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Expression level and distribution of HMGB1 in Sombati's cell model and kainic acid-induced epilepsy model

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Abstract. – OBJECTIVE: Observe the expression and distribution of HMGB1 in Sombati's cell model and kainic acid-induced epileptic rats' model.

MATERIALS AND METHODS: Dissociated hippocampal neurons from neonatal SD rats and cultured those for 9 days, then changed medium to Mg^{2+}-free medium for 3 hours to induce Sombati's cell model. The expression level of HMGB1 in the neurons was detected at 24h and 72h by Western Blotting. Appropriate kainic acid was injected into the lateral ventricles to induced epileptic rats' model *in vivo* trial, the expression level and distribution of HMGB1 at 24h and 72h were established by immunohistochemistry.

RESULTS: The expression level of HMGB1 showed significantly different between model group and control group both *in vitro* and *in vivo* trials. At 24h, the expression level of HMGB1 in the model group was lower than the control group ($p < 0.05$), and became higher than the control group at 72h ($p < 0.05$). From the *in vivo*-trial, a nucleus-to-cytoplasm translocation was also discovered.

CONCLUSIONS: This investigation indicates that HMGB1 plays a crucial role in the pathophysiology of epilepsy, by altering its quantity and distribution.

Key Words:
HMGB1, Sombati's cell model, Kainic acid-induced epilepsy animal model, Hippocampal neurons.

Introduction

Inflammation is considered to be a key factor in pathophysiology of epilepsy, the interaction between epilepsy and the immune system attracted public attention in past decades[1-4]. Some studies maintained that inflammation might induce epilepsy[5,6], while others suggested that epilepsy was the cause of inflammation[7,8]. Moreover, more and more inflammatory factors were discovered in the epileptic model brain tissue, such as tumor necrosis factor (TNF)[9], interleukin-1 (IL-1)[10], and so on.

Growing evidence demonstrated that brain inflammation played a crucial role in the pathophysiology of epilepsy[1]. High-mobility group box 1 (HMGB1), which is secreted by neurons, arouses scientists' attention because its crucial function during epilepsy[11,12]. Previous study suggested that HMGB1 might be a key mediator in inflammation[11]; however, the mechanism remained unclear.In this study, we tried to detect the expression level and distribution altering of HMGB1 in the Sombati's cell model and rat kainic acid-induced epileptic rats' model of intractable epilepsy and discussed the relationship between HMGB1 and epilepsy.

Materials and Methods

Ethic Statement

All rats were disposed according to the National Institutes of Health Guidelines (China) for the Care and Use of Laboratory Animals. This protocol was ratified by the Bioethics Committee of Guangxi Medical University.

Neural Cell Cultures and Sombati's Cell Production

Pure neuronal cultures were prepared by seeding hippocampal cells obtained from 1-day-old postnatal rats as precious discription[13]. Neurons were cultured in Neurobasal medium (2%B-27 supplement, 0.2 mol/L L-glutamate, and 98% neurobasal medium, all materials from GIBCO, Carlsbad, CA, USA) for 9 days before product-

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ing Sombati's Cell. The neurons were divided into model group (n=5) and control group (n=5). The model group were exposed to media without added Mg^{2+} for 3h as the standard treatment procedure and then returned to Mg^{2+} containing media, while the control group were culture by Mg^{2+} containing media overall the procedure[14].

Western Blotting

The protein levels of HMGB1 in these two groups were determined at 24h and 72h respectively. Cells were collected in Eppendorf tubes, centrifuged (1000 g for 5 min at 4°C), and resuspended in lysis buffer (50 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 4 lg/mL aprotinin and leupeptin, 1% sodium dodecyl sulfate). Fifty-to-Sixty micrograms of protein per lane were loaded. After electrophoresising in 12% SDS-polyacrylamide gels, protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Hybond ECL; Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% skimmed milk and then probed overnight with primary rabbit anti-HMGB1 monoclonal antibody 1:10000 (Abgent, Media, PA, USA) overnight at 4°C. After hatching in fluorescent goat anti Rabbit IgG 1:9000 (Lincoln, NE, USA) in 25°C for 90 min, the PVDF membranes were scanned by Licor Odyssey Infrared Imaging System (from Li-Cor Bioscience, Lincoln, NE, USA). Data are expressed as mean ± standard errors, values were considered significantly difference when $p < 0.05$.

Paracele-injection and Animal-Model Establishing

Thirty-two of adult male SD rats (200-250 g of body weight) were divided into model group and control group randomly. Normal saline or kainic acid (2 μg/kg) was injected (0.05 μL/min) in the left paracele of anesthetized mice.The grade of seizures were recorded using the standard of Racine[15], the rats suffered from epileptic seizure which reach IV-V level were considered to be epileptic.

Rats were killed and 4 um hippocampal paraffin sections were made at 24h and 72h after injection.

Immunohistochemistry

Immunohistochemistry was performed in the Histology And Embryology Research Laboratory of the Guangxi Medical University using the following commercially available anti-HMGB1 monoclonal antibody (1:400, from Abgent, USA) at 4°C after dewaxing and blocking by 10%normal goat serum (Zhongshan Golden Bridge, Beijing, China). Incubating with matching secondary antibodies of goat anti-rabbit IgG (Zhongshan Golden Bridge, Beijing, China) at 37°C for 30 min the next day was done subsquently, then used 3, 3'-diaminobenzidine (DAB) as selenium organic reagent. The positive cells were yellowish-brown stained while the negative cells were not using light microscope. Six visual field images were obtained in every section by the image acquisition system (Olympus, Tokjo, Japan) randomly and analyzed by Imagepro-Plus 6.0 automatically. The averaged optical density (mean OD) was obtained from the immunohistochemical assay accounts, and the average density was calculated.

Statistical Analysis

Data are expressed as mean ± standard errors, values were considered significant difference when $p < 0.05$.

Results

Expression Level of HMGB1 in Sombati's Cell's Model

The protein expression level of HMGB1 in the cell model was detected by Western blotting. The optical density of the model group was lower than the control group at 24h ($OD_{model\ group} = 0.3331 \pm 0.21602$, $OD_{control\ group} = 0.5359 \pm 0.22741$; $p = 0.021$, $p < 0.05$). Moreover, the expression level of HMGB1 in the model group turned higher than the control group ($OD_{model\ group} = 0.8570 \pm 0.2528$, $OD_{control\ group} = 0.4846 \pm 0.26209$; $p = 0.003$, $p < 0.05$) at 72h (Figure 1A and B). In model group, the OD value was much higher at 72h than 24h ($p < 0.05$) (Figures 1 and 2).

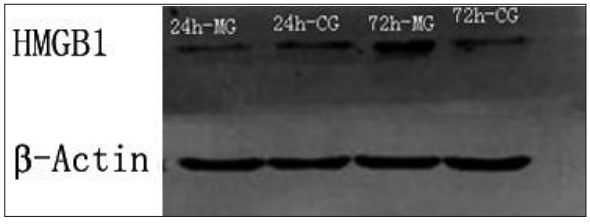

Figure 1. The Western blotting results of HMGB1 in Sombati's cell model at 24h and 72h. Same amount of β-actin was used as control. (MG: model group; CG: control group).

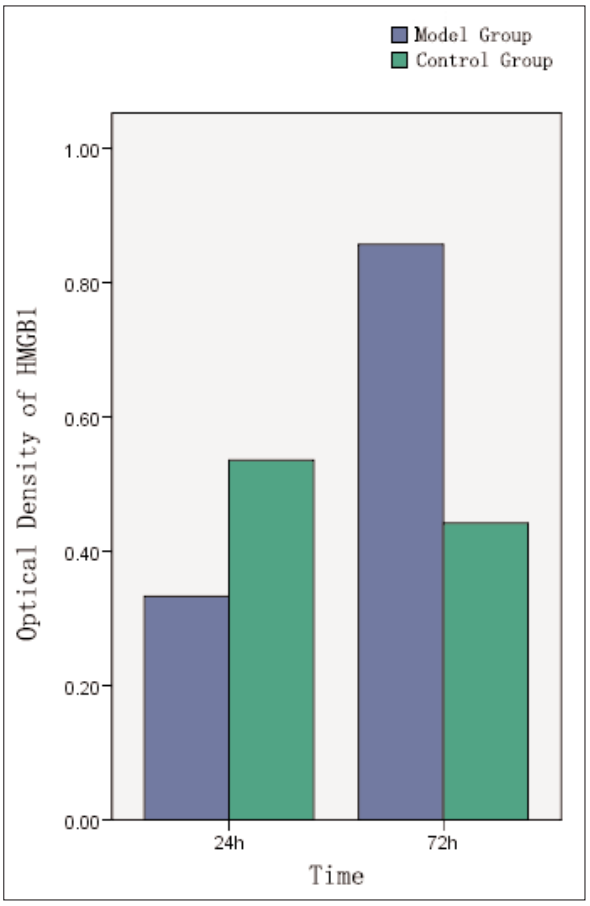

Figure 2. HMGB1 level at 24h and 48h in model cell and control cell after establishing Sombati's cell model. The optical density of the model group was lower than the control group at 24h (ODmodel group = 0.3331 ± 0.21602, ODcontrol group = 0.5359 ± 0.22741; $p = 0.021$, $p < 0.05$). The optical density of the model group was higher than the control group at 72h (ODmodel group = 0.8570 ± 0.2528, ODcontrol group = 0.4846±0.26209; $p = 0.003$, $p < 0.05$).

Expression Level and Distribution of HMGB1 in The Rats' Model of Kainic Acid-induced Epilepsy

The averaged optical density of the model group was lower than the control group significantly at 24h ($OD_{model\ group} = 0.1398 \pm 0.01801$, $OD_{control\ group} = 0.2154 \pm 0.02873$; $p = 0.000$, $p < 0.01$) and became higher than control group at 72h ($OD_{model\ group} = 0.3652 \pm 0.08330$, $OD_{control\ group} = 0.2354 \pm 0.00836$; $p = 0.012$, $p < 0.05$), which accorded with the results of cell's model (Figure 3). Moreover, there were more yellowish-brown staining in cytoplasm in the model group at 72h than at 24h, and no changes in the control group was found (Figure 4).

Discussion

HMGB1 was a kind of highly conserved protein that presented in nuclei of eukaryotic cells[16]. HMGB1 played a crucial role of binding DNA to make structure stable, like histone. Recent studies[17] demonstrated that HMGB1 was over expressed and released to extracellular fluid when cells were stimulated by endotoxin, stress, shock, infection and so on. Other studies[18,19] stated that such release could also be found in most necrotic cells. Wang et al[17] found that HMGB1 over expressed 8 hours after exposing to endotoxin, TNF, or IL-1 in cultured macrophages, indicating that HMGB1 was a late cytokine when inflammation occurs, which was different to early cytokines, such as TNF and IL-1. Moreover, Yu et al[20] found that IL-8 and TNF released when TLR2 or TLR4 on the surface had binded by HMGB1, and correspond inhibitors of such receptors paused the processes, indicating HMGB1 induced further inflammation by binding to cell

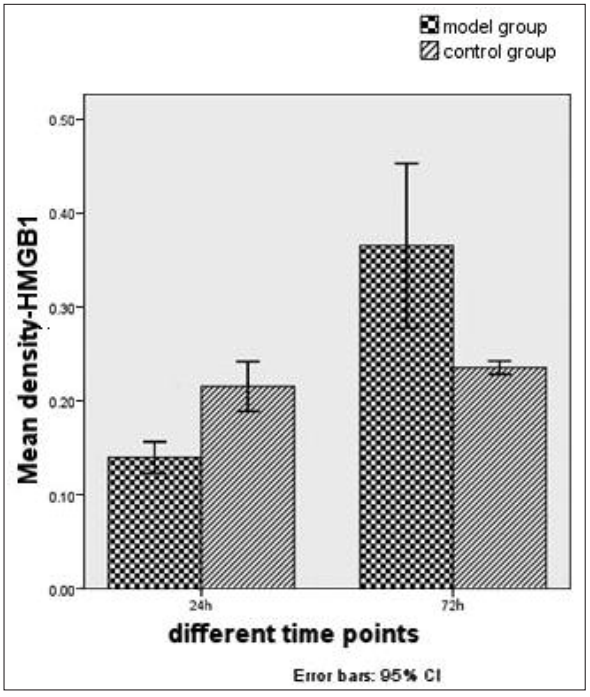

Figure 3. HMGB1 level at 24h and 48h in kainic acid-induced epileptic rats' group and normal rats' group. The averaged optical density of the model group was lower than the control group significantly at 24h (OD model group = 0.1398 ± 0.01801, OD control group = 0.2154 ± 0.02873; $p = 0.000$, $p < 0.01$). The averaged optical density of the model group was higher than the control group significantly at 72h (OD model group = 0.3652 ± 0.08330, OD control group = 0.2354 ± 0.00836; $p = 0.012$, $p < 0.05$).

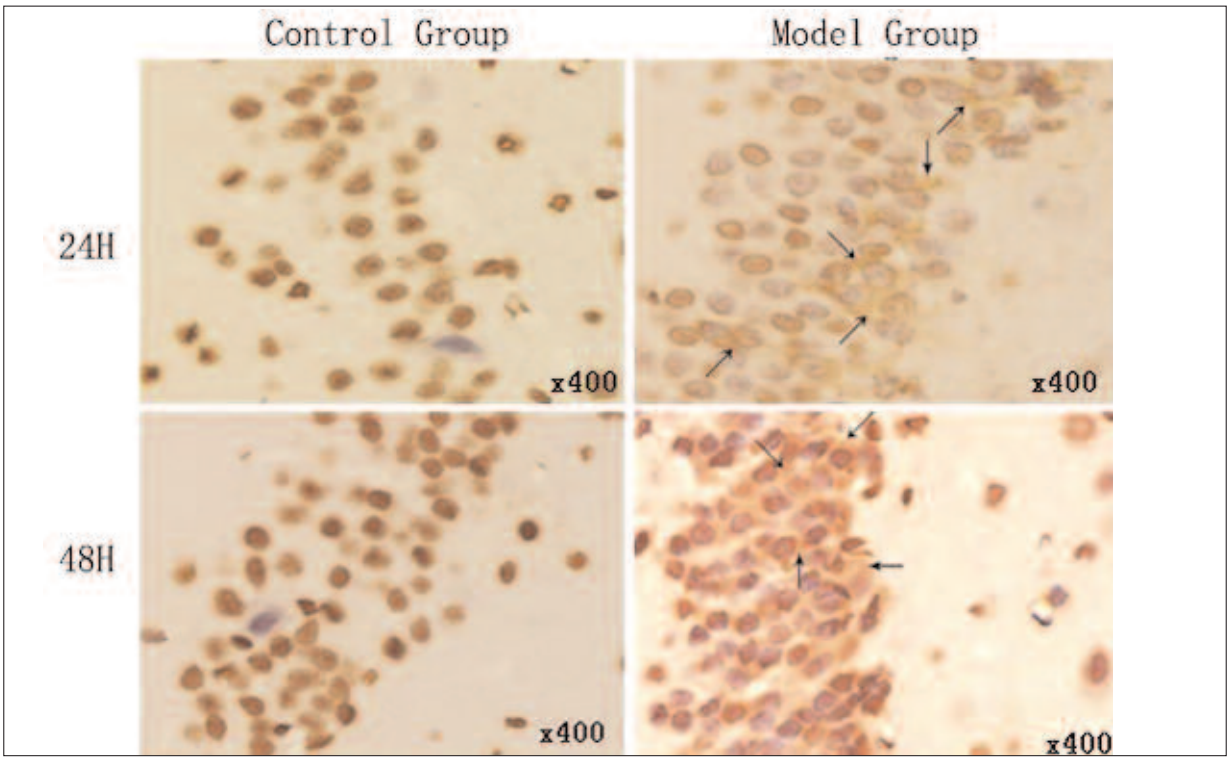

Figure 4. The distribution of HMGB1 at 24h and 48h in kainic acid-induced epileptic rats' group and normal rats' group under optical microscope with magnification of X400, a positive result was yellowish-brown stained. In the model group, vast majority of HMGB1 distributes in cytoplasm which are marked with dark arrowhead, while HMGB1 is confined to nucleus in control group.

surface receptors, resulting in movement of chemotactic cell and the releasing of pro-inflammatory cytokines[21].

Epilepsy which was considered to be a kind of chronic neurological disorders, was reported to be associated with infection. Multiple evidences supported that inflammation cause epilepsy, which is like other common autoimmune or infectious diseases[22], on the contrary, Vezzani et al[23] stated that cytokines, such as TNF-α, IL-6 and IL-1β[24,25], were significantly increase in animal brains after suffering from status epileptics[26,27], indicating epilepsy may also induce inflammation.

Level of early cytokines, like IL-1[8,28], TNF-α[29] and COX2[29,30] increase in the epileptic brain tissues of rats and patients, and these cytokines were confirmed to be epilepsy-associated cytokines by multiple studies. From our study, HMGB1, the late inflammatory cytokine, also increased at 72h in cultured neurons and rats' hippocampuses in epileptic model group, indicating HMGB1 is also an epilepsy-associated cytokine and be stirred under epileptic condition. Moreover, we also noticed that the optical density value was lower in model group than in control group at 24h both in cell's and animals' model, indicating epileptic cell release less HMGB1 in the early stage of epilepsy. Combining with the results at 72h, we can also conclude that excess HMGB1 has been synthesized between 24h and 72h, and vast majority of cytokines was reserved in cytoplasm rather than release outside, further release might occur late.

Some precious studies also concluded that classical cytokines were reasons of epilepsy, and Toll-like receptor 4 (TLR4), receptor for Advanced Glycation Endproducts (RAGE) and IL-1R were the receptors of HMGB1[20], as a result, whether such receptor participate in epilepsy is still unkown, and need more studies to focus on.

Conclusions

HMGB1 was a kind of late inflammatory cytokine and over expressed both in vivo and vitro epileptic model after cultivating for 72h. Our study indicated HMGB1 was a epilepsy-associated cytokine, further studies should focus on it.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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