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Icariin and icaritin ameliorated hippocampus neuroinflammation via mediating HMGB1 expression in social defeat model in mice

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ABSTRACT

Depression is a chronic, severe, and often life-threatening disease accompanied with impaired neurogenesis. Evidence showed that neuroinflammation played a key role in the process of depression. High mobility group protein box 1 (HMGB1) has been proved to function as a pro-inflammatory cytokine. In this study, we used a social defeat (SD) stress to induce inflammatory response, aiming to explore the relationship between HMGB1 and neuroinflammation. We found that the expression of HMGB1 decreased in mice exposure to SD stress, but showed a high expression of cytoplasmic HMGB1 and a high expression of RAGE, which could be rescued by ICA and ICT. So, we speculated that the translocation of HMGB1 from the nucleus to the cytoplasm might play an important role in neuroinflammatory process, and HMGB1-RAGE signaling was involved in this process. Furthermore, we also found that TLR4-XBP1s-ER stress related NF-κB signaling activation was also involved in HMGB1-related neuroinflammation. However, ICA and ICT treatment activated NF-κB signaling, and we also observed the translocation of HMGB1 into the nucleus and the increased number of neurons in mice hippocampus, indicating that the activation of NF-κB signaling might be related to neuroregeneration. Moreover, recombinant human HMGB1 protein (rHMGB1) pretreatment could suppress HMGB1-RAGE signaling and TLR4-XBP1s-ER stress related NF-κB signaling, resulted in a suppressed microglia activation in mice hippocampus. We supposed that ICA and ICT could ameliorate neuroinflammation in hippocampus via suppressing HMGB1-RAGE signaling and show neuroprotective effects via activating TLR4- NF-κB signaling at the same time, resulting in improving depressive behaviors in mice.

1. Introduction

Depression is a common but serious mood disorder that causes a persistent depressed mood, loss of interest or pleasure, and poor concentration, and what's worse, depression often leads to increased disability rate and suicide rate [1,2], resulting in a decreased quality of life. According to the World Health Organization (WHO), almost 1 million people take their own lives each year. Although much effort has been put into exploring the etiology and treatment of depression, the extant molecular mechanisms of this disease have not been completely deciphered yet, thus, it hinders the development of antidepressant treatment.

Meta-analysis revealed that depression had a strong relationship with immune dysregulation and inflammation [3–5]. Besides, in late-life major depressive disorder (MDD) patients, immunological markers, like tumor necrosis factor (TNF)-α, had a significant positive relationship with white matter hyperintensity volume [6]. Recent data also showed that neuroinflammation plays an important role in both normal brain functions and pathological brain process [7]. Human imaging studies showed a significant elevation of translocator protein (TSPO), a marker which used to measure microglia activation in vivo, in the prefrontal cortex, insula and anterior cingulate cortex (ACC) among depressive patients [8–10], offering a direct evidence that neuroinflammation is related to depression. Besides neuroinflammation, studies also indicated that depression occurs usually accompanied with neurogenesis suppression [11,12], and neurogenic effect was a common action of antidepressants [13,14], while acute or sub-chronic treatments didn't have such effect [14].

High mobility group protein box 1 (HMGB1) is a kind of highly conserved, ubiquitous protein which is widely expressed in most eukaryotic cells, including neural cells in human [15]. Evidence showed that HMGB1 played an important role in neurodegeneration diseases via mediating neuroinflammation [16,17]. Kim JB et al. found that HMGB1 linked exiteotoxicity-induced acute damage process with

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delayed inflammatory processes in the post-ischemic brain [18]. In a Parkinson's disease (PD) model, HMGB1 stimulated microglia to release inflammatory factors and caused chronic dopaminergic neurodegeneration which in turn activated microglia NF-κB pathway and NADPH oxidase [19]. Moreover, stress exposure also induced hippocampal microglia to release HMGB1, which increased the expression of the nucleotide-binding domain, leucine-rich repeat, pyrin domain containing protein 3 (NLRP3) and NF-κB protein [20]. As a sophisticated danger signal with pleiotropic functions, HMGB1 also promotes the regeneration process. *Hmgb1*$^{-/-}$ newborn mice died soon because of hypoglycaemia [21]. Dong, Y., et al. noted that HMGB1 didn't mediate the inflammatory process in spontaneous spinal cord regeneration, but promote central neural system (CNS) regeneration [22]. Fang, P., et al. found that HMGB1 could promote recovery from spinal cord injury (SCI) via enhancing neurogeneration and increasing angiogenesis [23]. A recent study also showed that HMGB1 could promote brain recovery by promoting neurotrophin expression [24].

To our knowledge, HMGB1 usually located in the nuclei and was involved in stabilization of nucleosomal structure, regulation of replication, and gene transcription [25]. While under stress situation, HMGB1 could be secreted actively by macrophages, monocytes and microglia to the cytoplasm or extracellular milieu [26]. Studies showed that HMGB1 was involved in lipopolysaccharide (LPS)-induced depressive-like behavior [27] and chronic unpredictable mild stress (CUMS)-induced depressive behaviors [28,29]. However, these studies focused on the correlation between HMGB1 and the depression model, and the exact role of HMGB1 played in depression still remains unclear.

Icariin (ICA) and icaritin (ICT) are major constituent of flavonoids isolated from *Herba Epimedii*, and ICT is one metabolite of ICA. Our group has demonstrated the anti-inflammation effects of ICA in LPS-induced lung inflammation via activating PI3K/Akt pathway and inhibiting NF-κB [30] and ICA exerted an antidepressant effect in CUMS rats via regulating hippocampal neuroinflammation and altering the expression of glucocorticoid receptor [31,32], while ICT could increase GR mRNA and BDNF mRNA in social defeat (SD) mice [33]. In this study, we focused on the anti-neuroinflammation effect of ICA and ICT in SD stress-induced chronic inflammation model in mice, aiming to confirm whether HMGB1 was involved in SD stress-induced depressive behaviors and the anti-neuroinflammation effect of ICA and ICT.

2. Material and methods

2.1. Animals

Male, 7-week-old C57 BL/6J mice and were purchased from Vital River Laboratories (Beijing, China). They were house in groups (5 per cage) under a 12/12 h light/dark cycle at controlled temperature (22 °C ± 2 °C), with standard food and water given ad libitum. After one-week period of the habituation, mice were subjected to the experiment. CD1 retired breeders (age of 8–9 months) were purchased from Vital River Laboratories (Beijing, China). They were housed individually and were used as resident aggressor. CD1 mice were screened for their aggressive behavior. Animal care and experimental procedures were carried out in accordance with the Experimental Animal Ethics Committee of Shanghai Medical College, Fudan University.

2.2. Social defeat stress

The SD stress was applied as described previously [34,35]. In brief, a C57 BL/6J mouse (intruder) was introduced into a home cage of a CD1 mouse (resident). These cages were divided into two parts by perforated Plexiglas dividers, and defeat sessions took place on one side of the divider. After a 5-min defeat session, the intruder was protected within the resident's cage by the divider for the remaining 24 h, while the intruder was still subjected to olfactory, visual and to some visible physical contact with the resident. For each defeat session, the experimental mouse encountered a new CD1 aggressor which lasted for 10 consecutive days. Control C57 BL/6J mice were housed in pairs, divided by a perforated Plexiglas divider, and were rotated to a new cage on a daily basis without any physical contact with their cage mates. During a whole period of SD stress, only food and water were available.

2.3. Social interaction test

Social interaction test was assessed on day 11 (24 h after the 10th defeat by the CD1 mouse) in a 42 cm × 42 cm open-field apparatus. The tested mouse was placed in the open-field with an empty wire-mesh cage (10 cm × 6 cm) located at one end, and its movement was tracked for 300 s ('no target' phase), then, the mouse was placed back into its home cage for 30 s. When the mouse was introduced back into the open-field for tracking another 300 s movement, the wire-mesh cage was placed a completely novel CD1 mouse ('target' phase). The duration of the tested mouse in the 'interaction zone' (defined as the 8 cm-wide area around the mesh cage) was measured by the automated video-tracking system based on the Ethovision XT software (Noldus Information Technology).

2.4. Treatment of the social defeat mice

Immediately following the social interaction test, house all susceptible C57 BL/6J mice singly in standard mouse cage with ad libitum access to food and water. The control C57 BL/6J was housed in the same condition. For pharmacological treatment, susceptible C57 BL/6J mice were divided into 4 groups randomly: SD stress group, Fluoxetine group, ICA group and ICT group. Each group was given NS (200 μL/mouse/day), fluoxetine (10 mg/Kg/day), ICA (20 mg/Kg/day), ICT (20 mg/Kg/day) respectively by gavage for 4 weeks (Fig. 1A). The control group was given NS (200 μL/mouse/day) for 4 weeks by gavage. Because of their bad water solubility, ICA and ICT were suspended in 0.5% carboxymethylcellulose Na (CMC—Na) and the doses we used

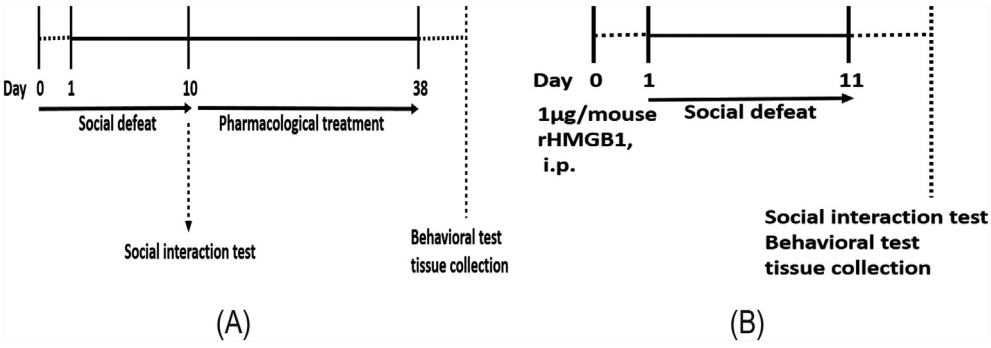

Fig. 1. The schematic presentations of each experimental design.

here were based on our previous studies [31,33,36]. After pharmacological treatment, the behavior tests were conducted immediately within 2 days. In other serious of experiments, recombinant human HMGB1 protein (rHMGB1) (1690-HMB-050, R&D Systems, Minneapolis, USA) was reconstituted at 200 μg/mL in PBS, and each mouse was injected i.p. with 1 μg rHMGB1 for 24 h before SD stress and the behavior tests was conducted immediately within 2 days (Fig. 1B).

2.5. Elevated plus maze (EPM) test

The mice were placed into the center of an elevated, plus-shaped (+) apparatus with two open and two closed arms (5 × 30 cm) one by one for 5 min. Two closed arms have 20-cm-high walls. The maze was elevated 40 cm above the floor. The time spent in the open or closed arms was measured by the Ethovision XT software.

2.6. Open filed test

The open field test (OFT) was conducted in an open box (50 cm × 50 cm × 50 cm, length × width × height). Mice were placed into the center of the arena and allowed to explore the apparatus for 5 min. The time spent in the center zone (covering 25 cm × 25 cm) were recorded by the Ethovision XT software.

2.7. Measurement of serum TNFα, IL-10 and HMGB1

Blood samples were collected by removal of eyeball prior to sacrificing, and serum was separated by centrifugation at 5000 rpm at 4 °C for 30 min, and was stored at −80 °C. Serum TNFα, IL-10 and HMGB1 were measured by sandwich ELISA based methods according to the manufacture instructions. ELISA kits for TNFα, IL-10 and HMGB1 were obtained from Anogen (MEC1003, Mississauga, Ontario, Canada), RayBiotech (ELM-IL10, Norcross, GA, USA) and Chondrex (6010, Redmond, WA, USA) respectively.

2.8. Immunohistochemistry

Mice were transcardially perfused with NS followed by 4% paraformaldehyde. Brains were removed and fixed in 4% paraformaldehyde for 24 h. Brain tissue was paraffin-embedded and cut into sections 4 μm thick. Sections were incubated with anti-MAP2 (1:100; ab32454, Abcam, Cambridge, UK) and anti-Iba1 (1:100; ab5076, Abcam) overnight at 4 °C, followed by anti-rabbit and anti-goat secondary antibody for 30 min at room temperature (RT). The striatum was observed by light microscopy (BX43, Olympus, Japan) and analyzed with Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

2.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNAs were synthesized using RevertAid First strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's protocol. Detection of mRNA was performed using Power SYBR™ Green PCR Master Mix (4,367,659, Thermo Fisher Scientific) and a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). Primers used for PCR amplification are listed in Table 1. The expression levels of mRNAs were normalized to GAPDH and were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.10. Nuclear and cytoplasmic extraction

Nuclear and cytoplasmic extracts were prepared using EqiQuick Nuclear Extraction Kit (OP-0002, Epigentek, NY, USA). Hippocampus tissues were collected and stored at −80 °C. LaminB1, nuclear protein (1:1000; 13435, Cell Signaling Technology, MA, USA), and β-actin, cytoplasmic protein (1:1000; 3700S, Cell Signaling Technology) were used as loading controls.

Table 1
Primers used for qRT-PCR analysis.

Primer	Sequence
TNFα RT F	CATCTTCTCAAAATTCGAGTGAC
TNFα RT R	TGGGAGTAGACAAGGTACAACCC
IL-1β RT F	TGGAAAAGCGGTTTGTCTTC
IL-1β RT R	TACCAGTTGGGGAACTCTGC
IL-6 RT F	GAGGATACCACTCCCAACAGACC
IL-6 RT R	AAGTGCATCATCGTTGTTCATACA
IL-10 RT F	CAACATACTGCTAACCGACTC
IL-10 RT R	AACTGGATCATTTCCGATAAG
Arginase-1 RT F	GTGAAGAACCCACGGTCTGT
Arginase-1 RT R	GCCAGAGATGCTTCCAACTG
CD206 RT F	CTTCGGGCCTTTGGAATAAT
CD206 RT R	TAGAAGAGCCCTTGGGTTGA
HMGB1 RT F	CACCGTGGGACTATTAGGAT
HMGB1 RT R	GCTCACACTTTTGGGGATAC
RAGE RT F	GATCCTGCCTCTGAACTCAC
RAGE RT R	AGAGGACCAGAGAAGGAAGTG
TLR4 RT F	CAGTTTCAATCGCATAGAGAC
TLR4 RT R	CCAACACATAAGGAGGTARRCAT
GAPDH RT F	AAATGGTGAAGGTCGGTGTG
GAPDH RT R	AGGTCAATGAAGGGGTCGTT

2.11. Western blot analysis

Hippocampus tissues were collected and lysed for protein expression analysis. The protein concentration was quantified using the BCA method (KeyGEN, Nanjing, China). Around 30 μg of protein per sample was used for western blot analysis and was separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferred onto a polyvinylidene fluoride membrane (Millipore, MA, USA), it was blocked with 5% non-fat milk for 1 h at RT. Immunoblots were incubated with different primary antibodies overnight at 4 °C. After washing with TBST, the membranes were incubated with secondary antibody at RT. Immunopositive bands were detected with enhanced chemiluminescence (ECL) and measured by ImageQuant™ LAS4000 mini system. Band intensities were quantified using Image J analysis software. Primary antibodies used here included anti-HMGB1 antibody (1:1000; 3935S, Cell Signaling Technology), anti-RAGE antibody (1:1000; ab3611, Abcam), anti-TLR4 (1:500; ab47093, Abcam), anti-BIP (1:1000; 3177S, Cell Signaling Technology), anti-p65 (1:1000; 4764S, Cell Signaling Technology), anti-IκB (1:1000; 4814S, Cell Signaling Technology), anti-p-IκB (1:1000; 9246S, Cell Signaling Technology), anti-XBP1s (1:1000; 12782S, Cell Signaling Technology).

2.12. Statistics

Our data was expressed as mean ± SEM. Statistical analysis was performed using the IBM SPSS Statistics 21. Multiple comparisons were evaluated by Least Significant Difference (LSD) after a one-way analysis of variance (ANOVA). A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. ICA and ICT could attenuate SD stress induced behavioral deficits in mice

SD is a kind of very potent stressor leading to varieties of behavioral effects, like social withdrawal, anxiety, reduced exploratory behavior [37,38]. As we can see, with the situation that the aggressor was absent, all the defeated mice spent the similar time in the interaction zone compared to the undefeated ones, however, when the aggressor was in the cage, the defeated mice spent less time in the interaction zone,

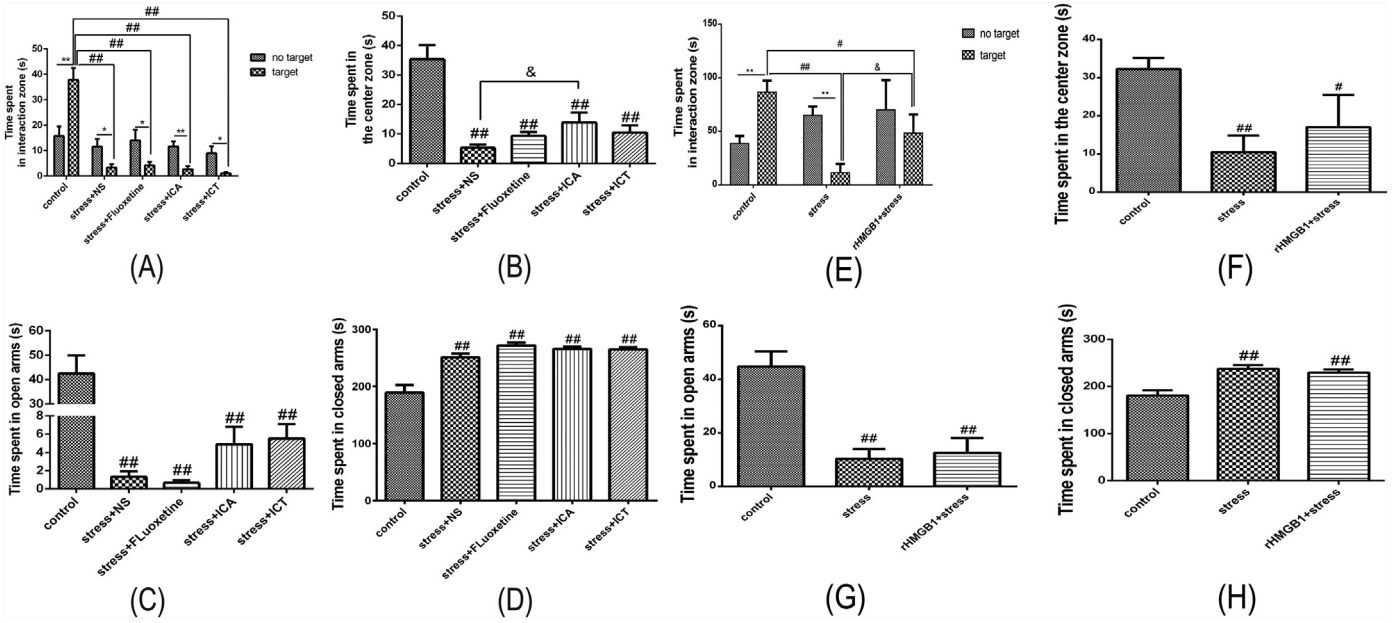

Fig. 2. Effects of ICA, ICT and rHMGB1 on behavioral performance of SD stress-treated mice. (A) and (E) SD induced social avoidance behavior in the social interaction test (n = 8–10 each group). (B) and (F) SD induced time spent in the center zone in the open field test (n = 8–10 each group). (C) and (G) SD induced time spent in open arms in the elevated plus maze (EPM) (n = 8–10 each group). (D) and (H) SD induced time spent tin closed arms in the EPM (n = 8–10 each group). (*p < 0.05, **p < 0.01, target vs no target; ##p < 0.01, defeated ones vs control; &p < 0.05, each group vs stress + NS).

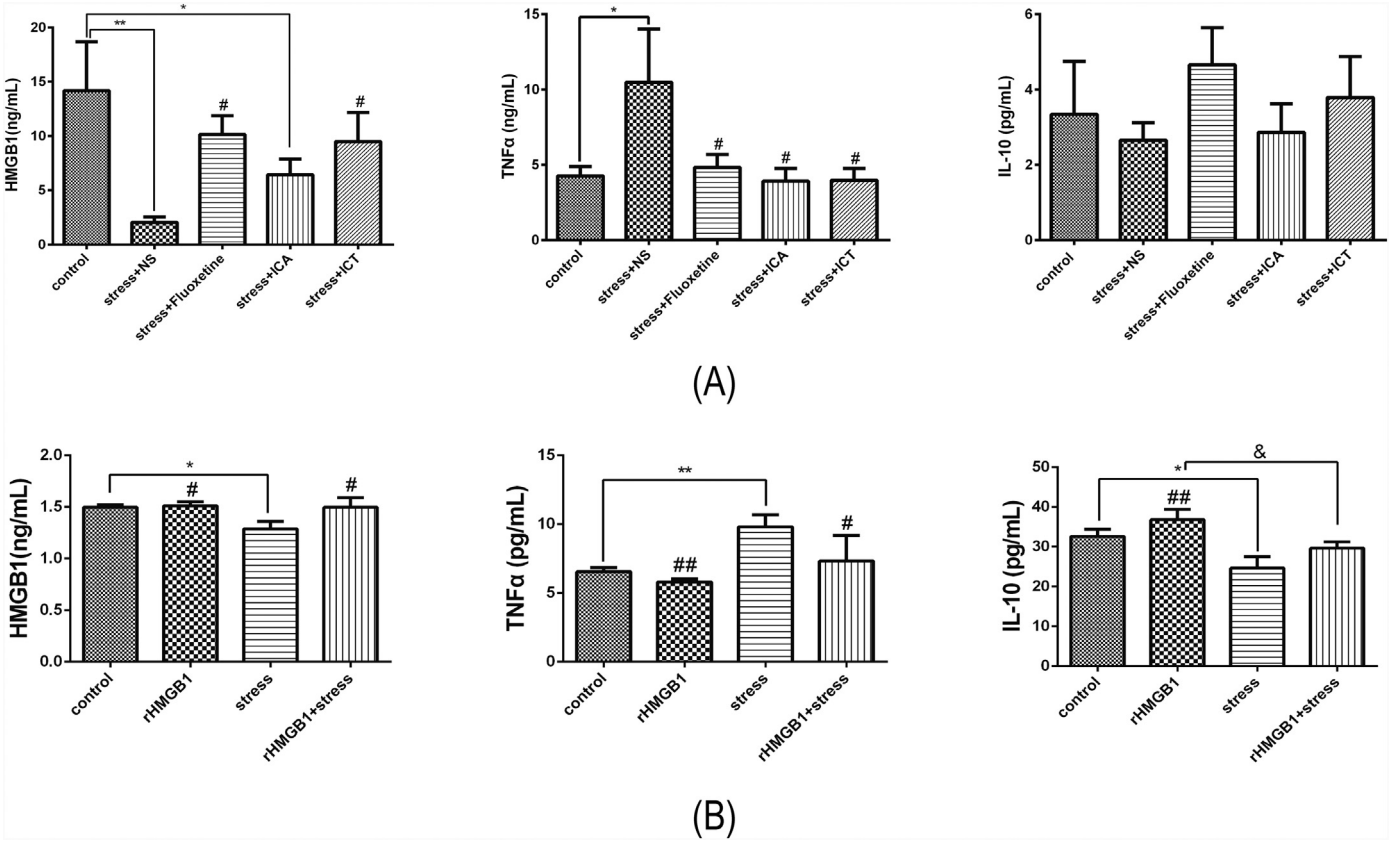

Fig. 3. The HMGB1, TNFα and IL-10 expression in mice serum. (A) Mice exposure to SD stress were administrated with NS, fluoxetine, ICA and ICT by gavage for 4 weeks (n = 8–10 each group). (B) Mice exposure to SD stress were pretreated with rHMGB1 or not (n = 8–10 each group). (*p < 0.05, **p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs stress + NS group or stress group; &p < 0.05, each group vs rHMGB1 group).

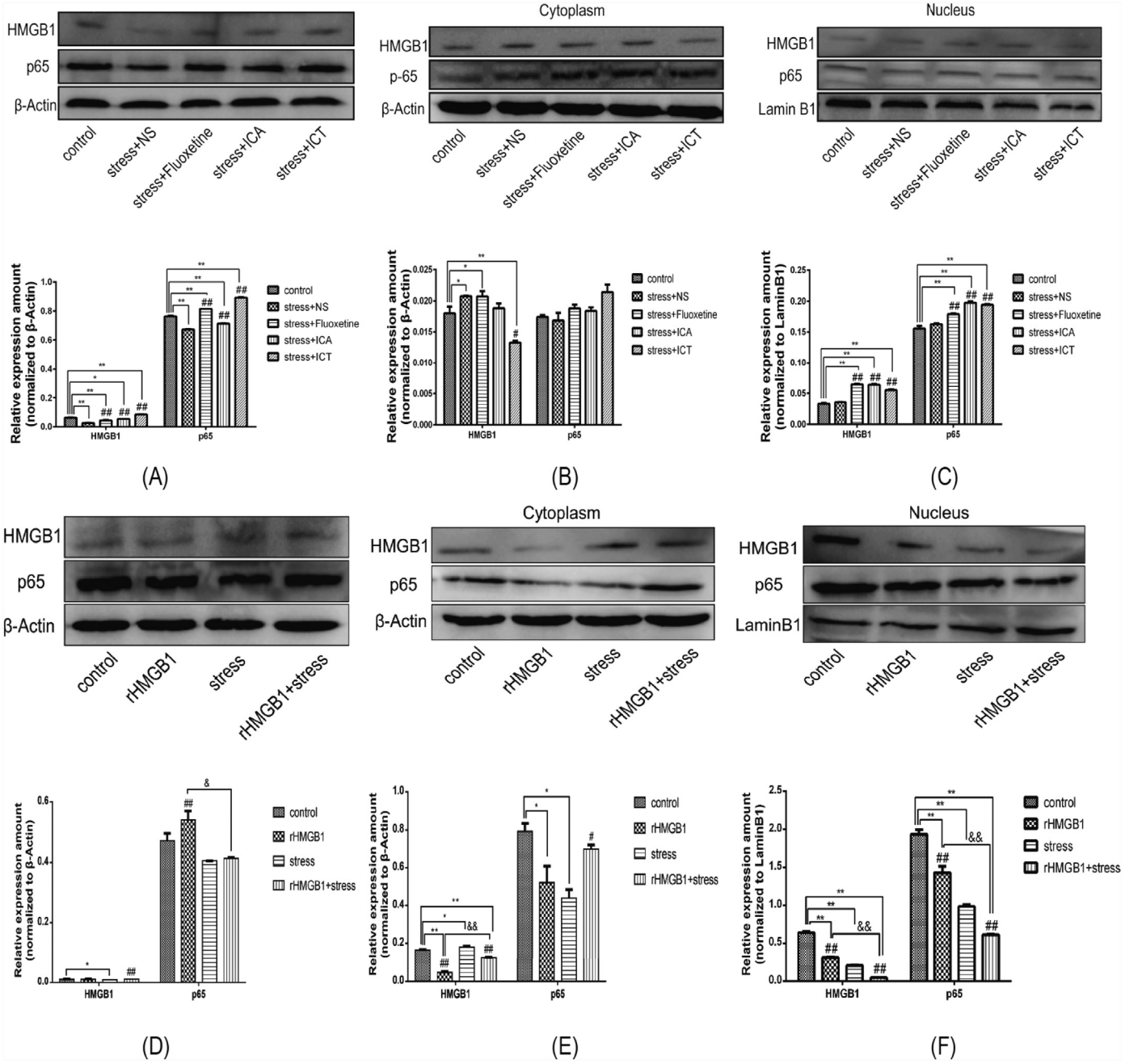

Fig. 4. The expression of HMGB1 and p65 in mice hippocampus. After different treatments, the total HMGB1 and p65 expression (A and D), the cytoplasmic HMGB1 and p65 expression (B and E) and the nuclear HMGB1 and p65 expression (C and F) of each group were measured by western blotting method ($n = 6$ each group). (*p < 0.05, **p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs stress + NS group or stress group; &p < 0.05, &&p < 0.01, each group vs rHMGB1 group).

compared to the undefeated ones, and the time itself spent in the interaction zone when the aggressor was absent, while the undefeated ones showed a greater interest in the aggressor (Fig. 2A). We next used the OFT and the EPM test to measure their locomotor behaviors and anxiety. As shown in Fig. 2B, the stress + NS group showed the least time spent in the center zone, while, ICA treatment could significantly increase the time ($p < 0.05$), and the other two drugs showed a similar effect. For EPM test, all the defeated mice showed a significant longer time spent in the closed arms compared to the control group ($p < 0.01$) (Fig. 2D), and for all the defeated mice, ICA and ICT treatment could increase the time spent in the open arms (data showed no difference) (Fig. 2C).

We also detect whether rHMGB1 pretreatment could change these mice's depressive like behaviors. As shown in Fig. 2E, when the aggressor was in the cage, all the defeated mice spent less time in the interaction zone compared to the undefeated ones, and compared to the stress group, rHMGB1 pretreatment spent more time in the interaction zone ($p < 0.05$). However, for the OFT and EPM test, the rHMGB1 + stress group showed a slightly improvement in anxiety behaviors compared to the stress group ($p > 0.05$) (Fig. 2F, G and H).

3.2. ICA and ICT could rescue SD stress induced TNFα and HMGB1 expression change in mice serum, so did rHMGB1 pretreatment

As shown in Fig. 3A, we can see that, compared to control group, mice exposed to SD followed with 4-week-NS treatment showed a

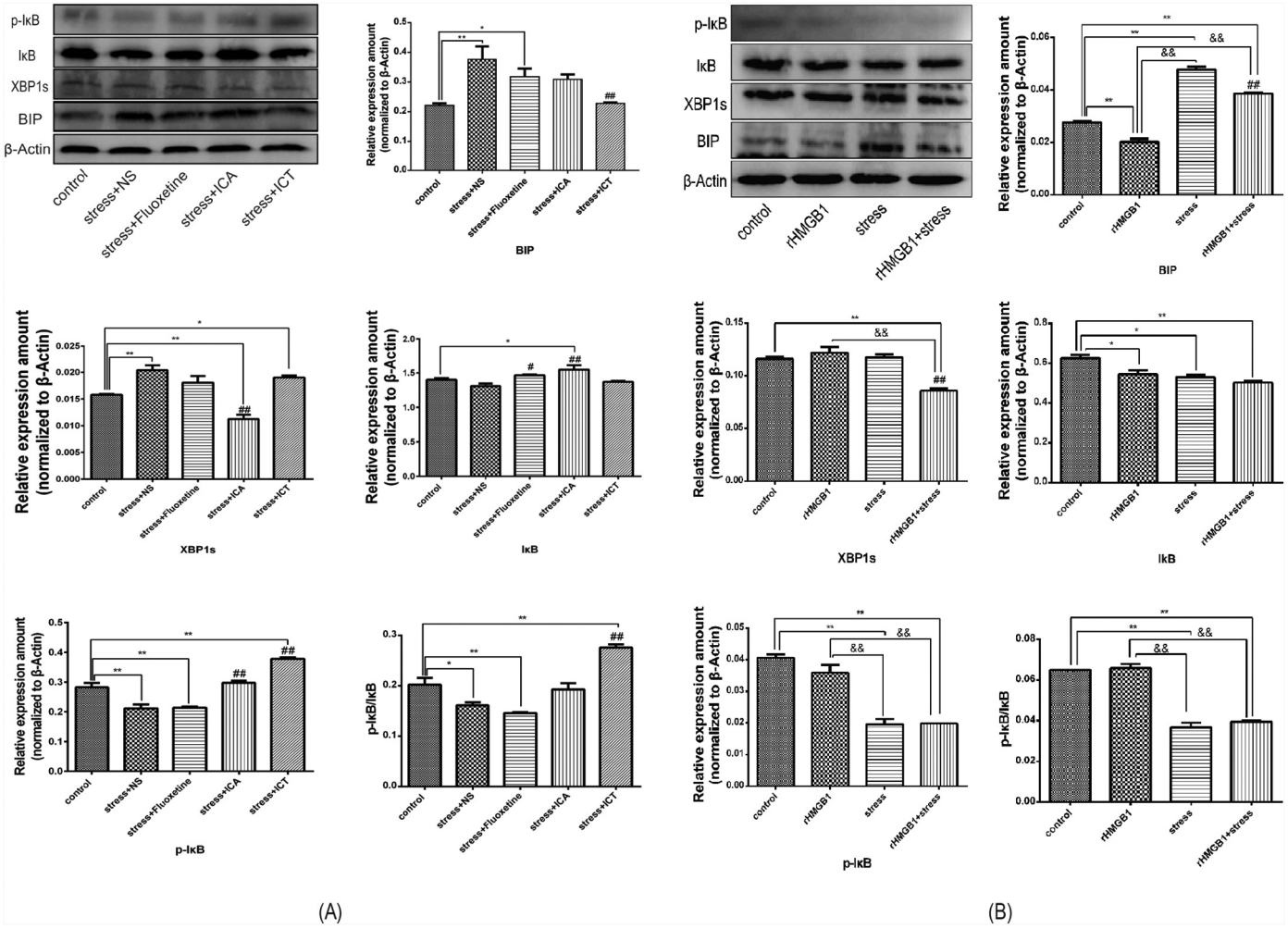

Fig. 5. The expression of BIP, XBP1s, IκB and p- IκB expression in mice hippocampus. (A) Mice exposure to SD stress were administrated with NS, fluoxetine, ICA and ICT by gavage for 4 weeks (n = 6 each group). (B) Mice exposure to SD stress were pretreated with rHMGB1 or not (n = 6 each group). (*p < 0.05, **p < 0.01, each group vs control; ##p < 0.01, each group vs stress + NS group or stress group; &&p < 0.01, each group vs rHMGB1 group).

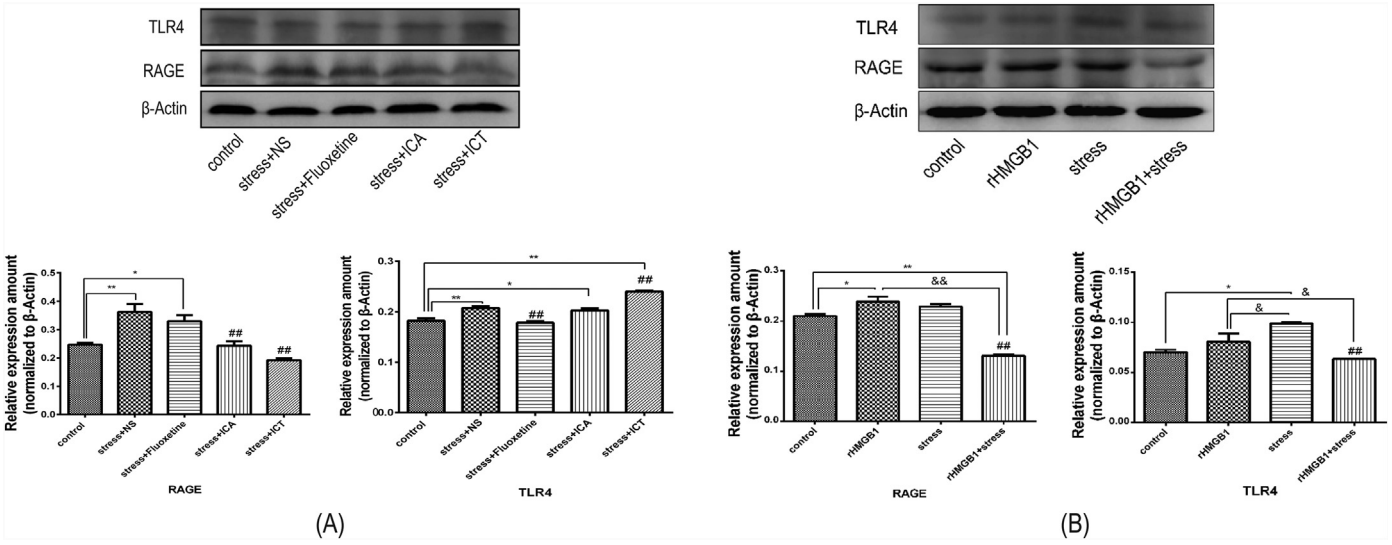

Fig. 6. The RAGE and TLR4 protein expression in hippocampus. (A) Mice exposure to SD stress were administrated with NS, fluoxetine, ICA and ICT by gavage for 4 weeks (n = 6 each group). (B) Mice exposure to SD stress were pretreated with rHMGB1 or not (n = 6 each group). (*p < 0.05, **p < 0.01, each group vs control; ##p < 0.01, each group vs stress + NS group or stress group; &p < 0.05, &&p < 0.01, each group vs rHMGB1 group).

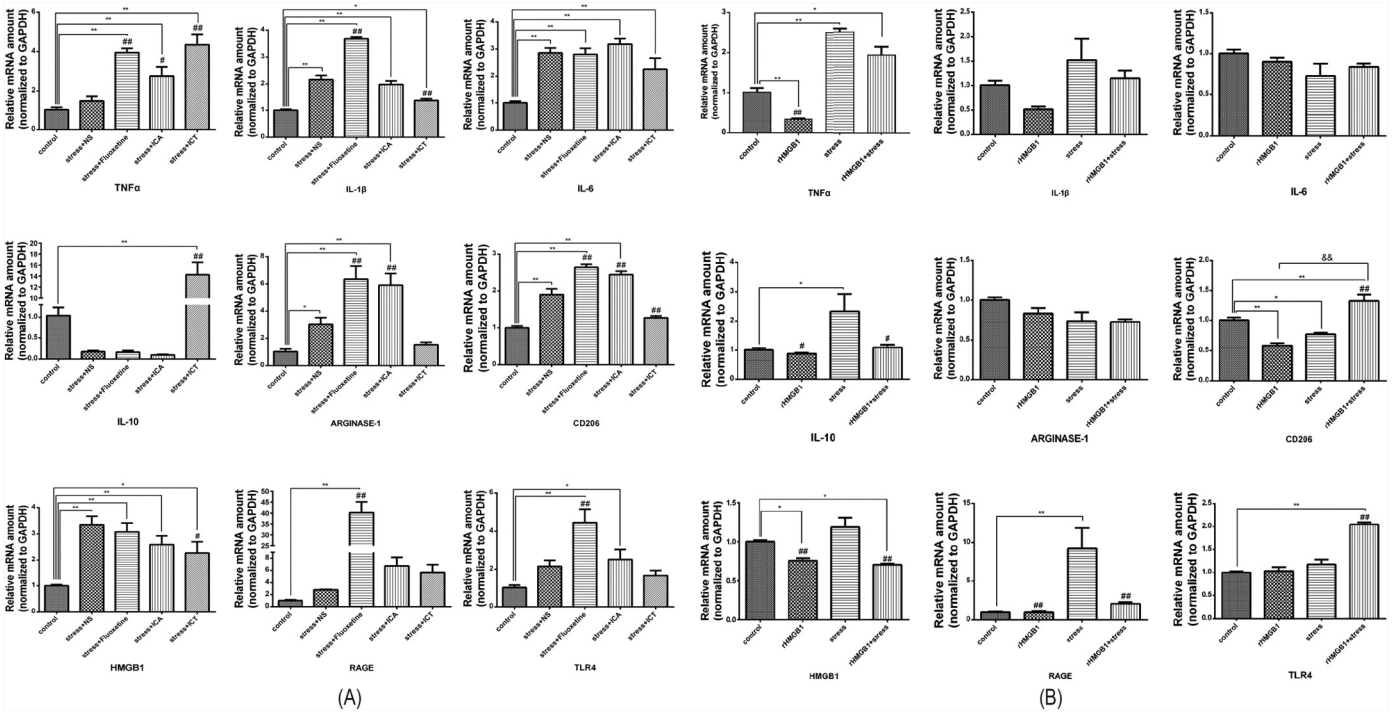

Fig. 7. The relative mRNA expression of M1 (TNFα, IL-1β and Il-6) cytokines, M2 (IL-10, Arginase-1 and CD206) cytokines, HMGB1, RAGE and TLR4 in mice hippocampus. (A) Mice exposure to SD stress were administrated with NS, fluoxetine, ICA and ICT by gavage for 4 weeks (n = 6 each group). (B) Mice exposure to SD stress were pretreated with rHMGB1 or not (n = 6 each group). (*p < 0.05, **p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs stress + NS group or stress group; &&p < 0.01, each group vs rHMGB1 group).

significant lower expression of HMGB1 in serum ($p < 0.01$), and a higher expression of TNFα ($p < 0.05$). We used fluoxetine as a positive control here, and we found that fluoxetine, ICA and ICT could all increase the HMGB1expression in serum in SD stress model, and decease the expression of TNFα (Fig. 3A). As for IL-10 expression in mice serum, fluoxetine, ICA and ICT could slightly increase its expression compared to the stress + NS group (data showed no difference) (Fig. 3A). Further, we used 1 μg/mL rHMGB1 i.p. for 24 h followed a SD stress, and the results showed that rHMGB1 + stress group and rHMGB1 group showed a significant higher expression of HMGB1 and a lower expression of TNFα expression in mice serum compared to the stress group (Fig. 3B).

3.3. ICA and ICT could increase the total and the nuclear HMGB1 and p65 expression in hippocampus

As shown in Fig. 4A, compared to the control, mice exposed to SD followed with 4-week-NS treatment showed a lower expression of the total HMGB1 and p65 ($p < 0.01$) which could be rescued by fluoxetine, ICA and ICT treatment ($p < 0.01$). As HMGB1 usually located in the nuclei, and could transfer to the cytoplasm under stress situation, we detected the nuclear and cytoplasmic expression of HMGB1in hippocampus. We can see that, in the cytoplasm, the stress + NS group showed a higher HMGB1 expression than the control group ($p < 0.05$), while in the nucleus, the HMGB1 expression showed no difference with the control group (Fig. 4B). Interestingly, compared to the NS treatment, fluoxetine, ICA and ICT treatment could increase the nuclear HMGB1 and p65 expression ($p < 0.01$) (Fig. 4C), while only ICT treatment could decrease the cytoplasmic HMGB1 expression effectively ($p < 0.05$) (Fig. 4B). We also found that rHMGB1 pretreatment followed a SD stress significantly increased the total HMGB1 expression in mice hippocampus than the stress group (Fig. 4D), and decreased HMGB1 expression both in the cytoplasm and the nucleus (Fig. 4E and F). Meanwhile, as shown in Fig. 4E and F, rHMGB1 pretreatment could increase the cytoplasmic p65 expression and decrease the nuclear p65 expression ($p < 0.05$). However, as shown in Fig. 4E and F, compared to the stress group, rHMGB1 alone showed a lower cytoplasmic HMGB1 expression and a higher nuclear HMGB1 and p65 expression ($p < 0.01$).

3.4. Endoplasmic reticulum stress related NF-κB signaling might be involved in SD stress-induced inflammatory response

Evidence showed that endoplasmic reticulum (ER) stress was induced under various inflammatory states [39,40], and was associated with NF-κB signaling [41]. Here, we further detected the expression of BIP (a marker protein of ER stress), XBP1s and IκB in mice hippocampus, and we found that, BIP and XBP1s were highly expressed in stress + NS group which could be reduced by pharmacological treatments (Fig. 5A). However, the activation of IκB was suppressed by the NS treatment, while ICA and ICT treatment could active IκB (Fig. 5A). Moreover, we found that rHMGB1 pretreatment followed a SD stress could reduce BIP and XBP1s expression compared to the stress group, but had no effect on IκB activation (Fig. 5B).

3.5. ICA and ICT could rescue SD stress induced RAGE protein expression increase in mice hippocampus

We also detected the expression of the two main receptors of HMGB1, the receptor of advanced glycation end products (RAGE) and toll-like 4 (TLR4), in hippocampus. As shown in Fig. 6A, we can see that, compared to the control group, SD + NS group showed an increase of RAGE and TLR4 protein expression ($p < 0.01$). However, the mRNA expression of RAGE and TLR4 showed a slightly increase in stress + NS group (Fig. 7A). Compared to the NS treatment, ICA and ICT treatment could decrease the expression of RAGE protein, and interestingly, ICT further increased TLR4 protein expression, while ICA had no effect on TLR4 protein expression (Fig. 6A). As shown in Fig. 7A, ICA and ICT treatment both had no effect on the expression of RAGE mRNA and TLR4 mRNA compared to the stress + NS group.

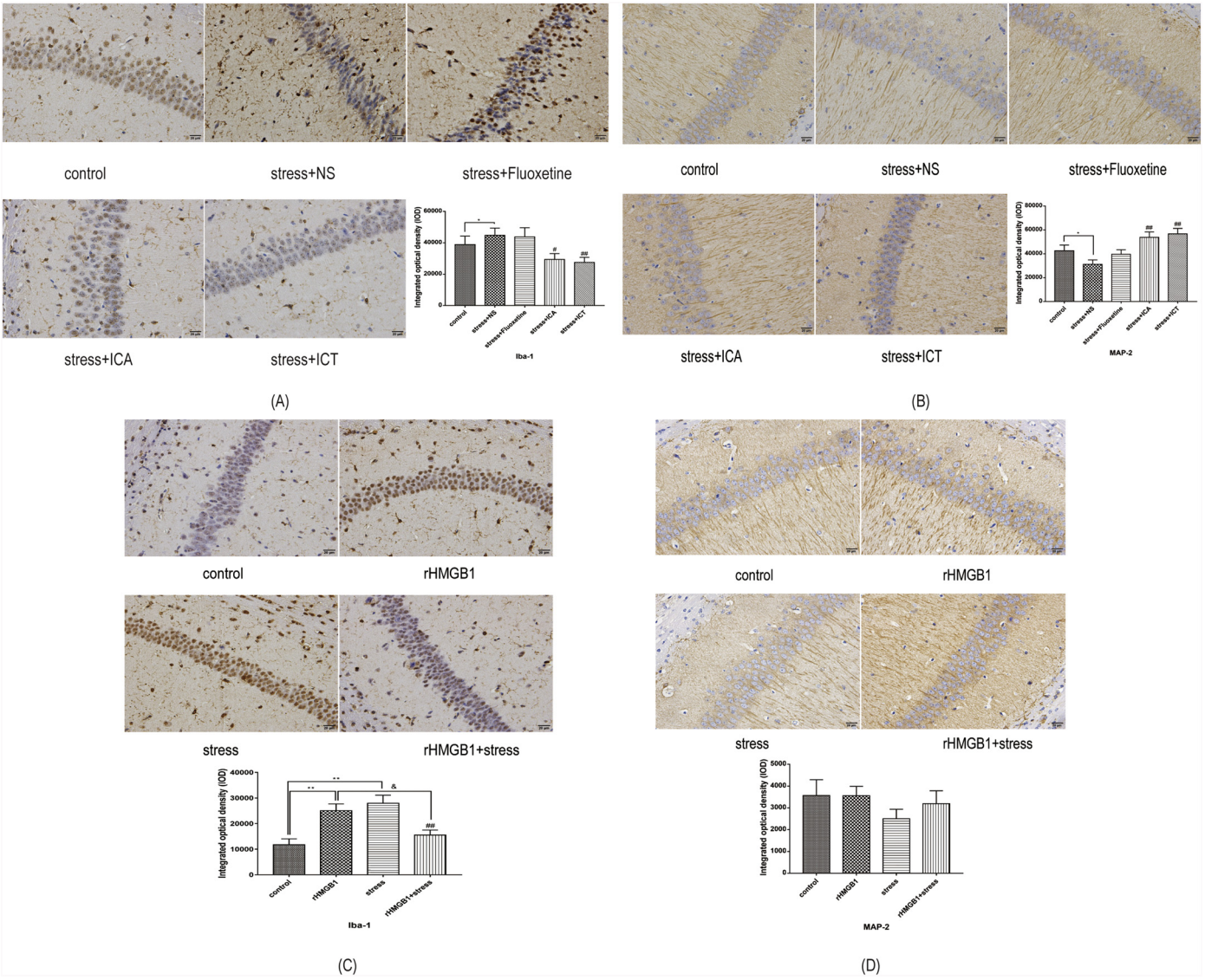

Fig. 8. The expression of Iba-1 and MAP-2 in mice hippocampus. (A) Mice exposure to SD stress were administrated with NS, fluoxetine, ICA and ICT by gavage for 4 weeks ($n = 4$ each group). (B) Mice exposure to SD stress were pretreated with rHMGB1 or not (n = 4 each group). (*p < 0.05, **p < 0.01, each group vs control; #p < 0.05, ##p < 0.01, each group vs stress + NS group or stress group; &p < 0.05, each group vs rHMGB1 group).

However, as shown in Fig. 6B, rHMGB1 pretreatment decreased both RAGE and TLR4 protein expression compared to both the stress group and rHMGB1 group in mice hippocampus ($p < 0.01$). What's more, rHMGB1 pretreatment showed a decrease in RAGE mRNA expression and an increase in TLR4 mRNA expression compared to the stress group (p < 0.01) (Fig. 7B).

3.6. ICT could reduce the SD stress induced increase of IL-1β mRNA expression in mice hippocampus

We detected the mRNA expression of M1 and M2 cytokines in hippocampus, and the results indicated that, compared to the control, the M1 cytokines, TNFα, IL-1β and IL-6 showed a higher expression in the stress + NS group, but for the M2 cytokines, only IL-10 showed a lower expression in the stress + NS group (Fig. 7A). Interestingly, as shown in Fig. 7A, fluoxetine, ICA and ICT treatment all increased the TNFα mRNA expression in hippocampus when compared to the stress + NS group ($p < 0.05$), and showed no difference on the expression of IL-6 mRNA. For IL-1β mRNA expression, ICT could decrease its expression (p < 0.01), while fluoxetine increased its expression (p < 0.01). What's more, compared to the stress + NS group, ICT markedly increase the IL-10 mRNA expression with a lower expression of ARGINASE-1 mRNA and CD206 mRNA, while fluoxetine and ICA had the opposite effect (Fig. 7A). We also found that in the hippocampus, pretreated with rHMGB1 could decrease the TNFα mRNA and IL-1β mRNA expression, compared to the stress group (data showed no difference), and rHMGB1 alone had the ability to decrease TNFα mRNA and IL-1β mRNA expression (Fig. 7B). For the M2 cytokines, compared to the stress group, rHMGB1 pretreatment showed a higher expression of CD206 mRNA and a lower expression of IL-10 mRNA expression (p < 0.05) (Fig. 7B).

3.7. ICA and ICT could alleviate SD stress induced higher expression of Iba-1 and lower expression of MAP-2 in mice hippocampus

We used the immunohistochemistry method to detect the expression of Iba-1 (the marker of microglia) and MAP-2 (the marker of neuron cell) in mice hippocampus. As we can see in Fig. 8A and B, NS treatment could increase the expression of Iba-1 and decrease the expression of MAP-2 in mice hippocampus, while, ICA and ICT had the ability to decrease Iba-1 expression and increase MAP-2 expression (Fig. 8A and B). As shown in Fig. 8C, compared to the stress group, rHMGB1

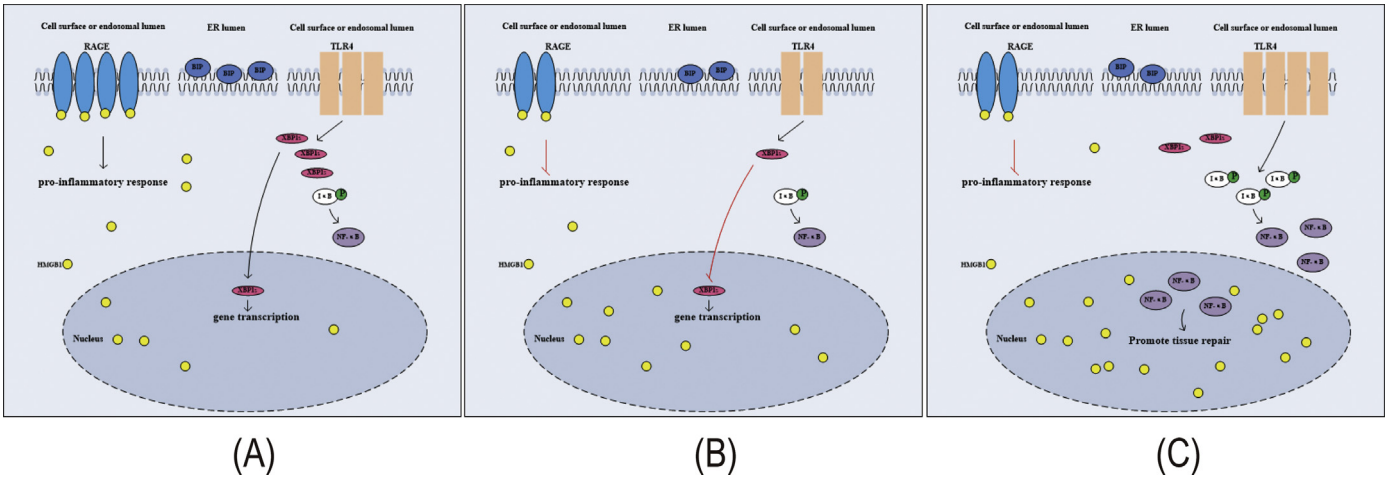

Fig. 9. The schematic diagram of the role of HMGB1 played in neuroinflammation. (A) SD could promote HMGB1 expression in the cytoplasm, activating HMGB1-RAGE signaling and TLR4-XBP1s signaling to increase pro-inflammatory cytokines in hippocampus. (B) rHMGB1 pretreatment could promote HMGB1 translocating into the nucleus, suppressing HMGB1-RAGE signaling and TLR4-XBP1s-ER stress related NF-κB signaling to decrease pro-inflammatory cytokines in hippocampus. (C) ICA and ICT could increase HMGB1 expression in the nucleus and decrease HMGB1 expression in the cytoplasm, suppressing HMGB1-RAGE signaling to reduce pro-inflammatory cytokines in hippocampus, and activating TLR4-NF-κB signaling to promote neuroregeneration.

pretreatment could decrease Iba-1 expression in mice hippocampus ($p < 0.01$), but had no effect on MAP-2 expression (Fig. 8D).

4. Discussion

In this study, we found that SD stress could induce an inflammatory response in mice, even after the stress was removed, the inflammation state also continued for 4 weeks. Moreover, we found that the cytoplasmic HMGB1 was critical for inflammatory response, while translocation from the cytoplasm into the nucleus was important for inflammation remission and might be correlated to tissue repair.

HMGB1 usually located in the nucleus. Once translocated to the cytoplasm or to the extracellular medium, it can acts as a damage-associated molecular patter (DAMP), altering nearby cells and the immune system to immediate danger, triggering inflammation [42]. In this study, mice exposed to SD stress appeared a high expression of cytoplasmic HMGB1 in the hippocampus, and at the same time, we can see a high TNFα expression in serum. Besides, in the hippocampus, the TNFα, IL-1β and IL-6 mRNA showed a high expression as well. These facts indicated that the translocation of HMGB1 from the nucleus to the cytoplasm might be related to the inflammatory response induced by stress. Next, we used rHMGB1 to further explore the mechanism, and we found that rHMGB1 alone could reduce the cytoplasmic HMGB1 expression in hippocampus, and reduce the TNFα expression in mice serum. Moreover, mice pretreated with rHMGB1 showed a lower expression of cytoplasmic HMGB1 in hippocampus and a lower TNFα expression in serum, when compared to the stress group. Taking together, we indicated that HMGB1 here could act as a DAMP to alert body about danger, and was bound up with inflammatory response in mice exposure to SD stress.

Numerous studies reported that high expression of HMGB1 had relationship with inflammation or depressive-like behavior [27,29], however, in our study, we found that the total HMGB1 expression was decreased in a SD stress, while pretreatment with rHMGB1 could attenuate the inflammatory response induced by SD stress in mice. Concerning the high expression of HMGB1 in the cytoplasm, we speculate that this phenomenon may be bound up with the location of HMGB1, but not the total expression of HMGB1. We found that ICA and ICT treatment showed a well anti-inflammatory effect here, and the mechanism may be that ICA and ICT could promote HMGB1 translocating from the cytoplasm to the nucleus.

It has been reported that RAGE, a pattern recognition receptor (PRR), binding a range of DAMPs including HMGB1, played a critical role in the innate immune system via binding to a range of DAMPs including HMGB1 to activate inflammatory responses [43]. In our study, the RAGE protein remained a high expression in the SD stress group, staying consistent with the high expression of pro-inflammatory cytokines. Moreover, no matter ICA and ICT treatment, nor rHMGB1 pretreatment, could reduce RAGE expression, when compared to the stress + NS group, or the stress group. Evidence showed that HMGB1-RAGE axis contributes to inflammatory response [44,45]. However, in our study here, we found that stress stimulation increased the expression of RAGE protein and decreased the total HMGB1 protein expression. Considering that the location of HMGB1 is related with inflammatory response, and we found that mice pretreated with rHMGB1 showed a lower expression of RAGE, we indicate that the high expression of RAGE protein might be related to the high expression of the cytoplasmic HMGB1.

Except RAGE, TLR4 is also a classic ligand of HMGB1, which was also reported to be linked with inflammation [46]. However, in this study, rHMGB1 pretreatment showed a lower TLR4 expression compared to the stress group. Evidence showed that TLR4 could activate NF-κB signaling [47,48], and ER stress took part in this progress [49,50]. We detected ER stress and NF-κB signaling here, and we can see, compared to the stress group, rHMGB1 pretreatment showed a lower expression of p65 in the nucleus and a higher expression of p65 in the cytoplasm. The ER stress was suppressed and XBP1s expression was decreased by rHMGB1 pretreatment. Basing on these results, we speculate that rHMGB1 pretreatment could suppress the TLR4 expression, then suppress the TLR4-XBP1s- ER stress related NF-κB signaling, appearing an anti-inflammatory effect. Unlike rHMGB1 pretreatment, ICA and ICT treatment increased TLR4 expression in SD stress model, and what's interesting, ICA and ICT treatment could activate IκB, increase the total p65 expression and the nucleus p65 expression. We think this might be attributed to the activation of TLR4-NFκB signaling, resulted in promoting p65 translocation to the nucleus. Previous studies showed that increased expression of p65 in the nucleus could promote tissue repair [23,51]. This might explain the effect of ICA and ICT on MAP-2 protein expression increase in hippocampus.

As we observed, the defeated mice showed a social withdrawal and a reduced exploratory behavior compared to the undefeated mice. Our team had previously identified the antidepressant effect of ICA and ICT in rats and mice [31–33], and we also found that ICA and ICT showed a better effect on attenuating depressive behaviors than fluoxetine here.

Moreover, rHMGB1 pretreatment could obviously increase the time that the defeated mice spent to interact with CD1 mice, improving the exploratory behavior. These findings indicate that ICA and ICT could attenuate the depressive behavior in mice via ameliorating neuroinflammation, which was related to the translocation of HMGB1.

5. Conclusion

We speculate that the neuroinflammation induced by SD stress showed a HMGB1-RAGE signaling activation and TLR4-XBP1s-ER stress related NF-κB signaling activation, which are related to the increased expression of HMGB1 in the cytoplasm. Prolonged neuroinflammation caused neural damage, which are detrimental to depressive syndromes (Fig. 9A). ICA and ICT could promote HMGB1 translocation to the nucleus, on the one hand, suppressing the HMGB1-RAGE signaling to anti-inflammation, on the other hand, might activating TLR4-NF-κB signaling, increasing p65 expression in the nucleus to promote neural regeneration (Fig. 9C). While rHMGB1 pretreatment also decreased the cytoplasmic HMGB1 expression, suppressing the HMGB1-RAGE signaling, and suppressed the TLR4-XBP1s-ER stress related NF-κB signaling (Fig. 9B). In another study, we found that the total HMGB1 was significantly increased in mice hippocampus which was administered by intraperitoneal injection of LPS for 24 h, and interestingly, we also observed a higher cytoplasmic HMGB1 expression and a lower nuclear HMGB1 expression [52]. This might indicate that HMGB1 increased at the initial stage to act as a DAMP, and as the damage lasted, on the contrary, HMGB1 decreased, and this might be a clue for neural damage, and the cytoplasmic HMGB1 is key for inflammatory response. ICA and ICT showed a good anti-neuroinflammatory effect and a neuroprotective effect here via mediating HMGB1 expression, thus improved depressive behaviors of the defeated mice, making them possible to act as a kind of antidepressant drug.

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