with/without curcumin treatment after KA induced epilepsy. (**B**), Quantification of the level of NLRP3 and cleavage of caspase-1 and IL-1β. (**C**), Neurons number in hippocampus was detected with H&E staining. The results represent the mean ± SEM for three experiments. P < 0.05 *vs*. KA group.

affecting neuronal excitability and seizure threshold *via* phosphorylating voltage-dependent and receptor-coupled ion channels [32, 33]. Viviani *et al.,* reported that IL-1β increases NMDA receptor function through NMDA receptor 2B subunit phosphorylation by Src, resulting in enhanced neuronal calcium influx and hyperexcitability [34]. As hippocampus is the second-richest area of IL-R1 in brain (next to hypothalamus), IL-1β mediated neuronal excitoxicity might markedly influence the neuron viability and seizure threshold in this area [35].

In addition to neurons, inflammation could contribute to epilepsy by affecting some other cells in brain, including astrocytes. Previous investigations revealed that IL-1R1 was rapidly increased not only in neurons post-seizures, but also later in astrocytes, indicating the role of IL-1β as a mediator of glioneuronal communications in epileptogenic tissues [36]. Both *in vitro* and *in vivo* studies suggested that astrocytes respond to IL-1β with elevated expression of HMGB1, a ubiquitous chromatin component that can aggravate the inflammatory response and tissue damage [37, 38]. Interestingly, the expression of P2X receptor has been reported in cultured astrocytes as well. In human fetal astrocytes, inhibition of P2Y receptor affects IL-1β signaling, resulting in the change of chemokine synthesis in astrocytes [39, 40]. Moreover, IL-1β could modulate the expression of Adenosine Kinase (ADK), providing a potential modulatory crosstalk between the astrocyte-based adenosine cycle and inflammation. Next step, we need further investigation on the effect of curcumin in other neural cells, including astrocytes, so as to better understand its mechanisms in epilepsy treatment [40].

In the past decades, inflammasome is reported to be involved in non-inflammation physiological processes. Zhou *et al.*, observed an increase in Reactive Oxygen Species (ROS) concentration following cellular stress leads to dissociation of thioredoxin-interacting protein (TXNIP) from oxidized thioredoxin-1 (Trx-1), subsequent association of TXNIP with NLRP3 and NLRP3 activation. TXNIP knockout or knockdown impairs caspase-1 activation, yet not completely, thus indicating that other regulators of the inflammasome activity or other pathways might function together with ROS production to initiate the inflammatory response [41]. Seizure-like activity stimulates voltage-gated and NMDA-depen-dent ion channels and elevate intracellular calcium lead to ROS production [41]. It could partially explain the activation of inflammasome induced by KA. However, the ROS level was not examined in the present study. For the next plan, we will determine whether ROS is upregulated in KA-induced epilepsy and employ antioxidants to further validate the role of ROS ininflammasome activation, exploring potential actives for epilepsy therapy.

The limitation of this study is that only KA induced epilepsy mice model was used here. Although KA is one of the first compounds employed to mimic temporal lobe epilepsy, it causes hippocampus-restricted injury [42]. Since that extrahippocampal injuries are also significant in temporal lobe epilepsy. Other epilepsy models should also be used in the future study, such as pilocapine model and kindling model [26].

CONCLUSION

To conclude, curcumin is a potent active natural component that ameliorates the cognition deficiency in KA induced experimental epilepsy rats through modulation ofNLRP3 inflammasome activation. Our study suggested that curcumin might be a potential candidate for clinical application of epilepsy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Animal Care Ethics and Use Committee of First Affiliated Hospital of Guangxi University of Chinese Medicine and performed in accordance with the guidelines of this Committee (Approval No. 2017-0143).

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are base of this research. All the animals used were in accordance with the US National Research Council's "Guide for the Care and Use of Laboratory Animals.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

FUNDING

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