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# HIGH-MOBILITY GROUP BOX1 PROTEIN PROMOTES NEUROINFLAMMATION AFTER INTRACEREBRAL HEMORRHAGE IN RATS

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**Abstract—High-mobility group box1 (HMGB1) protein is massively released into the cytoplasm and induces inflammation following various insults such as sepsis, acute cerebral ischemia, and pancreatitis. However, whether HMGB1 can act as an early proinflammatory cytokine to promote inflammation after intracerebral hemorrhage (ICH) is unclear. We explored this question using a rat model of collagenase-induced ICH. We found that HMGB1 was released into the cytoplasm soon after ICH. Administration of ethyl pyruvate decreased the level of HMGB1 and microglia around the hematoma. Ethyl pyruvate also ameliorated ICH-induced neuronal apoptosis, cerebral edema, and neurological impairment. These findings suggest that HMGB1 may act as an early proinflammatory cytokine within the neurovascular unit to mediate inflammation during the acute phase of ICH.** 

**Key words: intracerebral hemorrhage, high-mobility group box1, ethyl pyruvate, inflammation.**

## INTRODUCTION

Intracerebral hemorrhage (ICH) involves initial and secondary injury. Initial injury occurs due to direct mechanical force of the expanding hematoma. Secondary injury results when the hematoma releases its breakdown products, such as thrombin and ferrous iron, which activate inflammation after ICH (Wasserman and Schlichter, 2007; Moskowitz et al., 2010; Hwang et al., 2011). Substantial evidence indicates that inflammation contributes to brain injury during the acute phase of ICH; this inflammation involves the production of inflammatory cytokines, activation of brain microglia and migration of peripheral immune cells into the ipsilateral brain (Felger et al., 2010; Dénes et al., 2011; Bazan et al., 2012; Lambertsen et al., 2012; Ortega et al., 2012). ICH-related inflammation is associated with blood–brain barrier (BBB) disruption, cerebral edema, microglial activation, and cellular necrosis and apoptosis (Wang and Dore, 2007; Hwang et al., 2011). The significance of this inflammation in the complicated pathophysiology of ICH is widely recognized. However, which factors act as early proinflammatory cytokines after ICH remains unclear.

High-mobility group box1 (HMGB1) protein is a highly conserved nonhistone DNA-binding protein widely expressed in most eukaryotic cells, including neural cells (Apetrei et al., 2011; Gao et al., 2012; Li et al., 2012). Nuclear HMGB1 stabilizes nucleosome formation and regulates gene expression by facilitating transcription (Kojima et al., 2012; Zhang et al., 2012a).

HMGB1 is also an important proinflammatory cytokine that mediates inflammation following insults such as sepsis, acute cerebral ischemia, and pancreatitis (Hayakawa et al., 2010a; Ohnishi et al., 2011; Sansing et al., 2011). HMGB1 can be actively released into the cytoplasm by activated macrophages, myeloid dendritic cells, and natural killer cells (Hayakawa et al., 2008; Luan et al., 2010; Wang, 2010). HMGB1 can signal via various receptors such as the receptor for advanced glycation end products (RAGE), toll-like receptor-2 (TLR2), and toll-like receptor-4 (TLR4). These receptors are expressed not only in peripheral macrophages, but also in microglia and neurons in the central nervous system (Naglova and Bucova, 1999; del Zoppo, 2010; Kim et al., 2010). HMGB1 can bind to its receptors and activate a downstream pathway that triggers inflammation by upregulating interleukin beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and other cytokines (Kreutzberg, 1996; Yang et al., 2004; Kim et al., 2006; Qiu et al., 2008; Khasnavis et al., 2012).

Increasing evidence indicates that microglia are the innate immune cells of the central nervous system, and that they respond quickly to various injuries, even to minor pathological changes (Ransohoff et al., 2012;

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*Abbreviations:* BBB, blood–brain barrier; EP, ethyl pyruvate; HMGB1, high-mobility group box1; ICH, intracerebral hemorrhage; RAGE, receptor for advanced glycation end products; TLR4, toll-like receptor-4; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay.

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Sato et al., 2012; Zhang et al., 2012b). Microglia are known to initiate cerebral inflammation and induce brain cell apoptosis after various brain insults; they do so by releasing cytotoxic products, such as reactive oxygen and nitrogen species, proinflammatory cytokines, and proteases (Kreutzberg, 1996; Khasnavis et al., 2012).

Ethyl pyruvate (EP) is a stable and lipophilic ester derived from the endogenous metabolite pyruvic acid. The pharmacological effects of EP include the amelioration of redox-mediated damage to cells and tissues, inhibition of proinflammatory cytokine secretion, and inhibition of apoptosis (Yu et al., 2005; Kao and Fink, 2010; Su et al., 2011). EP acts via a HMGB1/TLR4/NF-κB-mediated pathway to decrease HMGB1 expression in the injured rat brain (Su et al., 2011).

The documented activity of HMGB1 as a proinflammatory cytokine in the brain prompted us to explore whether HMGB1 acted as an early proinflammatory cytokine to mediate inflammation during the acute phase of ICH. We tested this question using collagenase to induce ICH in rats.

## EXPERIMENTAL PROCEDURES

### Animals and treatment groups

All animal care and use procedures were in accordance with established regulations at the Sichuan University. Male Sprague–Dawley rats (270–320 g) were divided into the following groups: (1) EP group ($n$ = 33), in which EP was administered, ICH was induced and EP was administered at various time points afterward; (2) ICH group ($n$ = 36), which was treated in the same way as the EP group except that 0.9% saline was administered instead of EP each time; (3) sham group, which was subjected to the same procedures as the EP and ICH groups, except for ICH induction and EP administration. These procedures were described in greater detail in the following sections.

### ICH induction

Collagenase-induced ICH was induced in rats as previously described (Ohnishi et al., 2011). Rats were anesthetized with 3.6% chloral hydrate (l ml/100 g) and placed in a stereotaxic frame. Following disinfection and incision, a hole was drilled in the skull. Collagenase VII (Sigma C0773, 2.0 μl) was injected through a trace syringe into the striatum at 0.2 mm anterior, 3.0 mm lateral, and 5.5 mm ventral from the bregma. The syringe was kept in position for 2 min to prevent backflow of collagenase, then slowly removed. A thermo-regulated heating pad was used to maintain epidermal temperature at 37 °C.

### EP administration

EP dissolved in 0.9% saline solution (Sigma) was administered at a dose of 80 mg/kg via intraperitoneal (i.p.) injection 30 min before ICH induction, and then at 2, 24, and 48 h after induction.

### Immunohistochemical analysis

At the indicated times rats were killed by perfusion with saline and 4% paraformaldehyde. Brains were cleared of fat and water and embedded in paraffin. Sections 16 mm thick were cut onto slides. Sections were incubated overnight at 4 °C in a 1:500 dilution of polyclonal rabbit anti-HMGB1 primary antibody (Abcam, USA) and a 1:400 dilution of polyclonal rabbit anti-Iba1 (Abcam). Sections were then incubated for 15 min at room temperature with anti-rabbit secondary antibody (Abcam). Finally, the sections were observed by light microscopy and analyzed by Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) ($n$ = 3 for each time point).

### Immunofluorescence analysis

After depolarization and rehydration, sections were incubated overnight at 4 °C in a 1:500 dilution of polyclonal rabbit anti-HMGB1 primary antibody and a 1:400 dilution of polyclonal rabbit anti-Iba1. Sections were then incubated with secondary anti-rabbit antibody for 2 h at room temperature. Finally, the sections were observed by fluorescence microscopy and analyzed by Image-Pro Plus 6.0. Integrated optical density (IOD) was used to quantify positive cells (3 rat sections for each group).

### TUNEL staining

After depolarization and rehydration, sections were assayed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL; Roche). Coverslips were analyzed by microscopy ($n$ = 3 for each group); deep purple cells were TUNEL-positive cells.

### Western blotting

Rat striatum samples were analyzed by 12% SDS–polyacrylamide gel electrophoresis. Samples were homogenized for 30 min at 4 °C in lysis buffer containing a protease inhibitor cocktail. Homogenates were centrifuged at 13300 rpm for 40 min at 4 °C. Supernatants were mixed with loading buffer containing dithiothreitol (DDT) and boiled for 10 min. Samples were electrophoresed, and proteins were transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% milk for 24 h at 4 °C, then incubated for 2 h at 37 °C in a 1:1000 dilution of polyclonal rabbit anti-HMGB1 primary antibody and a 1:1000 dilution of polyclonal rabbit anti-β-actin (both from Abcam USA), which served as an internal control. The membranes were washed three times at room temperature at in TBST. The samples were then incubated for 1 h at 37 °C with a 1:10,000 dilution of HRP-conjugated secondary antibody. Finally, the blotted protein bands were visualized by enhanced chemiluminescence and analyzed by Image J 1.46 software (U.S. National Institutes of Health) ($n$ = 5 for each group).

### Brain water content

At 72 h after ICH induction, rats were decapitated under deep anesthesia. Ipsilateral brains were quickly harvested and weighed to obtain the wet weight. The samples were dried in an oven at 110 °C for 48 h, and weighed again to obtain the dry weight. Brain water edema was calculated using the formula ($n$ = 5 for each group):

$$\text{percentage of brain water} = [(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100\%.$$

### Evaluation of neurological impairment

Neurological impairment was assessed at 24 and 72 h after ICH induction using tests of postural reflex and forelimb placing ($n$ = 8 in each group for each test). Postural reflex was scored