Levetiracetam Reduces Early Inflammatory Response After Experimental Intracerebral Hemorrhage by Regulating the Janus Kinase 2 (JAK2)–Signal Transducer and Activator of Transcription 3 (STAT3) Signaling Pathway

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Background: Levetiracetam (LEV) is an antiepileptic drug that promotes recovery of neurological function by alleviating inflammatory reactions. However, it is not known whether it can improve secondary brain injury after intracerebral hemorrhage (ICH). The aim of this study was to determine whether LEV can reduce early inflammatory response after ICH in rats.

Material/Methods: An *in vitro* model of early inflammation was created by treating microglia cells with lipopolysaccharide (LPS). After exposure to various concentrations of LEV, the expression levels of NF-κB and STAT3 and inflammatory factors such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α in microglia were detected. *In vivo*, autologous blood was used to induce the rat ICH model. The effects of LEV on post-cerebral hemorrhagic inflammatory response were examined using neurobehavioral tests, FJC staining, brain water content testing, and analysis of protein expression levels of NF-κB, JAK2, STAT3, and inflammatory factors.

Results: LEV treatment significantly reduced the expression of inflammatory factors and protein expression levels of NF-κB and STAT3 in LPS-treated microglia cells (P<0.05). In male Sprague-Dawley (SD) rats, LEV treatment markedly decreased the volume of hematoma and the number of degenerative neurons (P<0.05). It also improved the neurological function and relieved brain edema. The protein expression levels of NF-κB, JAK2, and STAT3 were significantly lower in the ICH+LEV group than in the control group (P<0.05).

Conclusions: Our study suggests that treatment with LEV alleviates early inflammatory responses induced by ICH. Mechanistically, LEV inhibited the JAK2-STAT3 signaling pathway and reduced neuronal injury around the hematoma, and ameliorated brain edema, all of which promoted recovery of nerve function after hemorrhage.

MeSH Keywords: Cerebrovascular Disorders • Neurogenic Inflammation • Neuroprotective Agents • Signal Transduction

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Background

Intracerebral hemorrhage (ICH) refers to non-traumatic hemorrhage in the brain parenchyma. ICH is associated with high disability and mortality rates [1,2]. Currently, there is no effective treatment available, and patients are mainly given supportive therapy [3]. The incidence of ICH is likely to increase greatly with population aging. The prognosis of ICH is poor and there are no effective prevention and treatment strategies [4].

Numerous studies have recently shown that the inflammatory response plays an important role in secondary injury of ICH [5]. ICH is associated with activation and release of inflammatory cells such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. This results in death of nerve cells, tissue damage, destruction of the blood-brain barrier, and entry of peripheral immune cells such as macrophages and T cells into the central nervous system (CNS), which aggravates brain tissue damage [6].

Microglia are considered to be the earliest non-neuronal cells to respond to acute CNS damage [7]. The roles of activated microglia are twofold: pro-inflammatory (M1) and anti-inflammatory (M2) [8]. In the early stage of cerebral hemorrhage, M1-type microglia are involved, causing inflammatory reactions by secreting and activating pro-inflammatory factors such as TNF-α, IL-1β, superoxide, and various chemokines. The middle and late stage of ICH mainly involves M2-type microglia, which inhibit inflammatory reaction by secreting anti-inflammatory cytokines, promoting cell regeneration, tissue repair phagocytosis, and hematoma clearance [9,10]. Ultimately, this inhibits the release of pro-inflammatory cytokines from microglia, which is an important factor in alleviating neuro-inflammatory response after cerebral hemorrhage. This has a protective role of reducing secondary damage after cerebral hemorrhage.

The nuclear factor kappa-light-chain-enhancer of the activated B cells (NF-κB) signaling pathway contributes to the development of inflammatory reactions following ICH. NF-κB is a classical transcription factor abundant in various cells and can be activated by lipopolysaccharide (LPS). Also, NF-κB regulates the expression of M1-like pro-inflammatory phenotype in macrophages and microglia. Higher expression of pro-inflammatory cytokines, and cytokines such as IL-1β and TNF-α promotes activation of the NF-κB signaling pathway [11]. In addition, NF-κB is activated soon after ICH and is maintained in a high concentration for several days to weeks [12]. The STAT transcription factor family (STAT1-STAT6) is also activated by LPS, and the activated Janus kinase (JAK)-STAT signaling pathway plays a significant role in regulating cell proliferation, apoptosis, and immunity response, such as inflammatory response [13]. It has been reported that STAT3 regulates M1-like microglia by triggering the release of pro-inflammatory factors, which polarizes microglia to M1 phenotype [14]. In the ICH rat model, phosphorylated STAT3 is mainly expressed in microglia and macrophages. Inhibition of STAT3 decreases the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2, thus alleviating cell injury after hemorrhage [15].

Levetiracetam (LEV) is an acetyl pyrrolidine compound approved in 1999 for the treatment of adult seizures [16]. LEV is well tolerated by many patients. Administration of prophylactic antiepileptic drugs is one of the common treatments for cerebral hemorrhage. In a study of ICH patients, prophylactic use of LEV improved the prognosis. Moreover, LEV is more effective than phenytoin in preventing seizures, without affecting cognitive ability [17]. Also, LEV reduces production of inflammatory mediators by normalizing the resting membrane potential of astrocytes [18]. Recently, increasing attention has been paid to its anti-inflammatory functions. However, whether LEV has a protective effect on early ICH has not been reported. In this study, we explored the mechanisms of LEV in early inflammatory response following ICH.

Material and Methods

Animal and cells

Male adult Sprague-Dawley (SD) rats (n=110) weighing 250–280 g were obtained from the Experimental Animal Center of Qingdao University. The rats had free access to food and water in a controlled environment with a 12/12-h light/dark cycle. All animal experiments were approved by the Medical Ethics Committee of the Affiliated Hospital of Qingdao University and were conducted in accordance with its principles. BV2 cells were kindly provided by the Neurophysiological Laboratory of Qingdao Medical College.

Materials

LEV was purchased from Invitrogen (CA, USA) and LPS and Fluoro-Jade-C (FJC) dye were obtained from Sigma-Aldrich (St. Louis, MO, USA). MTT Cell Proliferation and Cytotoxicity Assay kits were purchased from Beyotime (Shanghai, China). Reverse Transcriptase kits and SYBR Fast qPCR mix were ordered from Takara (Kyoto, Japan). Mouse -IL-1β and Mouse-TNF-α qPCR primer were designed by Tsingke (Beijing, China). Rabbit polyclonal antibody, including NF-κB, STAT3, and JAK2, were purchased from Cell Signaling Technology (Beverly, MA, USA).

ICH model establishment and LEV administration

BV2 cells (immortalized microglia that are very similar to primary microglia in morphology, phenotype, and certain functions [19]) with good logarithmic growth phase were seeded

in 6-well plates at a density of 5×10^5 cells/well. The cells were cultured in a cell culture incubator for 24 h before discarding the old medium. LPS was then prepared in a serum-free cell culture medium at a concentration of 1 µg/ml [20] and we added 2 ml per well to the 6-well plates. The LPS-treated cell culture was incubated for 2 h. We administered 5 LEV dosages in PBS and the control, forming 6 groups: 0 µg/ml, 10 µg/ml, 30 µg/ml, 50 µg/ml, 100 µg/ml, and negative control, which were added to serum-free medium at a concentration of 1 µg/ml. Thereafter, RNA and protein were extracted after 24 h of incubation.

Autologous blood was used to induce the ICH rat model, as previously described [21]. First, the brain basal ganglia (taking the anterior fontanelle as the origin, right 0.2 mm, front 3.5 mm, and depth 5.5 mm) was located using a brain stereotaxic instrument, before drawing 50 µl of fresh blood from the rat tail artery using a micro-syringe. The syringe was vertically placed on the micro-injection pump and the fresh blood slowly injected into the cranial cavity at a speed of 1 mm/min for 5 min. Thereafter, the needle was placed in the rat's skull for 30 min to prevent blood reflux and to ensure formation of a hematoma in the brain. The needle was then slowly removed at the same speed. The bone hole was closed with bone wax, and the incision was disinfected and sutured. LEV can traverse the blood-brain barrier, so it was administered intra-peritoneally after it was dissolved in deionized water. The rats were randomly divided into 4 groups: a sham operation group, an ICH group, ICH+LEV (50 mg/kg) group, and a vehicle group (ICH+Veh). The doses of LEV were chosen based on typical doses in the published literature [22]. All the experimental rats were euthanized after 24 h to obtain brain samples for subsequent experiments.

Western blotting (WB)

Proteins extracted from brain tissues and cells were quantified using bicinchoninic acid (BCA) assay. The proteins were mixed with protein-loading buffer and heated. The protein samples were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% skimmed milk for 1.5 h, and incubated with primary antibody overnight at 4°C. The primary antibody dilution ratios were 1: 1000 for NF-κB, STAT3, and JAK2, while β-actin was diluted at a ratio of 1: 2000. The secondary antibody was incubated for 1.5 h at room temperature. Western blot data were quantified using Image J software.

Real-time quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated from the cell lines using TRIzol reagent. A reverse transcriptase kit was used to reverse transcribe the RNA into cDNA according to the manufacturer's instructions. A 1-µL aliquot of cDNA was amplified in a PCR reaction mixture containing SYBR Fast qPCR mix and 3.2 pmol of each specific primer for the target genes or reference gene (GAPDH). The relative mRNA expression was calculated using the comparative ΔΔCt method.

Neurobehavioral tests

A modified neurological severity score (mNSS) was used to evaluate neurological function of the rats at 24 h and 72 h after ICH. The test consisted of 5 parts: tail, walking, sensory, and balance beam tests, as well as loss of reflexes and abnormal movement. The range of scores was 0–18, with the normal value being 0 point and the highest value being 18 points. The higher the score, the more serious the neurological damage [23,24].

Brain water content

The rats were euthanized at 24 h or 72 h after ICH, and all brain tissues were quickly weighed as wet weight after removing the olfactory bulb, cerebellum, and brainstem. The dry weight was obtained after drying the brain tissues in an oven at 100°C for 24 h. Brain water content was calculated as (wet weight–dry weight)/wet weight×100%.

FJC staining

Fluoro-Jade (FJC) staining was performed on frozen sections of the brain tissues fixed in 4% paraformaldehyde. The brain sections of the rats were immersed in 80% ethanol containing 1% NaOH for 5 min. The samples were then immersed in 70% ethanol for 2 min, followed by 2 min in double-distilled water. Then, the brain sections were placed in 0.06% potassium permanganate for 10 min before being digested with distilled water for 2 min. The samples were then put into 0.0001% FJC solution (made from 0.1% acetic acid). Next, they were dipped in distilled water and dried in an oven at 50°C. The slices were then treated with dibutyl phthalate polystyrene xylene (DPX) sealing liquid, and the FJC-positive cells were counted under a fluorescence microscope.

Immunofluorescence (IF)

Paraformaldehyde (PFA) at 4% was perfused with brain sections using 10%, 20%, and 30% sucrose gradient, then frozen sections were made. The brain sections were first incubated with STAT3 and JAK2 rabbit antibody (1: 200) at 4°C overnight, followed by incubation with the secondary antibody (goat anti-rabbit) for 1.5 h at room temperature. The samples were then developed under a fluorescence microscope and imaged.
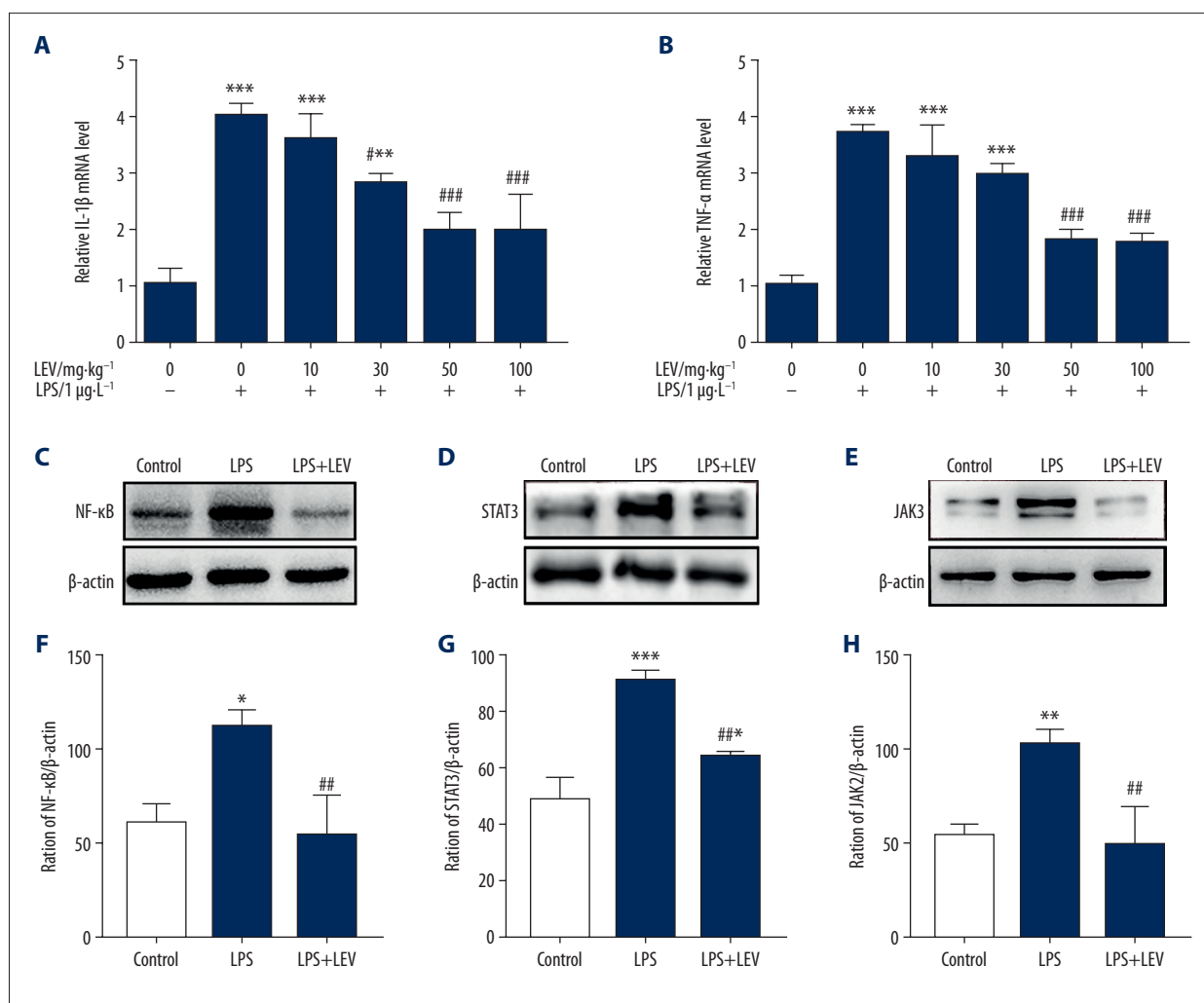
Figure 1. Effects of LEV on the inflammatory response in LPS-induced microglia. (**A, B**) Expression levels of IL-1β and TNF-α mRNA in various groups as detected by qPCR. (**C–E**) The protein expression levels of NF-κB, STAT3, and JAK2 in the 3 groups as detected by Western blot. (**F–H**) Quantitative analysis of NF-κB, STAT3, and JAK2 protein levels. Control – blank control group, LPS – only lipopolysaccharide stimulation group, LPS+LEV – treatment with levetiracetam after lipopolysaccharide stimulation group. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, as compared control group and # $P<0.05$, ## $P<0.01$, ### $P<0.001$, as compared with the ICH group; n=6 in each group).

Statistical analysis

GraphPad Prism 7.0 software was used for all data analysis. The results were recorded as mean±standard error of the mean (SEM). The data were statistically analyzed by using one-way analysis of variance (ANOVA) for multiple comparisons, followed by Tukey's post hoc test. Differences were considered to be statistically significant at $P<0.05$.

Results

LEV effectively alleviated the inflammatory damage of LPS-induced cell model

The mRNA expression levels of IL-1β and TNF-α in cells treated with 5 different doses of LEV (0 μg/ml, 10 μg/ml, 30 μg/ml, 50 μg/ml, and 100 μg/ml) are shown in Figure 1. Notably, mRNA levels of IL-1β (F=23.95, P<0.001) and TNF-α (F=37.85, P<0.001) were significantly increased in LPS-stimulated microglia compared with the negative control group. Treatment with increasing doses of LEV resulted in decreased mRNA levels of IL-1β and TNF-α in a dose-dependent manner (Figure 1A, 1B). At a concentration of 50 μg/ml LEV, the mRNA levels of IL-1β (q=8.993,
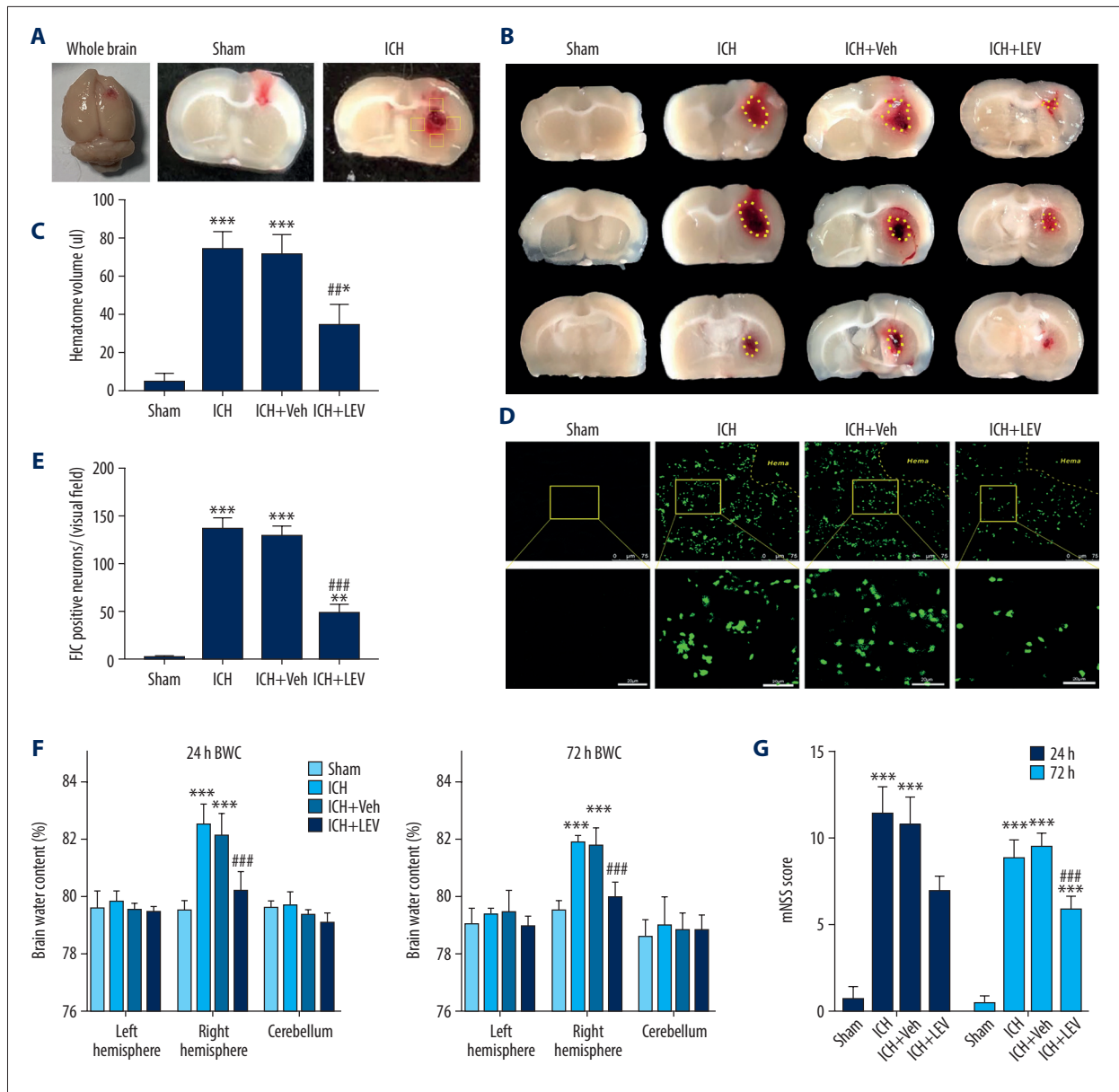
Figure 2. LEV decreased neuronal death, encephaloedema, and improved nerve function recovery after 24 hours of ICH. (**A**) Cerebral hemorrhage in SD rats; The yellow box marked perihematomal regions for subsequent experiments. (**B, C**) Hematoma volume measured at 24 hours after ICH. (**D**) FJC staining examined under a 400× confocal microscope. Green fluorescence indicates the FJC-positive neurons. (**E**) Quantitative analysis of the number of degenerative neurons. (**F**) Water content of the brain tissue. (**G**) The mNSS score of experimental rats. Sham – sham operation group; ICH – intracerebral hemorrhage group; ICH+Veh – intraperitoneal injection of levetiracetam solvent group after ICH; ICH+LEV – intraperitoneal injection of levetiracetam group after ICH. (* P<0.05, ** P<0.01, *** P<0.001, as compared to the control group and # P<0.05, ## P<0.01, ### P<0.001, as compared to the ICH group; n=6 in each group).

P<0.001) and TNF-α (q=11.18, P<0.001) were significantly lower than the 0 µg/ml LEV. However, there was no significant difference between 50 µg/ml and 100 µg/ml (IL-1β: q=0.01214, P>0.05; TNF-α: q=0.2187; P>0.05). Therefore, a concentration of 50 µg/ml was administered as the optimal LEV concentration for the reduction of expression of the pro-inflammatory factors. The expression levels of NF-κB (F=13.63, P<0.01), STAT3 (F=46.64, P<0.001), and JAK2 (F=15.1, P<0.01) protein were increased after LPS stimulation, but decreased after LPS stimulation with LEV (Figure 1C–1H).
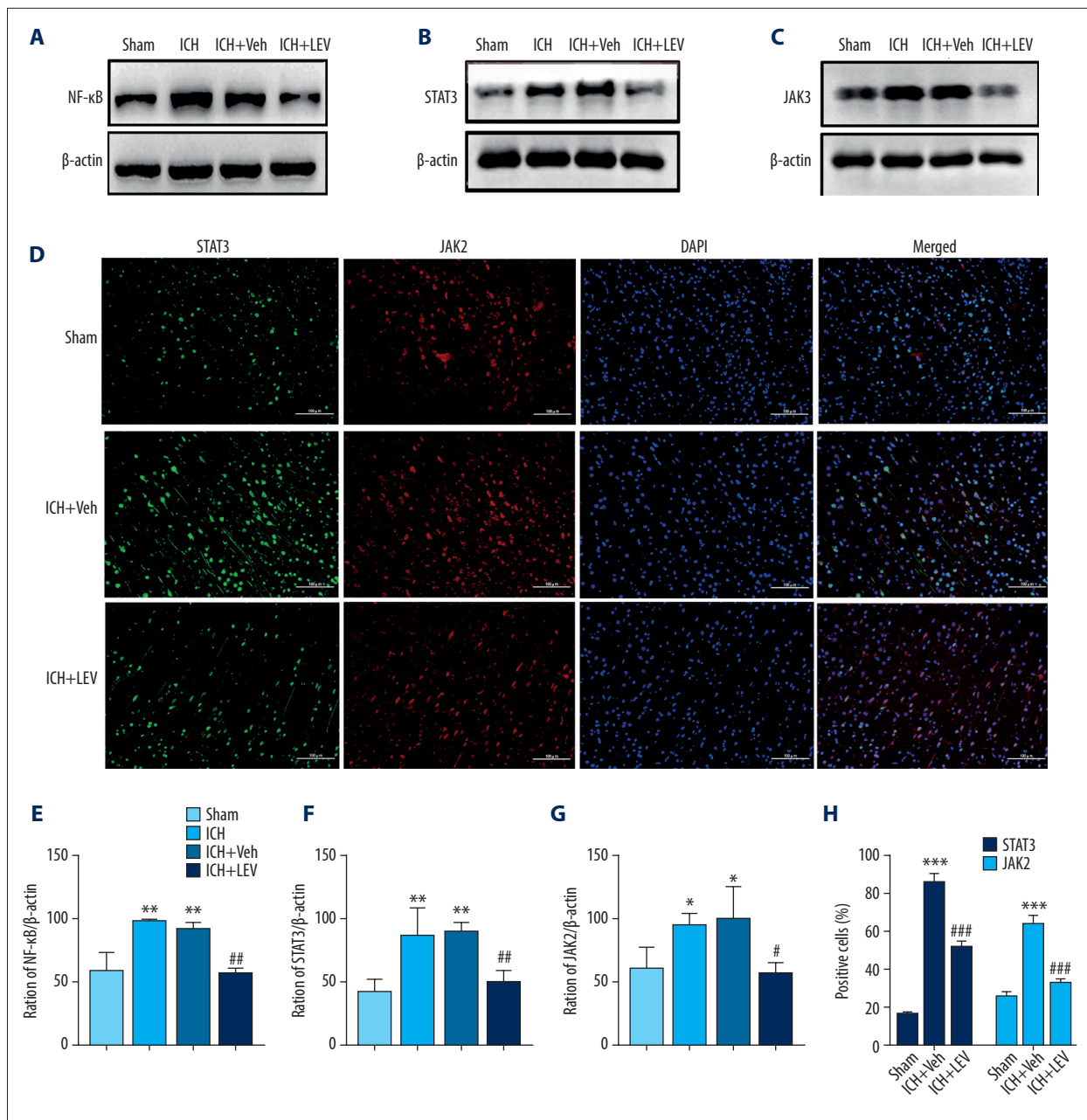
Figure 3. The protein expression levels of NF-κB, JAK2, and STAT3 were upregulated following ICH. (**A**) Analysis of NF-κB expression by Western blot. (**B, C**) Analysis of JAK2 and STAT3 expression by Western blot. (**E–G**) Quantitative analysis of NF-κB, STAT3, and JAK2 protein. (**D, H**) Immunofluorescence analysis of JAK2 and STAT3 by confocal microscopy. Green fluorescence shows STAT3-positive cells and red fluorescence shows JAK2-positive cells. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, as compared to the control group and # $P<0.05$, ## $P<0.01$, ### $P<0.001$, as compared with the ICH group; n=6 in each group).

LEV prevents nerve cell death and promotes recovery of nerve function after ICH

The results from the ICH rat model demonstrated that the sham group had normal structure without obvious hematoma, and there was no edema around the hematoma. On the contrary, the ICH group showed obvious hematoma as well as pronounced edema around the hematoma (Figure 2A). The results analysis of the Western blot, FJC staining, and immunofluorescence are indicated in perihematomal regions shown by the yellow box (Figure 2A). The hematoma volume was measured at 24 h after ICH. Similarly, quantitative analysis of hematoma volume showed that the volume in the ICH+LEV group was significantly reduced compared with the ICH group

(q=7.159, P<0.01) (Figure 2B, 2C). Figure 2D shows the neuroprotective effects of LEV measured after 24 h of FJC staining and imaged under a 400× confocal microscope. Fluoro-Jade tracer is a fluorescent dye that specifically stains degenerating neurons in the CNS [25]. Compared with the ICH group, there were fewer FJC-positive cells (green fluorescence) around the hematoma in the ICH+LEV group (q=14.32, P<0.001) (Figure 2E).

The brain water content of the ICH group was higher than in the sham group (24 h: q=10.68, P<0.001; 72 h: q=11.85, P<0.001), while that of the ICH+LEV group was lower than that of the ICH group (24 h: q=8.111, P<0.001; 72 h: q=9.291, P<0.001) (Figure 2F). The behavioral scores of SD rats at 24 h and 72 h after ICH are shown in Figure 2G. Notably, the ICH group had higher mNSS scores, indicating neurological deficits, compared with the sham group (24 h: q=21.97, P<0.001; 72 h: q=24.27, P<0.001). Compared with the ICH group, the mNSS score was lower in the ICH+LEV group (24 h: q=9.509, P<0.001; 72 h: q=9.439, P<0.001). Together, these results show that LEV prevents nerve cell death and promotes recovery of nerve function after ICH.

LEV upregulated the JAK2-STAT3 signaling pathway

Results showed that ICH upregulated protein expression of NF-κB (F=20.9, P<0.001), but the expression of this protein was not significantly different between the ICH and ICH+Veh groups (q=1.325, P>0.05) (Figure 3A). Following treatment with LEV, the protein level of NF-κB in ICH rats decreased (q=8.753, P<0.01) (Figure 3E), suggesting that LEV reduced the inflammatory response after ICH. The expression levels of JAK2 and STAT3 were assessed by Western blot images (Figure 3B, 3C) and immunofluorescence images (Figure 3D), which indicated that their expression levels in the ICH group were significantly higher than those in the sham group (WB: STAT3: q=7.061, P<0.01; JAK2: q=5.63; P<0.05; IF: STAT3: q=37.27, P<0.001; JAK2: q=19; P<0.001) (Figure 3F–3H). However, there was no significant difference in the expressions of JAK2 and STAT3 proteins between the ICH and ICH+Veh groups (STAT3: q=1.373, P>0.05; JAK2: q=0.3519; P>0.05) (Figure 3F, 3G). Interestingly, expression levels of JAK2 and STAT3 were lower in the ICH+LEV group compared with the ICH+Veh group (WB: STAT3: q=6.228, P<0.01; JAK2: q=5.035; P<0.05; IF: STAT3: q=18.39, P<0.001; JAK2: q=15.52; P<0.001) (Figure 3F–3H), indicating that LEV inhibits the JAK2-STAT3 signaling pathway.

Discussion

The aggregation and activation of inflammatory cells in the early stages of ICH is caused by entry of blood components into the brain parenchyma, causing severe inflammatory reactions [26]. It is widely accepted that this inflammatory response

during the acute phase of ICH is one of the most important causes of secondary injury. These inflammatory reactions are accompanied by activation of NF-κB around the hematoma as well as the expression levels of inflammatory factors such as IL-1β, IL-6, and TNF-α. The subsequent release of these inflammatory factors results in a positive feedback that aggravates the inflammatory response [27]. Microglia and the innate immune cells of the CNS form the first line of defense when intracerebral hemorrhage occurs [28]. Activation of microglia has dual effects in that it can enhance phagocytosis activity of the hematoma as well as trigger the production of transcription factors, thus increasing the expression of pro-inflammatory genes [5]. The JAK2-STAT3 signaling pathways have been reported to regulate inflammatory responses of microglia cells. Also, STAT3 polarizes the microglia to pro-inflammatory (M1) type [14]. On the other hand, LEV normalizes the resting membrane potential of astrocytes, thereby reducing the production of inflammatory mediators [18]. In this study, we explored the possible mechanism of action of LEV in the reduction of the inflammatory response of microglia after ICH, both *in vitro* and *in vivo*.

We stimulated the BV-2 cell line, an immortalized microglia cell line that is similar to primary microglia in morphology, phenotype, and function, using LPS to mimic the inflammatory model of microglia *in vitro* [19]. Our results indicated that exposure of BV-2 cells to LEV can effectively decrease the mRNA levels of IL-1β and TNF-α, which were increased by LPS stimulation. This effect is optimal at the dose of 50 μg/ml LEV. Notably, treatment of BV-2 cells with 50 μg/ml LEV significantly decreased protein expression of NF-κB compared with cells treated with only LPS. A similar finding was obtained in the protein expression of STAT3. These results indicate that LEV can alleviate the inflammatory response of microglia and reduce the release of inflammatory factors by inhibiting the expression of STAT3 *in vitro*.

Currently, the mature animal ICH model is established by treatment with autologous blood-induced and collagenase [21]. The autologous blood-induced model simulates the cytotoxicity and other inflammatory reactions that results when blood components enter the brain parenchyma [19]. Here, we used the autologous blood induction method to establish an ICH model in rats. Through neurobehavioral testing in rats, we found that the neurological function of LEV-treated rats was improved to some extent after 24 h of ICH. We also found that the brain water content was lower in LEV-treated rats relative to the ICH group, and the expression of NF-κB in LEV-treated rats was decreased. Damage to neurons around the hematoma can be alleviated by treatment with LEV, as evidenced by FJC staining. Our results show that LEV exerts an anti-inflammatory role in the early stages of ICH. It achieves this by upregulating the protein expression of JAK2 and STAT3 in the early

stage of ICH, while their levels decreased 24 h after LEV treatment, suggesting a relationship with inflammation reduction. The results of the present study show that LEV can reduce the inflammatory response and neuronal damage in early ICH by regulating the JAK2-STAT3 signaling pathway. As such, treatment with LEV can reduce encephaloedema and simultaneously improve neurological function after ICH.

Conclusions

LEV can regulate the activation of microglia and reduce inflammation by inhibiting the JAK2-STAT3 signaling pathway. Furthermore, LEV can inhibit inflammation and provide cerebral protection in early stages of ICH. Therefore, levetiracetam appears to have potential as a drug for controlling ICH. However, the long-term effects of LEV still require further exploration.

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