Department of Pharmacy[1], Department of Cardiology[2], the Second Hospital of Shandong University; Shandong Center for Drug Evaluation & Certification[3], Jinan, China

Acetylpuerarin protects against OGD-induced cell injury in BV2 microglia by inhibiting HMGB1 release

Deqing sun[1,#], aiying xue[2,#], xia xue[1], manru ren[3], jing wu[1], rongmei wang[1,*]

Received August 9, 2017, accepted September 11, 2017

** Corresponding author: Rongmei Wang, Department of Pharmacy, the Second Hospital of Shandong University, 247 Beiyuan Street, Jinan 250033, China*
sdeyyjk@126.com
[#]Co-first authors

Pharmazie 73: 92–97 (2018) *doi: 10.1691/ph.2018.7819*

High mobility group box 1 (HMGB1), a non-histone DNA-binding protein, is massively released into the extracellular space from neuronal cells after ischemic injury, initiates inflammatory response and aggravates brain tissue damage. Acetylpuerarin (AP), an acetylated derivative of puerarin, was reported to protect against cerebrovascular ischemia-reperfusion injury in rats through anti-inflammation. In the present study, we aim to investigate whether AP inhibited HMGB1 release in oxygen-glucose deprivation (OGD)-treated BV2 microglia. BV2 microglia viability after OGD with or without AP was measured by CCK-8 assay, apoptosis of BV2 microglia was determined by Hoechst 33258 staining and FITC-Annexin V/7-AAD staining. HMGB1 protein level and release was detected by western blotting and immunofluorescent FITC-staining. The results demonstrated that AP significantly rescued OGD-induced cell death and apoptosis in a dose-dependent manner. AP inhibited OGD-induced HMGB1secretion at the level of nuclear to cytoplasmic translocation, decreased cytoplasmic HMGB1 at protein level, and the effects showed dose-dependent. The findings suggest that AP can protect against OGD-induced cellular injury in BV2 microglia by inhibition of HMGB1 release.

1. Introduction

Stroke is one of the leading causes of death and adult disability throughout the world. Of the subtypes, ischemic stroke makes up 80% of the disability and death (Jia et al. 2011). In spite of substantial research efforts, a lack of specific and effective drugs is still a major problem for this obstinate illness.

Although the pathophysiological processes of stroke have been extensively studied, the mechanisms of brain injury are not fully understood. It is generally considered that acute ischemic stroke involves a complex array of pathophysiological processes, and many molecular mechanisms have been implicated in the processes, including glutamate excitotoxicity, ionic imbalance, oxidative stress and programmed cell death pathways comprising apoptosis, necroptosis and autophagy (Puyal et al. 2013; Quillinan et al. 2016). In addition, accumulating evidence shows the integral role of inflammatory response in the pathogenesis of ischemic stroke and secondary damage (Shukla et al. 2017; Vidale et al. 2017). Following transient or permanent middle cerebral artery occlusion (MCAO) in rats, acute inflammatory response occurs within hours and persists for days after the insult, and is currently thought to contribute to neuronal death (Toth et al. 2015).

High mobility group box1 (HMGB1), a non-histone DNA-binding protein, is implicated in the stabilization of nucleosomal structure and the facilitation of gene transcription (Naglova and Bucova 2012). Recently, HMGB1 has been characterized as a cytokine that initiates inflammatory response after tissue injury (Asavarut et al. 2013; Martinotti et al. 2015). HMGB1 normally resides in the nuclei of cells, but when cells are injured or underwent necrosis, HMGB1 can be secreted by activated macrophages/monocytes, or passively released by necrotic cells. Extracellular HMGB1 activates innate immune cells and endothelial cells to produce proinflammatory cytokines, chemokines, tissue factors, and adhesion molecules, thus amplifies an inflammatory response (Martinotti et al. 2015). Administration of HMGB1 to normal animals produces systemic inflammatory responses including fever, weight loss, acute lung injury, epithelial leakage syndrome, arthritis and organ failure; on the contrary, anti-HMGB1 treatment with antibodies, specific antagonists or other pharmacological agents, is beneficial in many preclinical inflammatory disease models, ameliorates severity of diseases and reduces mortality (Yang et al. 2013). In the central nervous system (CNS), HMGB1 is highly expressed in the nuclei of neurons and glial cells, and involved in the inflammatory process (Fang et al. 2012). HMGB1 is released into the extracellular space early after ischemic injury; the released HMGB1, in turn, increases brain levels of proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), thus inducing neuroinflammation and microglial activation in the post-ischemic brain and aggravating brain injury (Singh et al. 2016). Therefore, it is a promising strategy for the treatment of post-ischemic injury to inhibit the release of HMGB1.

Acetylpuerarin, an acetylated derivative of puerarin, is considered a potential drug for ischemic stroke. Previous studies have indicated

Table: Effects of AP on the viability of OGD injured BV2 microglia by CCK-8 assay (mean±SD, n=6).

Group	Viability (%)
Control	100 ± 6.01
OGD/R-6 h	70.89 ± 1.92 ++
OGD/R-12 h	67.24 ± 2.56 ++
OGD/R-24 h	65.27 ± 3.08 ++
OGD/R-48 h	65.18 ± 2.51 ++
OGD/R-24 h + AP-L (0.1 μM)	72.89 ± 3.24 *
OGD/R-24 h + AP-M (0.4 μM)	75.47 ± 4.16 **
OGD/R-24 h + AP-H (1.6 μM)	85.13 ± 3.08 **
OGD/R-24 h + EP (1 mM)	86.56 ± 3.87 **

The viability of the BV2 microglia without OGD injury is defined as 100%, AP at increasing concentrations (0.1, 0.4 and1.6 μM) and EP (1 mM) were treated before the OGD treatment and on the onset of reoxygenation.
++p<0.01 vs control group; *p<0.05, **p<0.01 vs OGD/R-24h group.

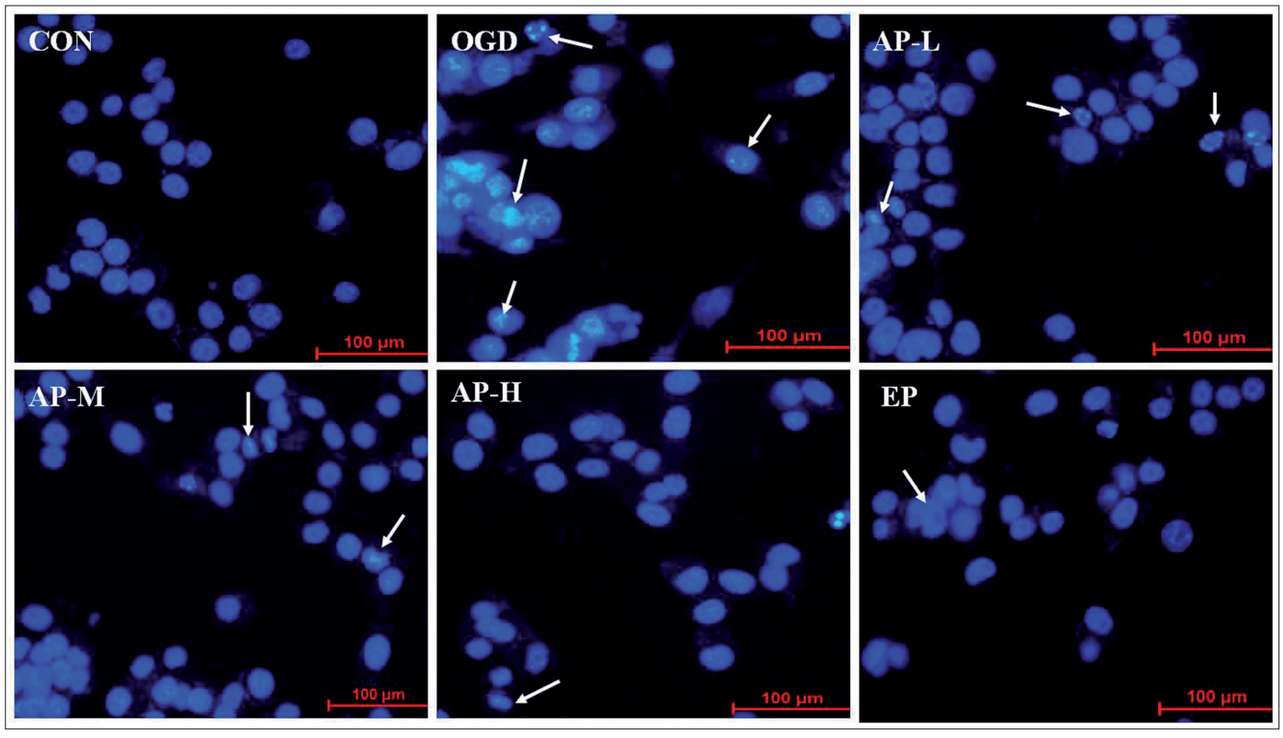

Fig. 1: Effects of AP on apoptosis in BV2 microglia by Hoechst 33258 staining.
BV2 microglia were pretreated with AP before the onset of OGD and then incubated with AP for an additional 24 h. The cells were then stained with Hoechst 33258, and observed under a fluorescent microscope at magnification of 200 ×. The scale bar is 100 μm. Arrows indicate apoptotic characteristics of the cells. CON, control cells; OGD, cells treated with oxygen-glucose deprivation/reoxygenation (OGD/R) for 2.5 h/24 h; AP-L, AP-M, AP-H represents 0.1, 0.4 and 1.6 μM AP treated BV2 microglia with OGD/R for 2.5 h/24 h, respectively; EP, EP (1 mM) treated BV2 microglia with OGD/R for 2.5 h/24 h.

the protective effect of AP against cerebrovascular ischemia-reperfusion injury in rats, and the mechanisms underlying this protection may involve increases in antioxidant enzyme, reductions in the damage induced by reactive oxygen species, decreases in the calcium concentration in neurons (Liu et al. 2007, 2013). Moreover, AP is found to inhibit the inflammatory response in the ischemic area after focal cerebral ischemia (Meng et al. 2013; Xiang et al. 2014). We suppose that AP protects against post-ischemic brain injury by inhibiting the release of HMGB1 into extracellular space. Glial cells such as astrocytes, oligodendrocytes and microglia play a beneficial role in neural cell viability and survival. In particular, microglia are involved in host defense and tissue repair in the CNS (Kraft and Harry 2011). In response to extracellular stimuli, microglia release pro-inflammatory mediators and cytokines to restore CNS homeostasis by clearing pathogens and infected cells (Zhao et al. 2017). However, overproduction of these inflammatory mediators under pathological conditions causes chronic neuroinflammation, promotes various neurodegenerative diseases, and eventually leads to neuronal cell death (Xiong et al. 2016). Therefore, the activated microglia are regarded as a marker of post-ischemic inflammation. Thus, control of microglial activation provides a potential therapeutic approach for treating ischemic cerebrovascular diseases.

To examine whether AP inhibits HMGB1 release from activated microglia, BV2 cells, a microglia cell line, were activated by *in vitro* oxygen-glucose deprivation/reoxygenation (OGD/R) treatment. The protective effects of AP against OGD injury and AP-mediated suppression of HMGB1 release were examined in the present study.

2. Investigations and results

2.1. *Effects of AP on OGD-induced cell death in BV2 microglia*

As estimated by CCK-8 assay, compared to those of control group, the cell viabilities markedly ($p<0.01$) decreased after OGD for 2.5 h followed by 6, 12, 24 and 48 h incubation with normal medium, which were 70.89±1.92 %, 67.24±2.56 %, 65.27±3.08 %, and 65.18±2.51 %, respectively.

To investigate whether AP protects BV2 microglia against injury after OGD, the cells were pretreated with different doses of AP prior to OGD and on the onset of reoxygenation, and the cell viability was measured 24 h after reoxygenation. As presented in the Table, compared to OGD/R-24 h group, AP significantly increased the viability of BV2 cells in a concentration-dependent manner. The findings suggest that AP can protect BV2 microglia against OGD-induced injury.

2.2. *Effects of AP on OGD-induced apoptosis in BV2 microglia*

To evaluate the effects of AP on OGD-induced apoptosis in BV2 microglia, we examined the morphologic changes by Hoechst 33258 staining. As shown in Fig. 1, the nuclei of BV2 microglia were round and homogeneously stained in the control group; however, the OGD injured cells exhibited evident apoptosis characteristics including cell shrinkage and membrane integrity loss or deformation, nuclear fragmentation and chromatin compaction of late apoptotic appearance; when the BV2 microglia were treated with different doses of AP (0.1, 0.4 and 1.6 μM) or EP (1 mM), the apoptotic morphologic changes were improved as compared with the OGD model group.

BV2 microglia were further stained with FITC-Annexin V/7-AAD, and analyzed by flow cytometric analysis. As shown in Fig. 2, the apoptosis rate of normal cells was only 2.7%, while the apoptosis of OGD injured BV2 cells significantly ($p<0.01$) increased to 28.6%. When treated with AP at the concentrations of 0.1, 0.4 and 1.6 μM, the percentage of apoptotic cells decreased to 19.7%, 15.1% and 12.7%, respectively, indicating that AP decreased the apoptosis in a concentration-dependent manner. In addition, AP at 1.6 μM showed similar protective effects with EP (1 mM) owning an apoptosis rate of 13.2%.

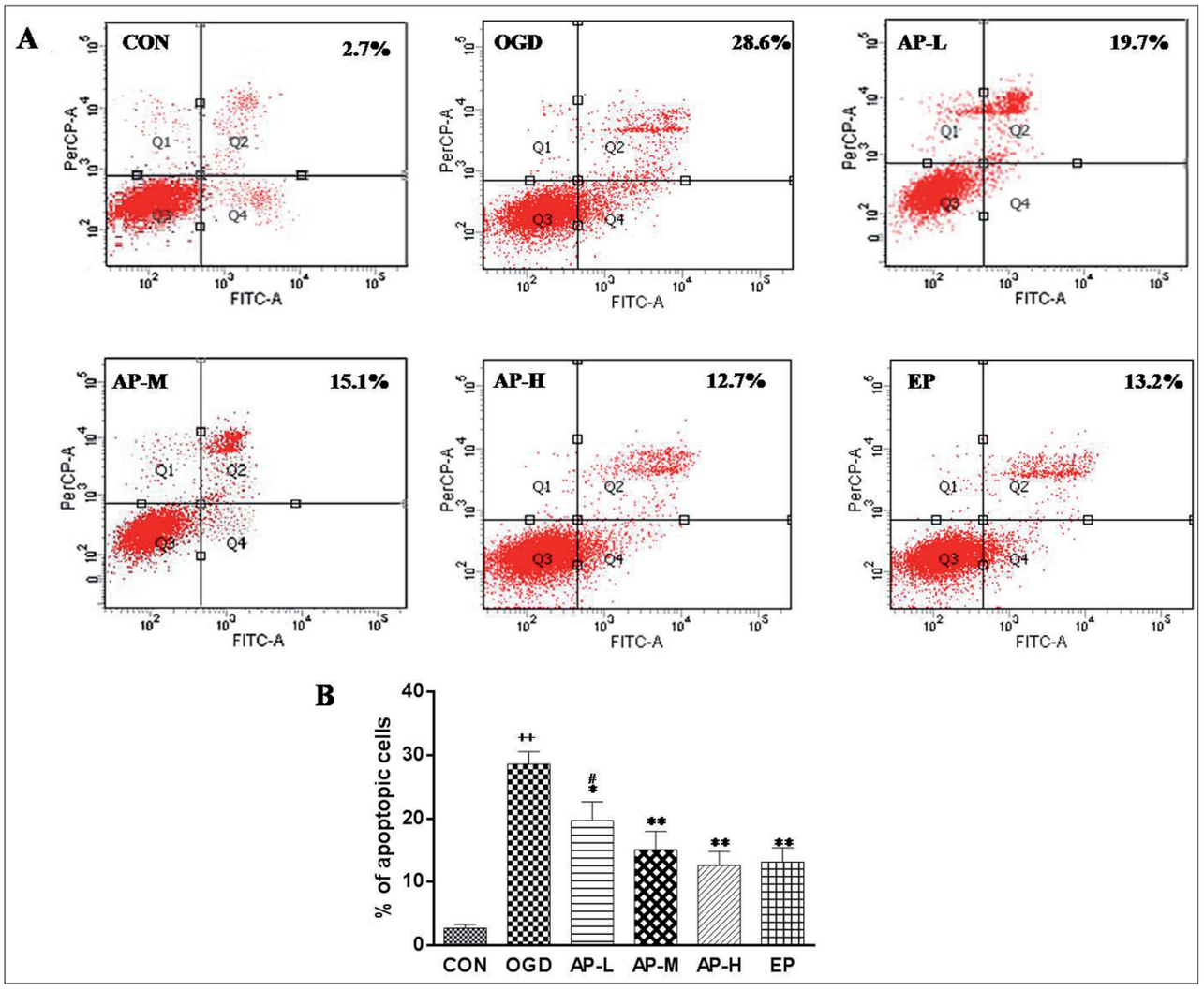

Fig. 2: Effects of AP on apoptosis in BV2 microglia after annexin-V/7-AAD staining by flow cytometric analysis.
BV2 microglia were pretreated with AP before the onset of OGD and then incubated with AP for an additional 24 h. The cells were subsequently harvested and stained with annexin-V/7-AAD.
(A) Flow cytometric analysis of apoptotic BV-2 cells.
(B) Graphical representation of the percentage of apoptotic cells result from (A). Mean ± SD, n=3. $^{++}p<0.01$ vs control group; $^{*}p<0.05$, $^{**}p<0.01$ vs OGD model group; $^{\#}p<0.05$ vs EP treated group. CON, control cells; OGD, cells treated with OGD/R for 2.5 h/24 h; AP-L, AP-M, AP-H represents 0.1, 0.4 and 1.6 μM AP treated BV2 microglia with OGD/R for 2.5 h/24 h, respectively; EP, EP (1 μM) treated BV2 microglia with OGD/R for 2.5 h/24 h.

2.3. Effects of AP on OGD-induced HMGB1 release in BV2 microglia

We first evaluated the cellular distribution of HMGB1 in BV2 microglia by immunofluorescent FITC-staining. As shown in Fig. 3, HMGB1 immunoreactivity was present in the nucleus but absent in the cytoplasm of control cells; on the contrary, HMGB1 was mainly localized in the cytoplasm (vesicle-like structures) of BV2 microglia subjected to OGD treatment; cells treated with different doses of AP or EP could retain HMGB1 in the nucleus, so HMGB1 immunoreactivity was highly represented in the nucleus, which was also present in the cytoplasm. The results indicated that AP could inhibit OGD-induced HMGB1secretion at the level of nuclear to cytoplasmic translocation in a dose-dependent manner.
We further analyzed the protein level of cytoplasmic HMGB1 by western blotting. As shown in Fig. 4, the HMGB1 was very poor in cytoplasmic extracts from normal cells; in contrast, the cytoplasmic HMGB1 level in the OGD model group was significantly upregulated at 24 h after incubation with normal medium, indicating the translocation of HMGB1 from nucleus to cytoplasm; AP significantly decreased cytoplasmic HMGB1 protein levels, and the effects were dose-dependent. The results indicated that AP inhibited the nuclear release of HMGB1 in BV2 microglia subjected to OGD treatment, and the findings agreed with the results of immunofluorescent FITC-staining.

3. Discussion

Microglia are the resident macrophages in brain, and they are now recognized as the prime cells in the immune defense and inflammatory response (Kraft and Harry 2011). Under physiological conditions, resting microglia play an important role in homeostasis within the CNS and in supporting neuronal cell function (Hu et al. 2014). Upon activation, the morphology of microglia altered, changing from resting microglia into activated microglia. Activated microglia produce pro-inflammatory mediators including nitric oxide (NO), prostaglandin E2 (PGE2), reactive oxygen species (ROS), and pro-inflammatory cytokines such as interleukin (IL)-1β, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) that function to restore CNS homeostasis by removing cell debris and restoring tissue integrity (Yenari et al. 2010). However, over-expression of these inflammatory mediators and cytokines under pathological conditions can cause neuronal cell death and brain injury. Furthermore, after stroke, microglia can die in the core whereas the number of brain mononuclear phagocytes

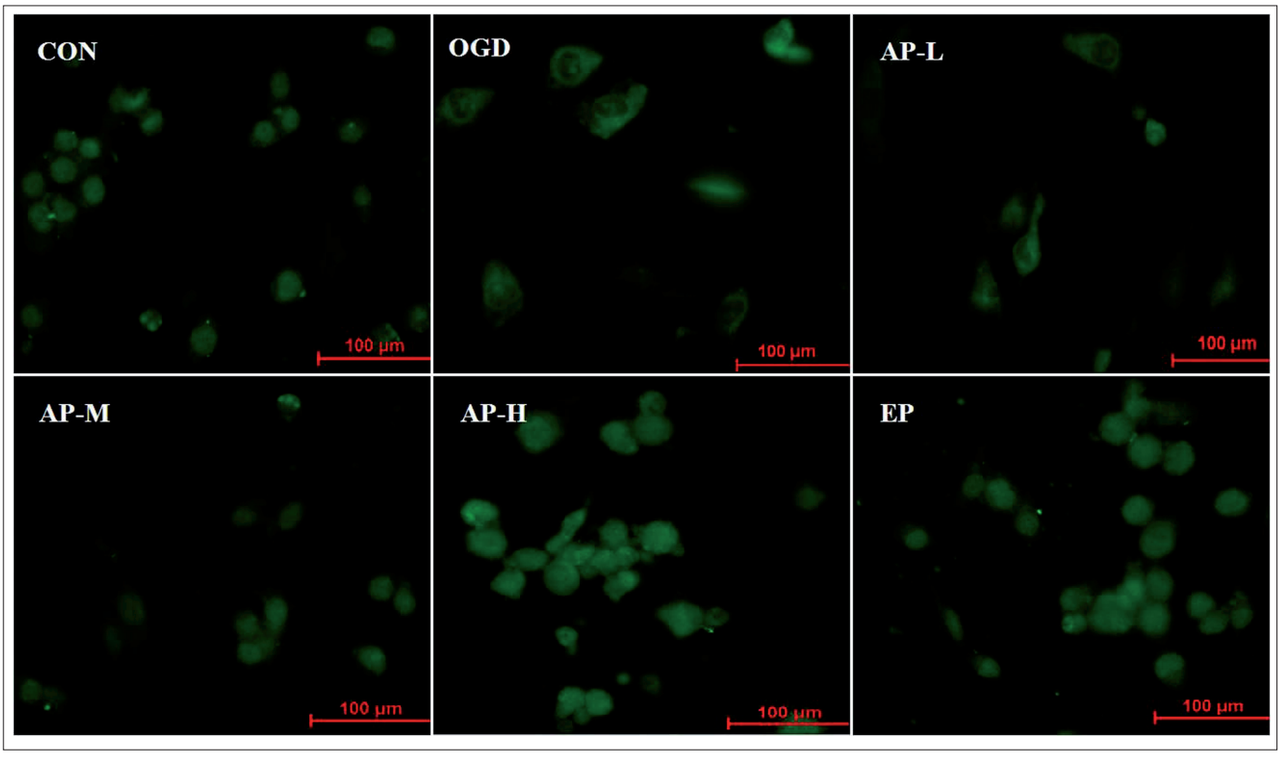

Fig. 3: Effects of AP on OGD-induced HMGB1 release in BV2 microglia by green immunofluorescent FITC-staining.
HMGB1 immunoreactivity was present in the nucleus but absent in the cytoplasm of control cells, while which was found in both nucleus and cytoplasm (vesiclelike structure) of OGD model microglia, indicating that HMGB1 is released from the nucleus into the cytoplasm in BV2 microglia after exposure to OGD injury; treatment with different doses of AP (0.1, 0.4 and 1.6 μM) and EP (1 μM) prior to OGD and on the onset of reoxygenation could retain HMGB1 in the nucleus, so HMGB1 immunoreactivity was highly represented in the nucleus.
The scale bar in the photograph is 100 μM. CON, control cells; OGD, cells treated with OGD/R for 2.5 h/24h; AP-L, AP-M, AP-H represents 0.1, 0.4 and 1.6 μM AP treated BV2 microglia with OGD/R 2.5 h/24 h, respectively; EP, EP (1 μM) treated BV2 microglia with OGD/R for 2.5 h/24 h.

is augmented by microglial proliferation/macrophage infiltration and most markers used are unable to discriminate them (Yenari et al. 2010). Taken together, activation of microglia plays an important role in the inflammatory response to ischemic stroke. Control of microglial activation and decreasing the number of pro-inflammatory factors could provide a potential therapeutic approach for treating cerebral ischemic diseases. Therefore, in this study, we selected BV2 cells, a microglia cell line, and prepared an OGD model to investigate the protective effects and molecular mechanism of AP, a potential drug for the treatment of ischemic stroke.

Inflammation is a classical host defensive response to infection and injury that has many beneficial effects. However, inappropriate inflammation is increasingly implicated in diverse disease states, including ischemic stroke. Focal cerebral ischemia evokes a robust inflammatory response that begins within a few hours of onset and typifies the secondary or delayed response to ischemia. It is generally accepted that inflammation in the post-stroke period contributes to ischemic brain injury (Denes et al. 2010). HMGB1 is now characterized as a cytokine-like mediator of systemic inflammation (Asavarut et al. 2013). In the pathophysiology of cerebral ischemia-reperfusion, HMGB1 is released from neurons early following ischemic injury, and acts as a mediator linking acute brain damage and subsequent inflammatory processes (Qiu et al. 2008; Fang et al. 2012). Neuronal cells subjected to OGD/R treatment can mimic cerebral ischemia-reperfusion *in vivo* (Alluri et al. 2015). In the present study, we successfully prepared an OGD model in BV2 microglia for the observation of HMGB1 release. Our finding of increased cytoplasic HMGB1 level in BV2 cells suggest that exacerbated HMGB1 release may be triggered by OGD treatment.

Immunofluorescent analysis revealed that HMGB1 was translocated from the nucleus to cytoplasm and further accumulated in the cytoplasm 24 h OGD treatment. However, the translocation of HMGB1 was markedly inhibited in AP-treated cells, indicating that AP suppresses HMGB1 translocation from nucleus to cytoplasm. Western blotting analysis showed that the protein level of cytoplasmic HMGB1 was significantly increased 24 h after OGD treatment, and AP treatments attenuated the increase in HMGB1 level in the cytoplasm of BV2 microglia. The findings indicated that AP protected against OGD-induced cellular injury in BV2 microglia *via* inhibition of HMGB1 release.

It is accepted that treatment with inhibitors of HMGB1 activity is beneficial in inflammatory disease models (Yang and Tracey 2010). Ethyl pyruvate (EP) is a simple aliphatic ester of pyruvic acid and has been demonstrated to be an inhibitor of HMGB1 (Shin et al. 2014). Accumulating evidence indicates the anti-inflammatory effects of EP in the post ischemic brain and in primary microglia cultures, and the mainly molecular mechanisms lie in the inhibition of HMGB1 phosphorylation and release (Shin et al. 2014; Kim et al. 2016). In addition, EP has been reported to inhibit HMGB1 expression in traumatic brain injury and in myocardial ischemia/reperfusion injury models (Su et al. 2011; Lin et al. 2015). Therefore, EP is considered to provide a therapeutic means of ameliorating inflammatory responses by suppressing HMGB1 expression and secretion. In this study, we compared the protective effects of AP and EP, and found that AP and EP contributed similar effects on inhibiting HMGB1 release. It is reported that serine phosphorylation of HMGB1 contributes to its translocation from the nucleus to cytoplasm (Taira et al. 2013). In the present study, we found that AP could inhibit HMGB1 secretion in activated microglia, however whether AP inhibits the phosphorylation of HMGB1 needs further study.

To the best of our knowledge, this is the first study to demonstrate the role of AP on HMGB1 expression and release. We can conclude that when activated by OGD treatment, BV2 microglia release HMGB1 from the nucleus to the cytoplasm; the released HMGB1, in turn, can further activate and injure the BV2 microglia, leading to the apoptosis and death of microglia. AP can protect against OGD-induced cellular injury in BV2 microglia, and the cytopro-

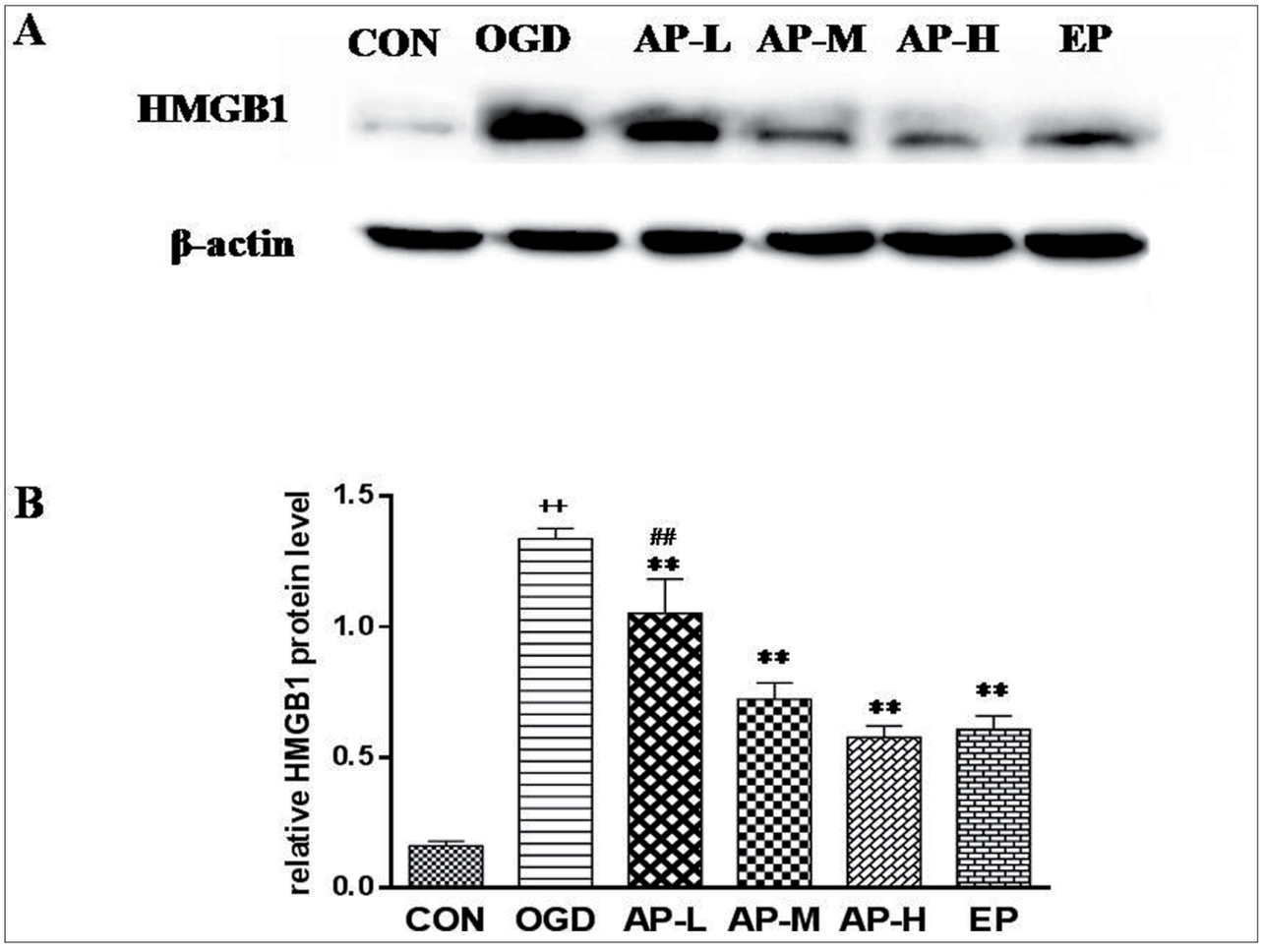

Fig. 4: Effects of AP on OGD-induced HMGB1 expression in BV2 microglia by western blotting.
(A) Protein levels of HMGB1 were determined by western blotting analysis. β-Actin was used as a loading control.
(B) The relative HMGB1 protein level normalized to β-actin level was detected. Mean ± SD, n=3. $^{++}p<0.01$ vs control group; $^{**}p<0.01$ vs OGD model group; $^{\#\#}p<0.01$ vs EP treated group. CON, control cells; OGD, cells treated with OGD/R for 2.5 h/24 h; AP-L, AP-M, AP-H represents 0.1, 0.4 and 1.6 μM AP treated BV2 microglia with OGD/R for 2.5 h/24 h, respectively; EP, EP (1 μM) treated BV2 microglia with OGD/R for 2.5 h/24 h.

tective mechanisms may be mediated by inhibition of HMGB1 expression and release in microglia, which leads to attenuation of OGD-induced necrosis and neuroinflammation.

4. Experimental

4.1. Materials

4.1.1. Drugs

AP sample (purity ≥ 98%) was a gift from Shandong Academy of Medical Sciences (Jinan, China) and was dissolved in DMSO at 1.0 mM as a stock solution. Ethyl pyruvate (EP, Batch No. E47808) was supplied from Sigma-Aldrich Company (Saint Louis, USA). The dilutions of the reagents were all freshly prepared before each experiment.

4.1.2. BV2 microglia culture and treatments

The BV2 mouse microglial cell line was a gift from Prof. Huiqing Liu (Institute of Pharmacology, School of Medicine, Shandong University, Jinan), and the experiment was conducted using protocols approved by the Shandong University Institutional Animal Care and Use Committee (Approval No. ECAESDUSM 201). Cells were kept at 37 °C in a humidified 5% CO_2/95% air incubator. The dissociated cells were cultured in high glucose DMEM medium (Gibco, NY, USA), supplemented with 10% fetal bovine serum (FBS). The medium was changed every two days.
BV2 microglia was randomly divided into 6 groups: control group (CON), OGD group (model group), OGD + AP groups (0.1, 0.4 and 1.6 μM) and OGD + EP group (1 mM). For the preparation of OGD model, BV2 microglia were washed with Hank's balanced salt solution (no glucose) for 4 times, incubated with 100 μL of the Hank's solution for 2.5 h at 37 °C in a special anaerobic jar (HP025, HiTech Photoelectricity Biotechnology Co.,Ltd, Guang Zhou, China) filled with 5% CO_2/95% N_2, and then incubated with 5% CO_2/95% air for an additional given duration. Microglia in the control group were treated with Hank's balanced salt solution containing glucose and incubated with 5% CO_2 and 95% air. In the AP or EP-treated groups, BV2 microglia were pretreated with AP or EP at an indicated concentration before the onset of OGD and then incubated with the drugs for an additional 24 h.

4.2. Methods

4.2.1. CCK-8 assay for the determination of cell viability

BV2 microglia seeded in 96-well plate (1×10^4 per well) were pretreated with AP before the onset of OGD and then incubated with AP for an additional 6, 12, 24 and 48 h, respectively. OGD-induced cell death was assessed by Cell Counting Kit-8 (CCK-8) according to manufacturer's instructions. The optical density (OD) was measured at a wavelength of 492 nm in a microplate reader (Therm MK3, Finland).

4.2.2. Hoechst 33258 staining

BV2 microglia were rinsed twice in 4 °C PBS and fixed in 4% formaldehyde at 4 °C for 10 min. After washing, the cells were incubated using Hoechst 33258 (Sigma, USA) staining at room temperature for 10 min in the dark. The apoptotic cells were visualized under a fluorescence microscope (Leica Microsystems Holdings GmbH, Germany). Cells were scored apoptotic if the nuclei presented chromatin condensation, marginalization or nuclear beading (Xue et al. 2014).

4.2.3. FITC-Annexin V/7-AAD staining

BV2 microglia seeded in 6-well plates (1.5×10^5 per well) were pretreated with AP before the onset of OGD and then incubated with AP for an additional 24 h. The cells were then harvested and washed with cold PBS. The cell surface phosphatidylserine in apoptotic cells was quantitatively estimated using Annexin V/FITC and 7-AAD

Apoptosis Detection Kit (Roche, USA) according to manufacturer's instructions. Cell apoptosis was analyzed on a FACScan flow cytometry (Becton Dickinson, USA). Annexin V-positive, 7-AAD-negative cells were scored as early apoptotic cells, and cells double-stained with both Annexin V and 7-AAD were considered late apoptotic cells. Normal cells were negative for both stains.

4.2.4. Immunofluorescent FITC-staining

For immunofluorescent detection of HMGB1 protein, cells were fixed in 4% paraformaldehyde (Sigma, USA) for 10 min and then blocked for 1 h in 5% BSA (Sigma, USA) diluted in 0.5%Tween-20/phosphate buffered saline (T-PBS) followed by permeabilization in PBS containing 10% of Triton X-100 for 30 min.
Cells were then incubated with primary antibody: polyclonal rabbit anti-HMGB1 antibody (1:500, Abcam, Cambridge, UK). Immunoreactivity was detected using a FITC-labeled secondary antibody (ZSGB Int, Beijing, China). Fluorescent images were obtained with a Zeiss Axio Observer Z1 invert microscope.

4.2.5. Western blotting analysis

The procedure for separation of nuclei and cytoplasm fraction of the cells was performed according to the manufacturer's instructions of Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China). The HMGB1 protein concentration was determined using a BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). The lysates were separated by SDS-PAGE on 12% Tris-Glycine gels and transferred to nitrocellulose membrane. The nonspecific binding of antibodies was blocked with 5% nonfat dried milk in PBS. The membranes were then probed with primary antibody of polyclonal rabbit anti-HMGB1 antibody (1:2000, Abcam, Cambridge, UK) overnight at 4 °C. After washing three times with TBS-T, the HRP-conjugated goat anti-rabbit second antibody (ZSGB-Bio, Beijing, China) was incubated with membranes for 1 h at room temperature. β-actin (Bioworld Technology, Minneapolis, MN, USA) was served as a loading control. The densitometry of the bands was performed using AlphaEaseFC 4.0 software (Alpha Innotech, San Leandro, CA, USA).

4.2.6. Data analysis and statistics

The results were expressed as the mean ± standard deviation (mean ± SD). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) test using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Results with $P<0.05$ were considered statistically significant.
Acknowledgments: This work was supported by Seed Fund of the Second Hospital of Shandong University (No. S2015010019) and Shandong Provincial Medical Science and Technology Development Program (No. 2016WS0336).

Conflicts of interest: None declared.

References

Alluri H, Anasooya SC, Davis ML, Tharakan B (2015) Oxygen-glucose deprivation and reoxygenation as an in vitro ischemia-reperfusion injury model for studying blood-brain barrier dysfunction. J Vis Exp: e52699.
Asavarut P, Zhao H, Gu J, Ma D (2013) The role of HMGB1 in inflammation-mediated organ injury. Acta Anaesthesiol Taiwan 51: 28-33.
Denes A, Thornton P, Rothwell NJ, Allan SM (2010) Inflammation and brain injury: acute cerebral ischaemia, peripheral and central inflammation. Brain Behav Immun 24: 708-723.
Fang P, Schachner M, Shen YQ (2012) HMGB1 in development and diseases of the central nervous system. Mol Neurobiol 45: 499-506.
Hu X, Liou AK, Leak RK, Xu M, An C, Suenaga J, Shi Y, Gao Y, Zheng P, Chen J (2014) Neurobiology of microglial action in CNS injuries: receptor-mediated signaling mechanisms and functional roles. Prog Neurobiol 119-120: 60-84.
Jia Q, Liu L, Wang Y (2011) Risk factors and prevention of stroke in the Chinese population. J Stroke Cerebrovasc Dis 20: 395-400.
Kim YM, Park EJ, Kim JH, Park SW, Kim HJ, Chang KC (2016) Ethyl pyruvate inhibits the acetylation and release of HMGB1 via effects onSIRT1/STAT signaling in LPS-activated RAW264.7 cells and peritoneal macrophages. Int Immunopharmacol 41: 98-105.
Kraft AD, Harry GJ (2011) Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity. Int J Environ Res Public Health 8: 2980-3018.
Lin Y, Chen L, Li W, Fang J (2015) Role of high-mobility group box-1 in myocardial ischemia/reperfusion injury and the effect of ethyl pyruvate. Exp Ther Med 9: 1537-1541.
Liu DM, Wang ZH, Liu L, Zhang XM, Lou FL (2013) Acetylpuerarin increases cell viability and reduces apoptosis in rat hippocampal neurons following oxygenglucose deprivation/reperfusion. Mol Med Rep 8: 1453-1459.
Liu R, Wei XB, Zhang XM (2007) Effects of acetylpuerarin on hippocampal neurons and intracellular free calcium subjected to oxygen-glucose deprivation/reperfusion in primary culture. Brain Res 1147: 95-104.
Martinotti S, Patrone M, Ranzato E (2015) Emerging roles for HMGB1 protein in immunity, inflammation, and cancer. Immunotargets Ther 4: 101-109.
Meng QH, Lou FL, Hou WX, Liu M, Guo H, Zhang XM (2013) Acetylpuerarin reduces inflammation and improves memory function in a rat model of Alzheimer's disease induced by Abeta1-42. Pharmazie 68: 904-908.
Naglova H, Bucova M (2012) HMGB1 and its physiological and pathological roles. Bratisl Lek Listy 113: 163-171.
Puyal J, Ginet V, Clarke PG (2013) Multiple interacting cell death mechanisms in the mediation of excitotoxicity and ischemic brain damage: a challenge for neuroprotection. Prog Neurobiol 105: 24-48.
Qiu J, Nishimura M, Wang Y, Sims JR, Qiu S, Savitz SI, Salomone S, Moskowitz MA (2008) Early release of HMGB-1 from neurons after the onset of brain ischemia. J Cereb Blood Flow Metab 28: 927-938.
Quillinan N, Herson PS, Traystman RJ (2016) Neuropathophysiology of brain injury. Anesthesiol Clin 34: 453-464.
Shin JH, Kim ID, Kim SW, Lee HK, Jin Y, Park JH, Kim TK, Suh CK, Kwak J, Lee KH, Han PL, Lee JK (2014) Ethyl pyruvate inhibits HMGB1 phosphorylation and release by chelating calcium. Mol Med 20:649-657.
Shin JH, Lee HK, Lee HB, Jin Y, Lee JK (2014) Ethyl pyruvate inhibits HMGB1 phosphorylation and secretion in activated microglia and in the postischemic brain. Neurosci Lett 558: 159-163.
Shukla V, Shakya AK, Perez-Pinzon MA, Dave KR (2017) Cerebral ischemic damage in diabetes: an inflammatory perspective. J Neuroinflammation 14: 21.
Singh V, Roth S, Veltkamp R, Liesz A (2016) HMGB1 as a key mediator of immune mechanisms in ischemic stroke. Antioxid Redox Signal 24: 635-651.
Su X, Wang H, Zhao J, Pan H, Mao L (2011) Beneficial effects of ethyl pyruvate through inhibiting high-mobility group box 1expression and TLR4/NF-kappa B pathway after traumatic brain injury in the rat. Mediators Inflamm 2011: 807142.
Taira J, Kida Y, Kuwano K, Higashimoto Y (2013) Protein phosphatase 2A dephosphorylates phosphoserines in nucleocytoplasmic shuttling and secretion of high mobility group box 1. J Biochem 154: 299-308.
Tóth M, Little P, Arnberg F, Häggkvist J, Mulder J, Halldin C, Gulyás B, Holmin S (2015) Acute neuroinflammation in a clinically relevant focal cortical ischemic stroke model in rat: longitudinal positron emission tomography and immunofluorescent tracking. Brain Struct Funct 221: 1279-1290.
Vidale S, Consoli A, Arnaboldi M, Consoli D (2017) Postischemic inflammation in acute stroke. J Clin Neurol 13: 1-9.
Xiang Y, Wei X, Chen L, Liu H, Liu X, Wang T, Zhang X (2014) Anti-inflammatory effect of acetylpuerarin on eicosanoid signaling pathway inprimary rat astrocytes. J Mol Neurosci 52: 577-585.
Xiong XY, Liu L, Yang QW (2016) Functions and mechanisms of microglia/macrophages in neuroinflammation and neurogenesis after stroke. Prog Neurobiol 142: 23-44.
Xue X, Yu JL, Sun DQ, Kong F, Qu XJ, Zou W, Wu J, Wang RM (2014) Curcumin induces apoptosis in SGC-7901 gastric adenocarcinoma cells via regulation of mitochondrial signaling pathways. Asian Pac J Cancer Prev 15:3987-3992.
Yang H, Antoine DJ, Andersson U, Tracey KJ (2013) The many faces of HMGB1: molecular structure-functional activity in inflammation, apoptosis, and chemotaxis. J Leukoc Biol 93: 865-783.
Yang H, Tracey KJ (2010) Targeting HMGB1 in inflammation. Biochim Biophys Acta 1799: 149-156.
Yenari MA, Kauppinen TM, Swanson RA (2010) Microglial activation in stroke: therapeutic targets. Neurotherapeutics 7: 378-391.
Zhao SC, Ma LS, Chu ZH, Xu H, Wu WQ, Liu F (2017) Regulation of microglial activation in stroke. Acta Pharmacol Sin 38:445-458.