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Progesterone suppresses estrogen receptor-mediated inflammatory pathways following intracerebral hemorrhage

Hengyang Ouyang[1], Xiaobing Zhou[2], Zhiming Zhang[3] and Lingfeng Lai[2]*

[1]Huankui Academy, Nanchang University, Nanchang, Jiangxi, China
[2]Department of Neurosurgery, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, Jiangxi, China
[3]Department of Neurosurgery, GaoXin Branch of The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China

Abstract: Intracerebral hemorrhage (ICH) is a highly fatal neurological disease with few successful treatments. The aim of the current study was to investigate the neuroprotection by progesterone and the related molecular mechanisms following ICH. Mice were treated with progesterone (8 mg/kg), estrogen receptor (ER) agonist-erteberel (10 nmol/2 μL), or ER-β-specific siRNA (si-ER-β, 6 nmol/2 μL). Neurological function, edema in the brain and inflammatory cytokine levels were tested. Progesterone significantly increased neurological function on day 1 to day 7 post-ICH and reduced cerebral water content compared to the control group on day 7. Progesterone also suppressed estrogen receptor beta (ER-β) and decreased inflammatory mediator levels such as prostaglandin E2 (PGE2), tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) in ICH-evoked brain tissues and in LPS-stimulated BV-2 microglial cells. These anti-inflammatory effects were inhibited by erteberel, indicating direct interaction with ER-β signaling. Furthermore, progesterone treatment inhibited the expression of Toll-like receptor 4 (TLR4) and nuclear factor kappa B (NF-κB) p65 via inhibition of ER-β. In summary, our findings show that progesterone is neuroprotective after ICH by modulating the ER-β/TLR4/NF-κB pathway and suggest its therapeutic value for managing post-ICH inflammation.

Keywords: Intracerebral hemorrhage; progesterone; ER-β; TLR4; NF-κB p65; inflammation; neuroprotection

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INTRODUCTION

Intracerebral hemorrhage (ICH) is a disabling and common subtype of stroke, often involving the basal ganglia and responsible for 15%-20% of strokes. It is highly morbid and fatal (Jain *et al*., 2021). The majority of survivors are left with lifelong neurological impairment and permanent disability (Zhang *et al*., 2021). The initial insult, or primary brain injury, is caused by abrupt laceration of cerebral vessels and results in compression of surrounding brain tissue by an expanding hematoma. The mechanical trauma causes elevated intracranial pressure and immediate loss of neurological function (Tschoe *et al*., 2020; Magid-Bernstein *et al*., 2022). Unfortunately, surgical removal of the hematoma in clinical practice has been of little value in maximizing neurological recovery. Instead, secondary injury due to post-hemorrhagic inflammation is largely responsible for contributing to the neuronal damage, disruption of the blood-brain barrier (BBB) and worsening of cerebral edema (Zhang *et al*., 2021). Although it is crucial, the mechanisms underlying this peripheral and central inflammatory response are not fully understood.

Intracal processes of neuroinflammation are triggered with hematoma formation and are one of the most critical contributors to secondary injury after ICH. These responses include activation of resident microglia and invasion of extravasating immune cells into the perihematomal brain tissues (Hsueh *et al*., 2021). Invading leukocytes and activated immune cells release proinflammatory mediatorse.g., tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), chemokines, reactive oxygen species and other cytotoxic substances-and intensify the inflammatory cascade. This leads to additional further BBB disruption, edema and neuronal damage (Zhang *et al*., 2019; Zhao *et al*., 2021). Against this background, inflammation-modulating approaches might represent a promising way to maximize functional outcomes in the context of ICH.

Progesterone is an endogenous neurosteroid with high expression in the central nervous system and is reported to have neuroprotective properties (Ghoumari *et al*., 2020). Earlier work suggests that progesterone can antagonize lipid peroxidation induced by reactive oxygen species, preserve BBB integrity, suppress inflammatory cytokine production and block neuronal apoptosis, finally with dampening of cerebral edema (Pabisz *et al*., 2024). Among the proposed mechanisms, downregulation of inflammation has been regarded as one of the primary mechanisms for its protective effect. Estrogen receptors (ERs), particularly the β isoform (ER-β), are also important in anti-inflammatory signaling (Fan *et al*., 2021). ER-β is largely confined to the brain, specifically areas such as cerebral cortex, hippocampus and in various populations of neurons and glia (Zuo *et al*., 2020; Takenawa *et al*., 2023). The role of ER-β in modulating inflammatory responses after ICH, nevertheless, is poorly understood. We hypothesize in this study that progesterone enhances ICH-

**Corresponding author:* e-mail: juan23769@163.com

induced neuroinflammation by downregulating ER-β-mediated signaling, thereby reducing proinflammatory cytokine production and improving neurological outcomes in a murine ICH model.

MATERIALS AND METHODS

Induction of ICH model and intraventricular injection

Intracerebral hemorrhage (ICH) model was induced as previously described protocol (Wang *et al*., 2022). The male C57BL/6 mice were divided into six experimental groups: sham, ICH + saline, ICH + progesterone, ICH + progesterone + ER-agonist (erteberel), ICH + progesterone + si-ER-β and ICH + progesterone + si-Con. Anesthesia was induced by intraperitoneal administration of ketamine (100 mg/kg) and thiazine (10 mg/kg). Body temperature was maintained at 37.0 ± 0.5 °C using a heating pad during the procedure.

Following scalp preparation, the mice were restrained in a stereotaxic apparatus in prone position. Artificial tear ointment was applied to prevent corneal desiccation. 30 μL of autologous arterial blood was drawn using non-heparinized capillaries and loaded into a 250 μL Hamilton syringe with a 27-gauge needle. With Bregma as a reference point, a burr hole was drilled in the cranium 0.8 mm in front of and 2.0 mm to the side of Bregma. A cranial high-speed drill was utilized to drill 1 mm through the skull. The blood was stereotactically injected into the basal ganglia of the right (3 mm below dura) at a rate of 2 μL/min. After injection, the needle was not removed until 5 min to prevent backflow and then slowly withdrawn. Burr holes were occluded with sterile medical bone wax and scalp incision with sutures. Sham mice were treated similarly without blood injection. Postoperative recovery was carried out on a 37 °C heating pad. All operations were carried out in an ultra-clean UV-sterilized environment under conditions of asepsis.

Progesterone (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline solution containing 1% dimethyl sulfoxide (DMSO) and 30% polyethylene glycol (PEG). ICH mice received intraperitoneal injections of progesterone (8 mg/kg) or vehicle at 1 hour after surgery and subcutaneously at 6, 24 and 48 hours (Liu *et al*., 2022). For intralateral ventricle injections, 10 nmol/2 μL of ER-agonist erteberel (LY500307, Selleck), si-ER-β (6 nmol/2 μL), or si-Con (6 nmol/2 μL) in vehicle was injected after scalp incision as previously reported (Yan *et al*., 2021). Animal experiments followed NIH and Chinese guidelines and were approved by the Animal Ethics Committee of the First Affiliated Hospital of Nanchang University, Jiangxi Medical College (Approval No. CDYFY-IACUC-2024-0XX).

Brain water content measurement

For cerebral edema evaluation, brain water content was determined by the wet/dry weight technique. Mice were deeply anesthetized and sacrificed at 24 and 72 h following ICH. Brains were rapidly excised and divided into five regions: bilateral cortex, bilateral basal ganglia and cerebellum. The sample was weighed for wet weight and subsequently dried in an oven at 100 °C for 24 h to be acquired in dry weight. Water content was determined as Brain water content (%) = [(wet weight - dry weight)/wet weight] × 100.

Neurological deficit assessment

Neurological function was assessed using the modified neurological severity score (mNSS) on days 1, 3 and 7 post-ICH. Testing was conducted by a blinded investigator. The mNSS is a test of motor, sensory, reflex and balance functions and ranges from 0 (normal) to 18 (maximum deficit).

Cell culture, transfection and treatment

BV2 murine microglial cells (ATCC) were cultivated in T25 flasks using DMEM supplemented with 10% fetal bovine serum (Gibco, USA) at 37 °C. The cells were subcultured when they reached 80–90% confluency. The cells were plated in six-well plates at 2×10^5/mL for transfection and transfected at 40–50% confluency with si-ER-β or si-Con (100 μM) and Lipofectamine 3000 (Invitrogen, USA). At six hours post-transfection, medium was replaced with complete DMEM and incubation was maintained for the next 48 h. Treatment included exposure of cells to LPS (1 μg/mL), progesterone (100 mM), or erteberel (6 nM) for 24 h before further investigation.

Enzyme-Linked immunosorbent assay (ELISA)

Blood was collected from orbital sinus and centrifuged at 3000 rpm to obtain serum. TNF-α, IL-1β and PGE2 concentrations were measured by using ELISA kits of JianCheng Bioengineering Institute (Nanjing, China) following the instructions provided. Absorbance at 450 nm and the concentration of chemokines were determined from duplicate well absorbance using a logistic curve-fitting algorithm.

Immunohistochemistry

Paraffin-embedded brain tissues were deparaffinized, rehydrated and subjected to antigen retrieval using citrate buffer under boiling conditions. Sections were treated using a PV9000 immunohistochemistry kit (Servicebio) and further incubated overnight at 4 °C with anti-ER-β antibody (1:200, Cell Signaling Technology, USA). Sections were washed and incubated for 30 min at room temperature with the secondary antibody. Color development was carried out using diaminobenzidine (DAB) and nuclei were stained with hematoxylin. After dehydration and lysis, slides were mounted for microscopic examination.

Western blots analysis

Protein lysates were extracted from brain tissue and BV2 cells and were quantified by BCA assay (Pierce Biotechnology, USA). Equal amounts of protein

concentrations were resolved by 12% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk and incubated at 4 °C overnight with primary antibodies to ER-β (1:1000), TLR4 (1:2000), NF-κB p65 (1:2000) and GAPDH (1:3000) (Abcam). The signals were visualized following incubation of the membranes with HRP-conjugated secondary antibodies for 1 hour using an ECL detection system (Pierce Biotechnology, USA).

Immunofluorescence staining

BV2 cells or paraffin-embedded brain sections were fixed in 4% paraformaldehyde overnight at 4°C. The samples were treated overnight with primary antibodies against TLR4 (1:200, Cell Signaling Technology) and NF-κB p65 (1:200, Abcam). PBS washing and incubation with fluorescent-conjugated secondary antibodies (1:200, Abcam) were conducted for 2 hours at room temperature. Images were taken using a fluorescence microscope (Leica Microsystems).

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS Statistics 17.0. Results are presented as mean ± SD. Between-group comparisons were performed by ANOVA with post hoc analysis by Tukey's test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Progesterone attenuates neurological deficits and brain edema after ICH

To identify the neuroprotective effect of progesterone following intracerebral hemorrhage (ICH), neurological dysfunction was assessed by the modified neurological severity score (mNSS) on day 1, day 3 and day 7 following ICH. Mice with ICH had significantly greater neurological deficit scores compared to sham group mice. Progesterone treatment reduced these scores significantly within the 7-day follow-up period (fig. 1A). Because cerebral edema is a major cause of post-stroke injury, brain water content was measured. Progesterone-treated mice showed significantly reduced brain water content compared with saline-treated mice at day 7 after ICH (fig. 1B).

To determine the anti-inflammatory potency of progesterone, the levels of pro-inflammatory cytokines were measured. ICH resulted in severe increase in levels of PGE2, TNF-α and IL-1β, which were notably repressed by progesterone administration (fig. 1C). Western blot analysis also demonstrated a remarkable upregulation of ER-β expression in brain tissues of ICH model mice when compared to sham group, which was notably repressed by progesterone treatment. Immunohistochemical staining further confirmed these findings, showing strong ER-β immunoreactivity in the perihematomal regions of ICH and ICH + vehicle groups, whereas weaker staining was observed in sham and ICH + progesterone groups (fig. 2A). These findings indicate that progesterone mitigates neurological damage and brain edema, in part by virtue of its anti-inflammatory activity and inhibition of ER-β expression (fig. 2B).

Progesterone inhibits ER-β expression and decreases neuroinflammation in BV-2 cells

To investigate underlying mechanisms in vitro, BV-2 microglial cells were stimulated with LPS, which markedly increased ER-β expression and elevated PGE2, TNF-α, and IL-1β. Progesterone treatment reversed these changes, suppressing ER-β and pro-inflammatory cytokine production (fig. 3A and 3B). ELISA confirmed that progesterone significantly reduced LPS-induced IL-1β and PGE2, highlighting its anti-inflammatory role (fig. 3C and 3D). These findings further corroborate the anti-inflammatory effect of progesterone on microglial-mediated neuroinflammation.

ER-β activation reverses the neuroprotective effects of progesterone

To further determine whether ER-β mediates the anti-inflammatory effects of progesterone, either the ER-β agonist erteberel, ER-β-targeting siRNA (si-ER-β), or control siRNA (si-Con) was administered by intracerebroventricular injection. Western blotting assured successful modulation of ER-β expression (fig. 4A). ELISA detection indicated that erteberel-activated ER-β abrogated the inhibitory effects of progesterone on PGE2, TNF-α and IL-1β in ICH mice, whereas ER-β silencing significantly augmented the anti-inflammatory effect of progesterone (fig. 4B). In vitro, ER-β activation increased ER-β and its associated cytokines in LPS-treated BV-2 cells but inhibited them after transfection with si-ER-β (fig. 4C and 4D). These findings suggest that ER-β is the mediator of neuroinflammatory response and that progesterone suppresses its protective effect by down-regulating ER-β at least in part.

Progesterone represses TLR4/NF-κB signaling through suppression of ER-β in vivo

Given the role of TLR4/NF-κB signaling in neuroinflammation, we next determined whether progesterone suppresses this pathway through blockade of ER-β. Western blotting of brain tissue revealed that ICH resulted in significant upregulation of TLR4 and nuclear NF-κB p65 expression. Progesterone treatment suppressed these increases, which were reversed by ER-β activation by erteberel. In contrast, si-ER-β enhanced the inhibitory effect of progesterone on TLR4 and NF-κB p65 expression (fig. 5A and 5B). The results suggest that progesterone inhibits the TLR4/NF-κB pathway by suppressing ER-β expression *in vivo*.

Progesterone suppresses ER-β to repress TLR4/NF-κB signaling in BV-2 cells

To further confirm these results in vitro, BV-2 cells were treated with LPS and progesterone alone or in combination

with ER-β agonist or si-ER-β. Nuclear NF-κB p65 concentration was prominently increased in LPS-stimulated cells and the elevation was considerably inhibited by progesterone. ER-β activation negated progesterone's suppressive actions, while ER-β silencing reinforced them (fig. 6A and 6B). These results reaffirm that progesterone inhibits ER-β expression to suppress the activation of the TLR4/NF-κB pathway to mediate anti-inflammatory actions on microglial cells.

DISCUSSION

Intracerebral hemorrhage (ICH) is the most fatal form of stroke, with a poor prognosis and limited therapeutic options. Despite much work done on its pathophysiology, therapy for ICH is still far from optimal. Therefore, it is imperative to discover novel therapeutic targets. Neuroinflammation, primarily caused by activated microglia, plays a pivotal role in the secondary brain injury unfolding after ICH. New evidence suggests that suppressing microglia-mediated inflammatory responses could potentially offer neuroprotection by limiting ICH-related brain damage (Hei *et al*., 2022). Progesterone, a neurosteroid with known roles in the CNS, has been reported to play protective as well as repair functions in the brain, spinal cord and peripheral nerves following injury.

In the present study, we investigated the neuroprotective actions of progesterone following ICH by dissecting its regulatory functions in ER-β-induced neuroinflammation and the downstream TLR4/NF-κB signaling pathway. The present paper is the first to demonstrate that progesterone inhibits neuroinflammation following ICH through downregulation of ER-β and inhibition of activation of the TLR4/NF-κB pathway.

Progesterone has previously been shown to possess antioxidant, anti-inflammatory and anti-apoptotic actions (Lee *et al*., 2023). In addition to its classic role in reproduction, progesterone is synthesized and secreted in the nervous system and has profound effects on the structure and function of neurons (Kabe *et al*., 2018). It was recently shown that progesterone improves cognitive and motor functions, reduces cerebral edema and suppresses inflammation in models of traumatic brain injury (Dhote *et al*., 2022).

Consistent with such findings, the present results showed that progesterone significantly improved neurological scores and reduced brain water content in ICH model mice, suggesting significant neuroprotective activity. Because cerebral edema is a feature of secondary brain injury, triggering neuroinflammation and apoptosis of neurons (Youssef and Wen, 2021), these findings further support progesterone's ability to counteract ICH-evoked injury.

Pro-inflammatory cytokines, such as TNF-α and IL-1β, are key mediators of neuroinflammation and are mainly released from activated microglia. Elevated IL-2 enhances neuronal damage and causes edema in various CNS disorders (Shao *et al*., 2020; Zheng *et al*., 2023), while IL-1β promotes significantly after brain injury and inflammation (Zhang *et al*., 2019). In some instances, prostaglandin E2 (PGE2) is a significant promoter of inflammation, enhances vascular permeability and causes the recruitment of neutrophils (Linke *et al*., 2017). Our study confirmed that progesterone significantly reduced the level of PGE2, TNF-α and IL-1β in ICH brain tissue and LPS-stimulated BV-2 cells, indicating that progesterone indeed suppresses neuroinflammatory responses.

The traditional mechanism of steroid hormones is to bind to intracellular receptors, including estrogen receptors and exert transcriptional regulation of target genes (Thomas, 2022). Our study showed that progesterone suppressed ER-β expression in both in vivo and in vitro neuroinflammation models. ER-β activation blocked, while ER-β silencing enhanced, progesterone’s anti-inflammatory effects, indicating that ER-β is essential for its anti-inflammatory and neuroprotective actions.

Besides, the TLR4/NF-κB pathway is an established mediator of inflammation after cerebral hemorrhage (Du *et al*., 2024; Eisa *et al*., 2023; Liang *et al*., 2022). TLR4 upregulation following ICH is linked with increased cytokine secretion and inflammatory cell invasion and NF-κB activation facilitates the transcription of inflammatory genes (Tian *et al*., 2022; (Wang *et al*., 2023). In the present study, progesterone reduced TLR4 and NF-κB p65 expression in ICH brain tissue and BV-2 cells and ER-β activation reversed this. Conversely, ER-β suppression increased the progesterone inhibition of TLR4/NF-κB signaling. The findings reveal a novel mechanism by which progesterone suppresses neuroinflammation via ER-β regulation of the TLR4/NF-κB pathway.

CONCLUSION

In conclusion, our study provides rigorous evidence that progesterone exhibits neuroprotective effects following ICH by inhibiting ER-β expression and downregulating subsequently the TLR4/NF-κB pathway. All these findings provide a hopeful therapeutic intervention for ICH. However, more work remains to be done to unravel the mechanism involved in how progesterone influences ER-β activity and to validate these findings in the clinic.

Ethical approval

All animal experiments followed the NIH Guide for the Care and Use of Laboratory Animals and relevant Chinese guidelines, with approval from the Animal Ethics Committee of the First Affiliated Hospital of Nanchang University (Approval No. CDYFY-IACUC-2024-0XX).

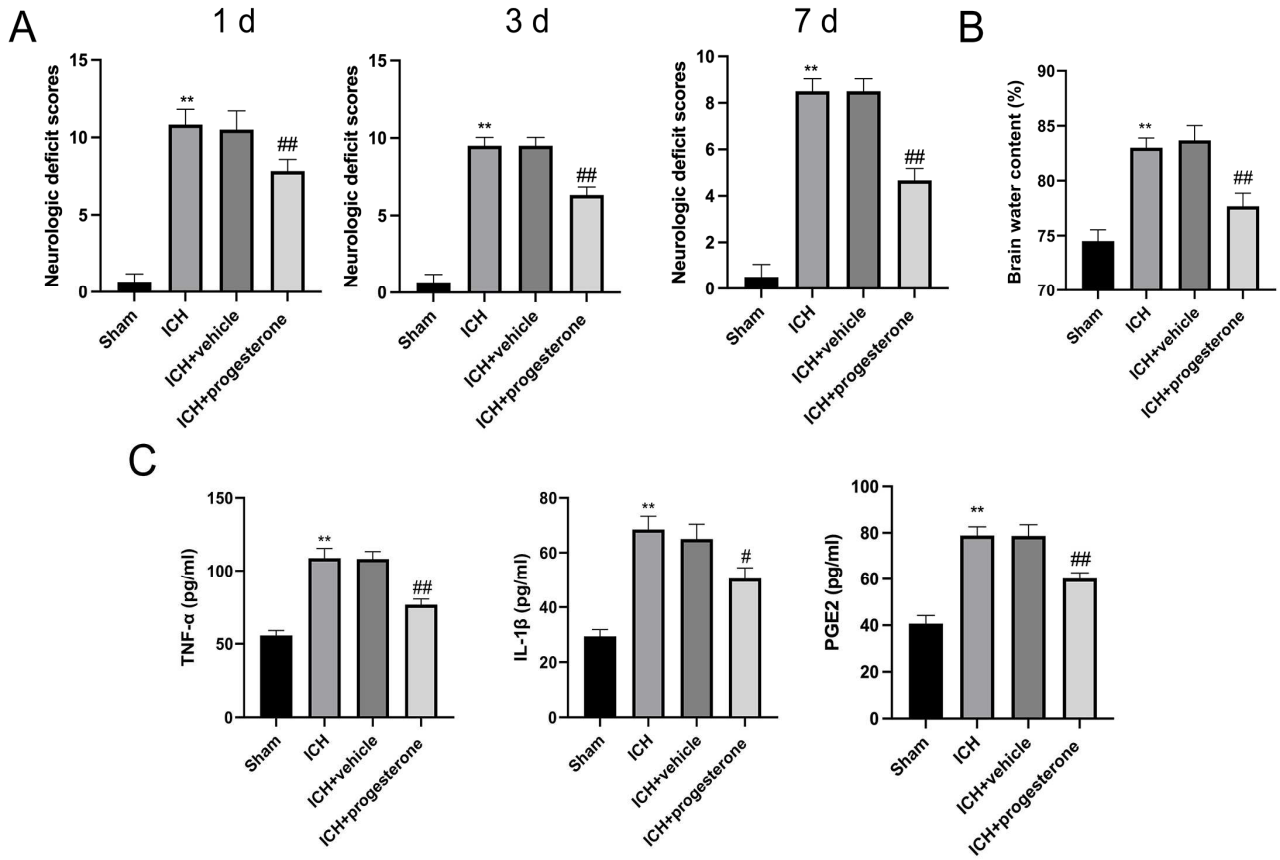

Fig. 1: Progesterone attenuated neurological deficits and reduced brain edema after ICH. (A) Neurologic deficit scores on day 1, 3 and 7. (B) Brain water content on day 7 after ICH. (C) ELISA measurement of PGE2, TNF-α, and IL-1β levels in serum from ICH mice. $^{**}P<0.01$ *vs*. sham group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs*. ICH + vehicle group. n = 6 per group.

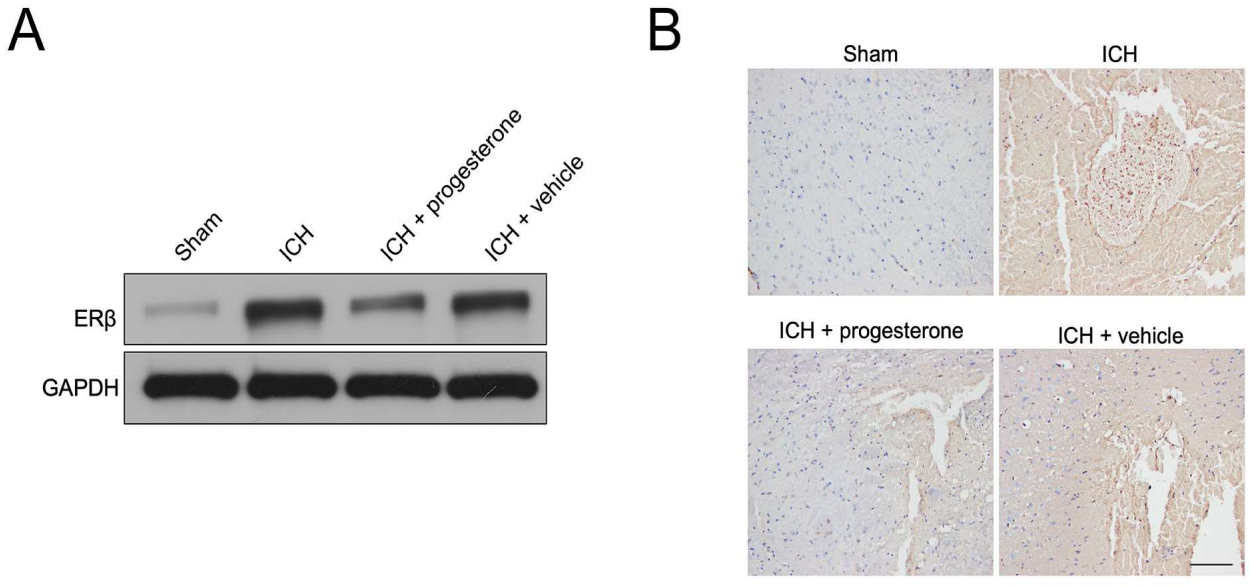

Fig. 2: Progesterone down-regulates ER-β expression. (A) Western blot detection of ER-β expression in brain tissues of ICH mice. (B) Immunohistochemistry of ER-β in brain tissues of ICH mice.

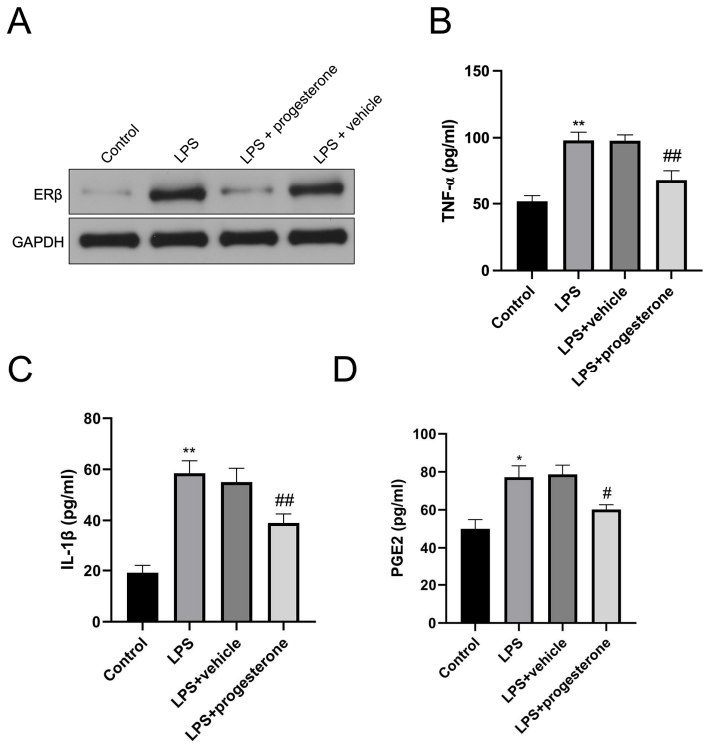

Fig. 3: Progesterone down-regulates ER-β expression in LPS-treated BV-2 cells. (A) Western blot detection of ER-β expression in LPS-treated BV-2 cells. (B) ELISA measurement of PGE2, TNF-α, and IL-1β levels in culture supernatant from LPS-treated BV-2 cells. $^{**}P$<0.01 *vs*. control group. $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs*. sham group.

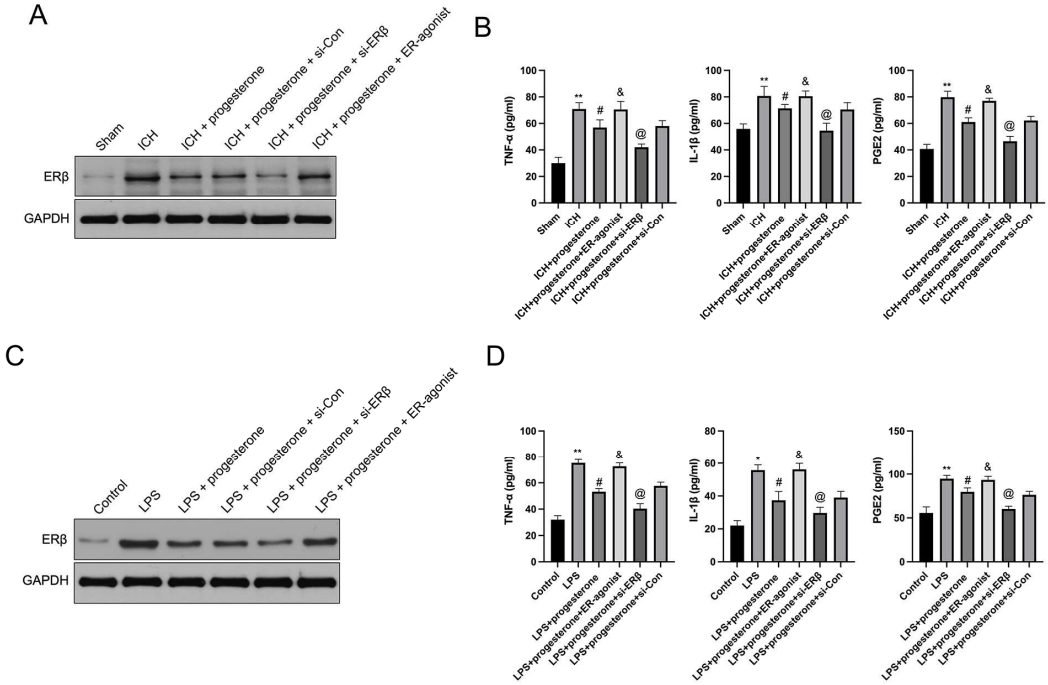

Fig. 4: ER-β activating reverses the neuroprotective effect of progesterone treatment. (A) ER-agonist Erteberel, si-ER-β, or si-Con were injected into the lateral ventricle after scalp incision. Western blot determination of ER-β expression in brain tissues of ICH mice. (B) ELISA measurement of PGE2, TNF-α, and IL-1β levels in serum from ICH mice. $^{**}P$<0.01 *vs*. sham group, $^{\#}P$<0.05 *vs*. ICH group, $^{\&}P$<0.05 *vs*. IHC + progesterone group, $^{@}P$<0.05 *vs*. IHC + progesterone group. (C) ER-agonist treatment or si-ER-β transfection were performed in LPS-treated BV-2 cells. Western blot detection of ER-β expression. (D) ELISA detection of PGE2, TNF-α, and IL-1β levels in culture supernatant from LPS-treated BV-2 cells. $^{**}P$<0.01 *vs*. control group, $^{\#}P$<0.05 *vs*. LPS group, $^{\&}P$<0.05 *vs*. LPS + progesterone group, $^{@}P$<0.05 *vs*. LPS + progesterone group.

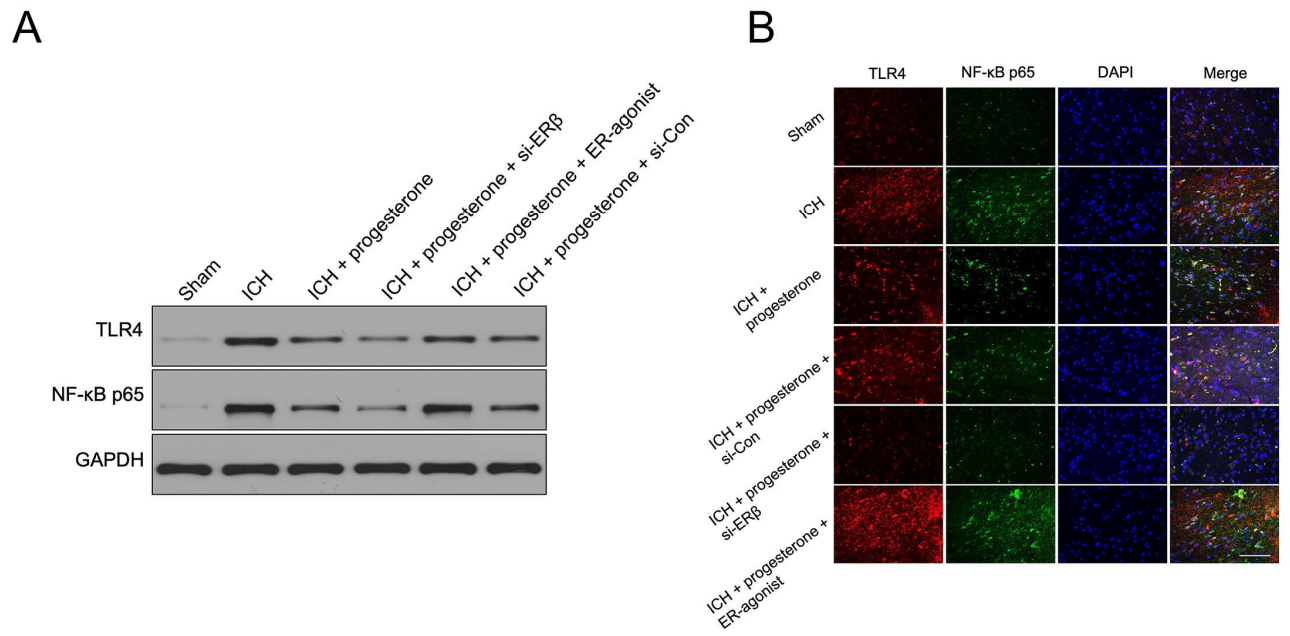

Fig. 5: Progesterone inhibits ER-β expression to inactivate TLR4/NF-κB pathway *in vivo*. (A) ER-agonist Erteberel, si-ER-β, or si-Con were injected into the lateral ventricle after scalp incision. Western blot determination of TLR4 and NF-κB p65 expression in brain tissues of ICH mice. (B) Immunofluorescence staining analysis of TLR4 and NF-κB p65 expression in brain tissues of ICH mice. Scar bar: 200 μm.

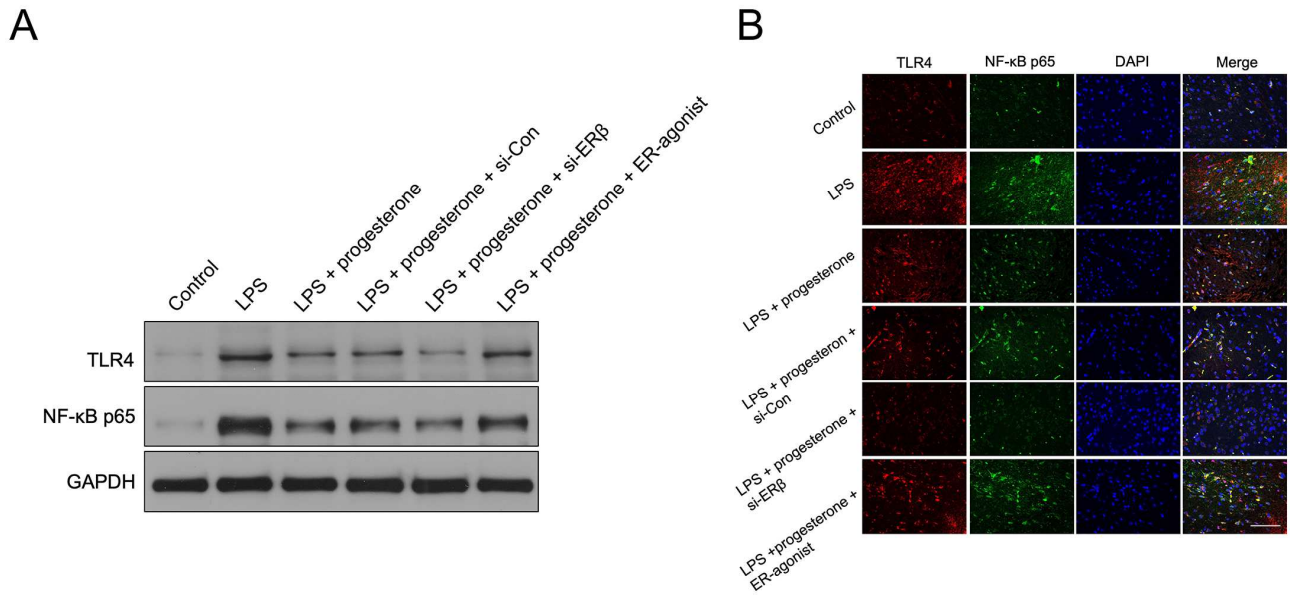

Fig. 6: Progesterone inhibits ER-β expression to inactivate TLR4/NF-κB pathway in BV-2 cells. (A) BV-2 cells were treated with ER-agonist Erteberel or transfected with si-ER-β, following by LPS or progesterone treatment. Western blot detection of TLR4 and NF-κB p65. (B) Immunofluorescence staining of TLR4 and NF-κB p65 in BV-2 cells. Scar bar: 200 μm.

Conflict interests

The authors declare no conflict of interest.

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