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HMGB1 SUPPRESSION CONFERS NEUROPROTECTION AGAINST STROKE IN DIABETIC RATS

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Abstract

Objectives: Diabetes is a principal risk factor for stroke, and results in poorer neurological outcome after stroke. High mobility group box-1 (HMGB1) was recently reported to mediate an increased inflammatory response through receptors for advanced glycation end products (RAGE) and Toll-like receptors (TLR). In this study, we investigated how blocking HMGB1, using glycyrrhizin, may reduce inflammation following ischemic stroke in a diabetic model. Methods: The effect of glycyrrhizin on non-stroke diabetic rats was assessed, following diabetes induction by streptozotocin. This was followed with the analysis of glucose, HMGB1 and cytokines levels. We then sought to determine the impact of HMGB1 suppression with glycyrrhizin after a stroke using transient occlusion of the middle cerebral artery. Infarct volume, HMGB1 levels, expression of TLR and RAGE and their subcellular pathways were measured. Results: In the non-stroke state, glycyrrhizin did not reduce glucose levels in diabetic rats, but significantly reduced HMGB1 and interleukin (IL)-1β levels. Furthermore, glycyrrhizin decreased the infarct volume after focal cerebral ischemia in diabetic rats (140±79 mm^3 versus 224±68 mm^3; p=0.03). HMGB1 secretion from the ischemic brain and expression of TLR2, TLR4 and RAGE were reduced after glycyrrhizin treatment. Intracellular signaling pathways related to HMGB1 receptors, such as NF-κB, ERK, and Akt, were reduced after HMGB1 inhibition using glycyrrhizin. Discussion: Our results indicate that HMGB1 suppression by glycyrrhizin reduces the inflammatory response in diabetes with or without stroke. We conclude that HMGB1 may be a good candidate to target for the inhibition of the inflammatory response after stroke.

Keywords

• HMGB1 • Glycyrrhizin • Ischemic stroke • Inflammation.

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Abbreviations

AGE - Advanced glycation end products
RAGE - Receptor for advanced glycation end products
HMGB1 - High mobility group box-1
TLR - Toll-like receptor
SDS-PAGE - Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
IL - Interleukin
TNF - Tumor necrosis factor
ERK - Extracellular-signal-regulated kinase
ANOVA - Analysis of variance

Introduction

Diabetes is a major clinical problem. Moreover, it is strongly associated with an increased risk of major vascular diseases such as stroke and myocardial infarction [1,2]. With diabetes, the future risk of stroke is two times higher, and the long-term outcome after an ischemic stroke is poorer than without diabetes [2]. Furthermore, approximately one third of ischemic stroke patients experience hyperglycemia even though they do not have any history of diabetes [3], and hyperglycemia during an acute stroke period is associated with a poorer outcome [4]. In this context, several earlier experimental studies have suggested various pathological mechanisms as potential causes of increased tissue damage in subjects with diabetes or hyperglycemia. These include, increased inflammation caused by oxidative stress [5], and impaired vascular reactivity associated with endothelial dysfunction [6,7].

Advanced glycation end products (AGE), which are produced in hyperglycemic conditions, bind to their surface receptor, called the receptor for advanced glycation end products (RAGE). Once bound, they induce various pro-inflammatory cytokines, including high mobility group box-1 (HMGB1) [8]. HMGB1 signals through Toll-like receptor 2 (TLR2), TLR4, and RAGE, which amplify the inflammatory reaction [9,10]. The HMGB1 signals are effectively down-regulated by glycyrrhizin, a natural compound derived from licorice root, which was used as a drug to treat patients with hepatitis in Japan [11], because glycyrrhizin directly binds to HMGB1 and inhibits its function [12]. Based on this knowledge, we hypothesized that blocking HMGB1 by using glycyrrhizin, may reduce inflammation following ischemic stroke in a diabetic model.

Materials and methods

Animal models and treatment groups

Diabetes was induced by a single injection of intraperitoneal streptozotocin (60 mg/kg) to male Sprague-Dawley (SD) rats, weighing 299 ± 24 g (Orient Bio, Korea). To confirm whether diabetes was successfully established, blood was drawn at 0, 3, and 7 days after

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injection. Only rats with the glucose levels of over 300 mg/dL were accepted as subjects for this experiment. Body weights were measured daily. A total of 42 rats were used in this study.

First, to verify the effects of glycyrrhizin on diabetes without stroke, normoglycemic and streptozotocin-induced diabetic rats were divided into three groups: 1) DM-GCZ – diabetic rats treated with glycyrrhizin; 2) DM – diabetic rats treated with normal saline; and 3) control – normal control rats treated with normal saline. Depending on the group either glycyrrhizin (10 mg/kg) or normal saline was administered daily intraperitoneally for 7 days after injection of streptozotocin.

Then, we moved on to next experiment regarding the effect of glycyrrhizin on diabetic rats with a stroke. Diabetic rats were subjected to focal cerebral ischemia using the transient middle cerebral artery occlusion procedure 7 days after streptozotocin injection. The focal cerebral ischemia model was induced by blocking the lumen of the middle cerebral artery using the intraluminal suture-induced ischemic technique, as described previously [13]. Briefly, male Sprague-Dawley rats were anesthetized using 2% isoflurane in a gas mixture of 70% nitrogen oxide and 30% oxygen. Rectal temperature was maintained consistently at 37.0°C during the procedure using a heating blanket. The left common carotid artery and external carotid artery were exposed and ligated. A 3-0 monofilament nylon suture was gently inserted and the left internal carotid artery was temporarily ligated to avoid further bleeding from the puncture site. After closure of the incision site, the animal was kept in a cage to recover from the anesthesia. One hour after the occlusion, the nylon suture was removed to recover the blood flow. Normoglycemic and diabetic rats were divided into three groups after focal cerebral ischemia: 1) DM-GCZ – diabetic rats treated with glycyrrhizin; 2) DM – diabetic rats treated with normal saline; and 3) control – normal control rats treated with normal saline. Glycyrrhizin or saline was injected twice immediately after reperfusion and then at 24 hours. Finally, at 48 hours after ischemia was induced, the rats were anesthetized and sacrificed.

All procedures were carried out after receiving institutional approval (No. 12-0192) in accordance with the National Institutes of Health Guide of the Institutional Animal Care and Use Committee of the Biomedical Research Institute at Seoul National University Hospital. Every effort was made to minimize animal suffering and to limit the number of animals used.

Infarct volume assessment

At 48 hours after ischemia was induced, the brains (n=9 per group) were removed after cardiac perfusion-fixation with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline. Using a freezing microtome, brains were cut into 30 µm coronal sections. Twelve brain sections were mounted on glass slides and processed for Nissl staining. Infarct volumes were measured using a computer-based image analyzer (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

Serum measurement of HMGB1 and cytokines

To measure the level of HMGB1, 500 µl of blood samples (n = 4 per group) were taken from the rat tail vein 7 days after diabetes induction and 2 days after ischemia was induced. The samples were centrifuged at 15,000 rpm at 4°C for 20 min, and 200 µl of the supernatant was separated. To separate 20 µg of serum proteins, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used and transferred to nitrocellulose membranes. The blots were blocked with 5% skim milk in Tris-buffered saline in 0.1% Tween 20 at room temperature for 1 hour, incubated with rabbit anti-HMGB1 primary antibody (Abcam, Cambridge, UK) of a 1:1000 dilution at 4°C overnight, and then treated with anti-rabbit secondary antibody of a 1:10000 dilution for 1 hour at room temperature. The intensity of the blots was measured by Image J 1.44 (National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). The serums acquired (n = 3-4 per group) were also used to measure the level of interleukin IL-1β and tumor necrosis factor α (TNF-α) using the Multiplex assay (Millipore, Billerica, MA, USA).

Western blotting

Rat brains were homogenated and centrifuged at 13,000 rpm for 10 min at 48 hours after ischemia was induced (n = 2 per group). Samples of proteins (20 µg) were separated by SDS-PAGE and analyzed by Western blot using primary antibodies to extracellular-signal-regulated kinase (ERK) 1/2, p-ERK1/2, Akt, p-Akt, p65, p-p65, and IκB. All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-b-actin antibody (Santa Cruz Biotechnology, CA, USA) was used as the control. Image J was used as the image analysis program.

Immunohistochemistry

At 48 hours after ischemia was introduced, rat brains (n = 3 per group) were perfused and fixed with 4% paraformaldehyde at 4°C overnight. Brain sections were prepared using a cryostat (Leica CM 1900, Leica Biosystems, Germany) and stained with primary antibodies (anti-TLR2, Santa-Cruz Biotechnology; anti-TLR4, Santa-Cruz Biotechnology; anti-RAGE, Abcam) and with a fluorescently-tagged secondary antibody. The thickness of the tissue was 40 µm. To counterstain the nuclei, DAPI was used. Slides were analyzed with a fluorescence microscope (Leica DM5500 B, Leica Microsystems, Germany).

Statistical analysis

Quantitative data were expressed as the mean ± standard deviation. Significance was determined using theStudent's *t*-test or by one-way analysis of variance (ANOVA), depending on which was deemed appropriate. P values <0.05 were considered significant. Data analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Glycyrrhizin did not directly affect the glycemic status in diabetic rats

Glycyrrhizin was administered daily to diabetic rats to demonstrate an anti-inflammatory effect by HMGB1 suppression. After diabetes induction, using streptozotocin injection, blood glucose levels were significantly elevated (control group: 105 ± 17 mg/dl; DM group: 386 ± 124 mg/dl; n = 9 per group) and body weight loss was also significant (control group: 332 ± 25 g; DM group: 303 ± 29 g) after 3 days

(Figure 1). When treated with glycyrrhizin, there were no differences in the blood glucose levels and body weights compared to the DM group.

Glycyrrhizin reduced HMGB1 and cytokine levels in diabetic rats

Serum HMGB1 and cytokine levels were measured 7 days after streptozotocin injection. Serum IL-1β levels were increased after diabetes induction (Figure 2B). Serum HMGB1 and TNF-α levels showed an increasing tendency without statistical significance (Figure 2A, C). After glycyrrhizin treatment, serum HMGB1 and IL-1β levels were significantly decreased (Figure 2A, B).

Glycyrrhizin decreased ischemic damage after transient MCAO in diabetic rats

To investigate the neuroprotective effect of glycyrrhizin after cerebral infarction, we induced focal cerebral ischemia and reperfusion in diabetic rats and treated them with glycyrrhizin. After diabetes induction, brain infarct volume was significantly increased (Figure 3A, B). With glycyrrhizin treatment, there was a significant reduction in infarct volume (control, 152 ± 76 mm^3; DM, 224 ± 68 mm^3; DM-GCZ, 140 ± 79 mm^3; $p < 0.05$).

Glycyrrhizin suppressed HMGB1 secretion from the ischemic brain

To analyze the effects of glycyrrhizin on HMGB1 release from ischemic brain tissue, we measured HMGB1 levels in the serum and in the brain 2 days after ischemia. Serum HMGB1 levels were elevated in diabetic rats, whereas brain HMGB1 levels were decreased after the ischemic insult (Figure 4A, B). Glycyrrhizin significantly reduced the serum HMGB1 levels and restored the brain HMGB1 levels, which implies that glycyrrhizin inhibits brain release of HMGB1.

Reduction of HMGB1 receptor expression after glycyrrhizin treatment

Immunofluorescence staining was performed to evaluate the expression levels of HMGB1 receptors on the border-zone of the cerebral infarct (Figure 5). Two days after ischemia was induced, the TLR2, TLR4, and RAGE positive cells

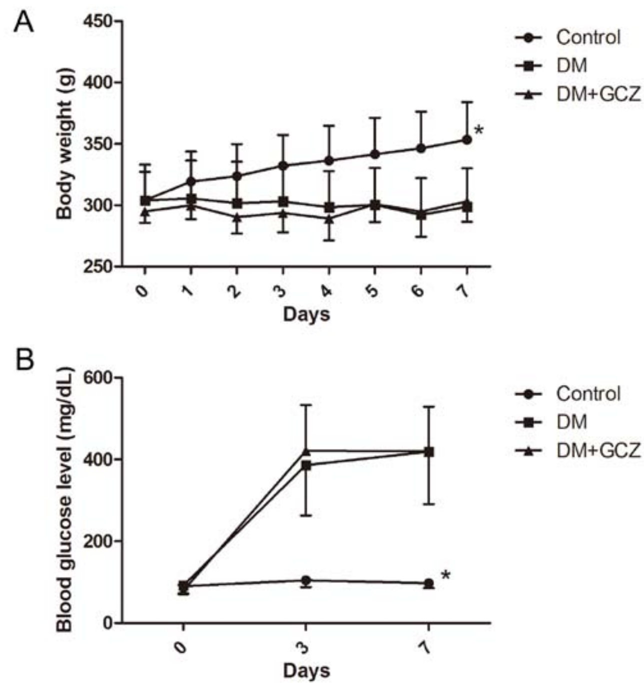

Figure 1. Daily changes of body weight (A) and blood glucose levels (B) of diabetic rats. The symbols and bars represent the mean ± standard deviation of 9 rats per group. *$p<0.05$ versus other groups.

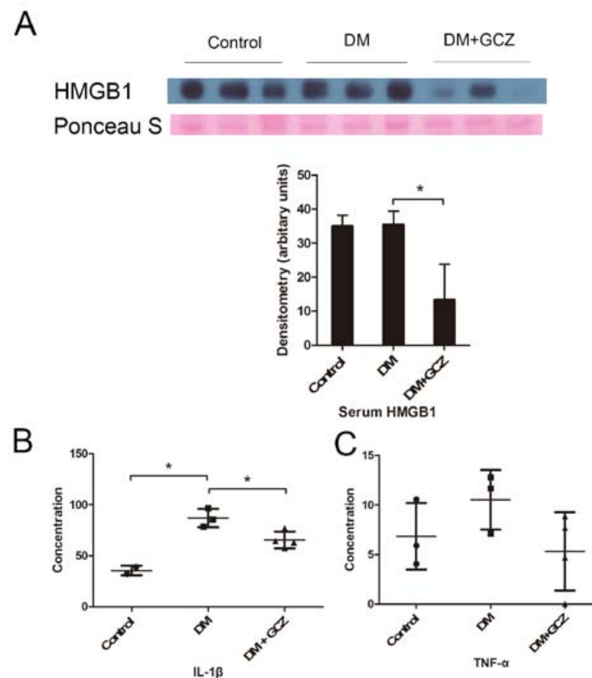

Figure 2. Serum level of HMGB1 and cytokine were suppressed by the administration of glycyrrhizin. (A) Serum HMGB1 level of diabetic rats 7 days after injection of streptozotocin; n=4 per group. (B) Serum IL-1β (C) Serum TNF-α; n=3~4 per group.

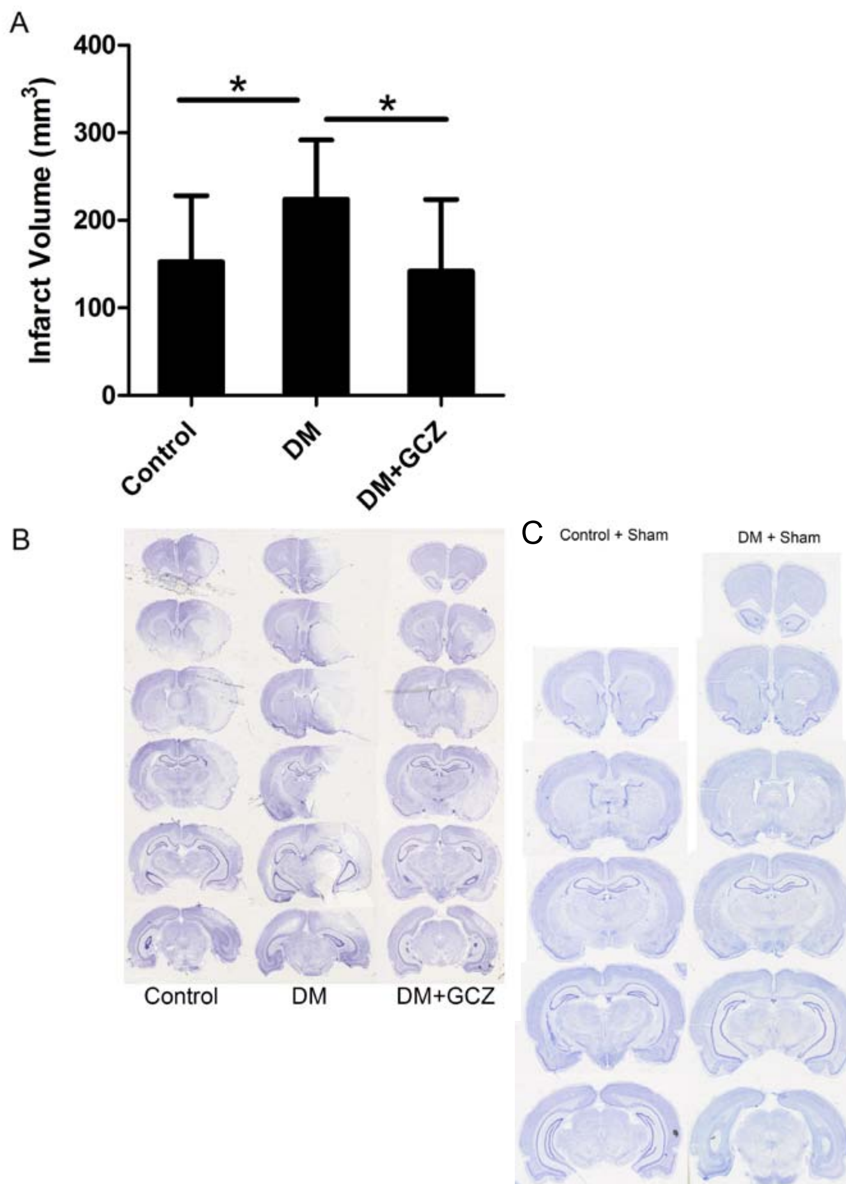

Figure 3. Effect of glycyrrhizin on ischemic damage of diabetic rats. (A) Infarct volume after transient middle cerebral artery occlusion. The symbols and bars represent the mean ± standard deviation of 9 rats per group; *p<0.05 versus other groups. (B) Representative brain slices from each group: control, DM – diabetic rats, DM-GCZ – diabetic rats treated with glycyrrhizin. (C) Representative brain slices after sham surgery that did not show any cerebral infarcts.

were elevated in diabetic rats, and glycyrrhizin reduced their expression.

Glycyrrhizin suppressed inflammation after ischemic insult

At 48 hours after ischemia was induced, Western blotting was done to investigate the effect of glycyrrhizin on the signaling pathways (Figure 6). The expressed levels of phosphorylated ERK were markedly decreased after glycyrrhizin treatment and the levels of phosphorylated Akt were slightly lowered. Phosphorylated p65 levels were also decreased.

Discussion

Our study revealed that hyperglycemia leads to the production of pro-inflammatory cytokines and makes the brain vulnerable to ischemic assault. Furthermore, our experiments demonstrate that the administration of glycyrrhizin reduces inflammatory milieu (without lowering the glucose levels) and protects against ischemic insult.

When localized ischemia occurs by occlusion of the middle cerebral artery, brain tissue undergoes necrosis, which subsequently induces an inflammatory response [14]. Recent studies report that HMGB1 could be elevated under various sterile ischemic conditions. Systemic release of HMGB1 during acute ischemic stroke and its impact on long-term outcome is reported [15,16].

In diabetic patients, plasma HMGB1 concentrations were significantly elevated [17]. Initially, hyperglycemia induces various molecular reactions resulting in production of AGE and radical oxygen species [18,19]. AGE can bind to their receptors called RAGE, which results in the release of pro-inflammatory stimuli, including HMGB1. Besides, as HMGB1 signals through multiple receptors, including RAGE, TLR2 and TLR4, it can initiate and mediate the amplification of inflammation in hyperglycemic circumstances [8]. The levels of TLR2 and TLR4 were elevated in type I and type II diabetes, which can induce an exaggerated response after cerebral ischemia [17,20]. The present study also supports that HMGB1 and inflammatory cytokines were elevated in the hyperglycemic status and TLR2, TLR4 and RAGE were increased in the rat brain after cerebral ischemia.

TLR, representative pattern recognition receptors, recognize a variety of pathogens or host-derived ligands, including HMGB1 [21]. Most TLR induce signal transduction via the myeloid differentiation protein 88 (MyD88), subsequently activating NF-κB-related genes, which encode various inflammatory cytokines [22]. Another HMGB1 receptor, RAGE, is activated via a variety signaling pathways, including NF-κB [23], ERK [24], and PI3K/Akt [25]. Through these receptors, HMGB1 can function as a complex and potent pro-inflammatory

cytokine. As presented in our study, glycyrrhizin could block these signaling pathways by directly binding to HMGB1. Additionaly, RAGE gene expression is dependent on NF-κB [26], which could be blocked by glycyrrhizin.

The central role of glycyrrhizin in inflammation and ischemia make it important to control its release from necrotic cells, modification by inflammatory signaling, and binding to its receptors. Previous studies made various efforts to develop an efficient therapeutic strategy. Treating with short hairpin RNA, anti-HMGB1 antibody, HMGB1 box A, or soluble RAGE reduced the infarct size and inflammatory response after cerebral ischemia [27,28]. However, until now, these approaches were far beyond application to clinical research. Takii *et al.* noted that glycyrrhizin binds directly to HMGB1, reduces DNA-binding abilities, and prevents phosphorylation of HMGB1 [29]. Recently, Mollica *et al.* also reported the inhibitory effect of glycyrrhizin by using NMR and fluorescence studies [12]. They identified that glycyrrhizin interacts with the hydrophobic chains located at the fold of the HMGB1 box A.

Glycyrrhizin is a natural compound found in licorice root, which has long been used as a drug to treat patients with hepatitis in Japan [11]. Former studies have shown that glycyrrhizin has an antidiabetic effect on both type I and type II diabetic rats [29,30]. However, this study failed to lower blood glucose levels, which may be due to the relatively short induction period. Therefore, we concluded that the neuroprotective effect of glycyrrhizin is not due to hyperglycemic control, but due to the control of the inflammatory status.

Because glycyrrhizin has long been used as a sweetener and ingredient of herbal medicine due to its safety, we are anticipating its potential therapeutic application in hyperglycemia after acute ischemic stroke.

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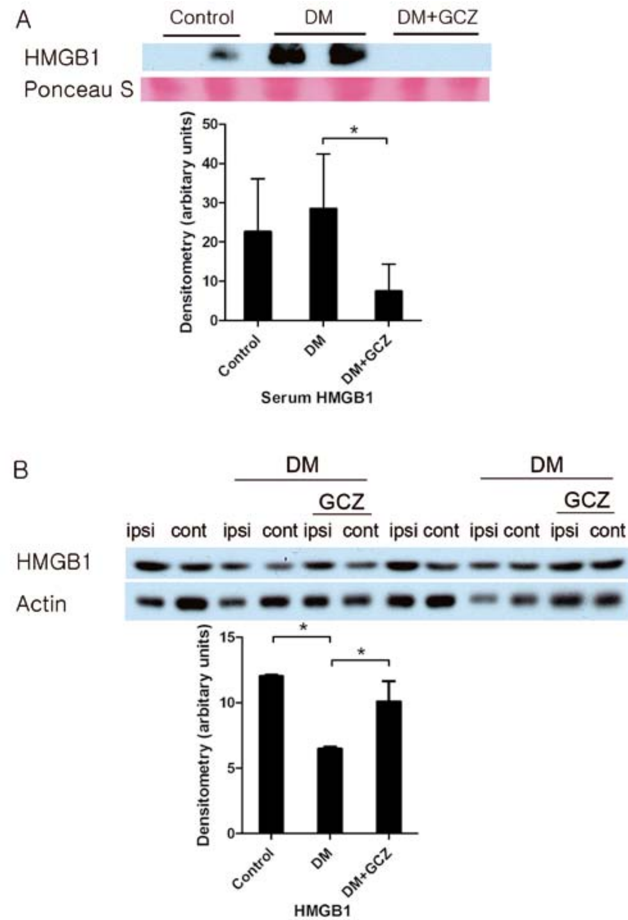

Figure 4. Cross-section of a propagating wavefront at several time points. The points of maximum curvature at each time, which determine the centerline of the CC, are circled.

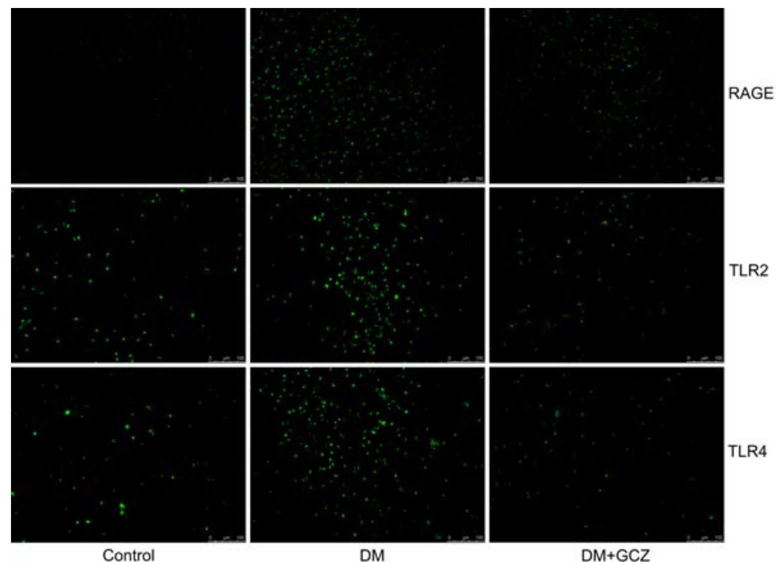

Figure 5. Reduction of HMGB1 receptor expression after HMGB1 inhibitor application. Enhanced expression of RAGE, TLR2, and TLR4 on diabetic rat brain was suppressed by glycirrhyzin; n=3 per group.

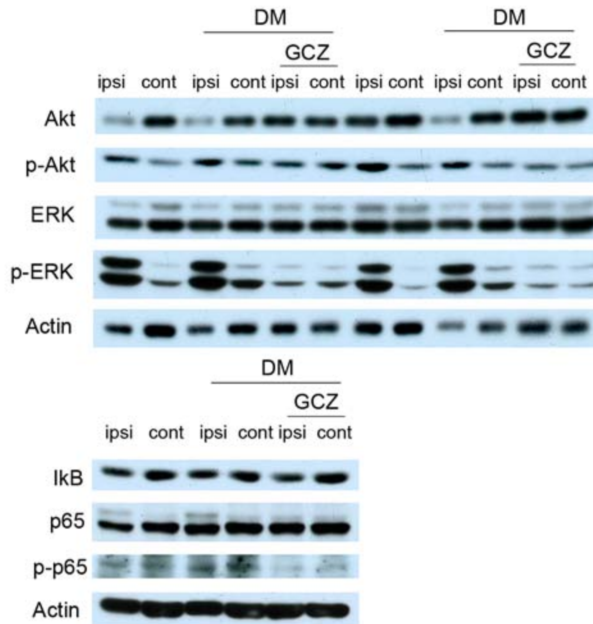

Figure 6. Western blot of transient middle cerebral artery occlusion model on diabetic rats. Western blot of ERK1/2, p-ERK1/2, AKT, p-AKT, p65, p-p65, and IκB; n = 2 per group.

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