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Chapter

MECHANISM OF EXACERBATION OF NEURONAL DAMAGE AFTER CEREBRAL ISCHEMIA: PROTECTIVE EFFECTS OF A WATER-SOLUBLE EXTRACT FROM CULTURE MEDIUM OF *GANODERMA LUCIDUM* MYCELIA

Naohiro Iwata, Mari Okazaki and Yasuhide Hibino*
Pharmaceutical Sciences, Josai University,
Sakado-shi, Saitama, Japan

ABSTRACT

The mortality of cerebrovascular diseases is decreasing worldwide, but the prevalence is increasing and associated with a considerably poor quality of life. Moreover, diabetes mellitus damages the macrovasculature and microvasculature, increasing the incidence of cerebrovascular diseases several times. Rapid deterioration of brain injury has been shown in the presence of both diabetes and cerebral ischemia, but the mechanism is poorly understood. The treatment of cerebral infarction is extremely challenging because pharmacotherapy has limitations in terms of indication time-frame, and treatment options are limited. Therefore, primary prevention of cerebral infarction is important. We focused on oxidative stress and inflammation, which are commonly involved in both diabetic diseases and transient ischemic attack, and investigated food products for prophylaxis as alternatives of pharmacological agents, and reported the efficacy and safety of extracts from culture medium of *Ganoderma lucidum* mycelia (MAK). MAK has been consumed for many years as a food for specified health uses; it is derived from hot water-soluble extraction from the fragments of whole culture medium of *Granoderma lucidum* mycelia after a specific time period, and has been shown to suppress blood glucose elevation and has antioxidative activity. This review initially addresses the involvement of oxidative stress, inflammatory response, and high mobility group box 1 (HMGB1) as an etiology of brain injury deterioration in the combined condition of diabetes and transient ischemic attack, and thereafter the mechanism of brain-protection activity by MAK.

* Corresponding author: seitaib@josai.ac.jp.

Keywords: cerebral ischemia, diabetes mellitus, water-soluble extract from culture medium of *Ganoderma lucidum* mycelia (MAK), oxidative stress, inflammatory

ABBREVIATIONS

AA	ascorbic acid
AGEs	advanced glycation end products
Bax	Bcl-2-associated X protein
BBB	blood brain barrier
CAT	catalase
CHOP	C/EBP homologous protein
COX-2	cyclooxygenase-2
CRP	C-reactive protein
CSF	cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DHA	dehydroascorbic acid
DHE	dihydroethidium
DM	diabetes mellitus
DPPH	1,1-diphenyl-2-picrylhydrazyl
dROMs	derivatives of reactive oxygen metabolites
Edaravone	1-phenyl-3-methyl-5-pyrazolone
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase 1/2, p44/42 MAPK (Thr202/Tyr204)
ETN	etanercept
GFAP	glial fibrillary acidic protein
GSH	glutathione
GLUT	glucose transporter
GPx	glutathione peroxidase
HIF	hypoxia inducible factor
HO-1	hemeoxygenase-1
Hsp70	heat shock protein 70
HMGB1	high mobility group box 1
Iba1	ionized calcium-binding adaptor molecule 1
ICAM-1	intercellular adhesion molecule-1
IDF	international diabetes federation
IL-1β	interleukin-1 beta
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MAK	water-soluble extract from culture medium of *Ganoderma lucidum* mycelia
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion

MCAO/Re	middle cerebral artery occlusion/reperfusion
MCP-1	monocyte chemotactic protein-1
MMP-9	matrix metalloproteinase-9
MPO	myeloperoxidase
MTT	3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-*2H*-tetrazolium bromide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NeuN	neuron specific nuclear protein
NF-κB	nuclear factor-kappa B
NMDA	*N*-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
non-DM	non-diabetes mellitus
O_2	superoxide anion radical
OH	hydroxyl radical
ONOO	peroxynitrite
PC12	pheochromocytoma 12
PKC	protein kinase C
p38 MAPK	p38 mitogen-activated protein kinase
QOL	quality of life
RAGE	receptor for advanced glycation end products
rHMGB1	recombinant HMGB1
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	real-time reverse transcription-polymerase chain reaction
SOD	superoxide dismutase
STZ	streptozotocin
SVCT2	sodium-dependent vitamin C transporter 2
TIA	transient ischemic attack
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TNFR	tumor necrosis factor receptor
tPA	tissue-type plasminogen activator
TTC	2,3,5-triphenyltetrazolium chloride
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labelling
XBP	X-box binding protein

1. INTRODUCTION

Cerebrovascular diseases include intracerebral hemorrhage, subarachnoid hemorrhage and cerebral infarction, which are subdivided into atherothrombotic cerebral infarction, cerebral embolization, and lacunar infarction, accounting for a substantial proportion of all cerebrovascular disease [1]. On a worldwide basis, the mortality of cerebrovascular diseases

is decreasing, but the prevalence is increasing, and it has become a social problem because these diseases worsen the quality of life (QOL) in patients. The brain accounts for 2% of total body weight, but consumes 20% of total oxygen consumption. This characteristic may lead to the production of reactive oxygen species (ROS), which accounts for 2 to 5% of oxygen consumption. Moreover, the brain includes neuronal cells, glial cells, and endothelial cells. While glial cells and endothelium are relatively resistant to ischemia (hypoxia and hypoglycemia), neuronal cells and glial cells are extremely vulnerable. Transient ischemic attack (TIA) is defined as reversible brain hypoperfusion, whose signs and symptoms resolve within 24 hr, but may be associated with neuronal death even in transient ischemia because of the susceptibility to ischemia. Moreover, repetitive ischemia is more likely to result in cerebral infarction [2], and apoptosis of neurons because of oxidative stress at ischemia and reperfusion [3].

The report in 2014 by the International Diabetes Federation (IDF) shows that diabetes affects 386.7 million people, 8.3% of the world adult population, and 4.90 million people died of diabetic complications. Chronic hyperglycemia increases oxidative stress such as ROS and reactive nitrogen species (RNS), and is a major risk factor for cerebral ischemia and atherosclerosis [4-7]. Previous studies in type 1 and 2 diabetic animal models have shown that concomitant diabetes worsens ischemic brain injury [8-10]. In the clinical setting, stroke is 2/3-fold more likely to develop in patients with diabetes and is associated with poor outcome and increased mortality due to unexplained causes [11, 12].

Previously, we focused on oxidative stress in both disorders of diabetes and transient ischemic attack; explored food products for the prophylaxis as alternatives of pharmacologic agents; and evaluated the efficacy and safety [8]. Investigation of several dozen natural compounds in foods with hypoglycemic and antioxidative activities resulted in identifying water-soluble extract from the culture medium of *Ganoderma lucidum* mycelia (MAK) after a specific time-period of culture. In addition, with the use of a model of transient cerebral ischemia in rats with streptozotocin (STZ)-induced diabetes mellitus (DM), cerebral injuries were shown to be enhanced as compared with non-diabetes mellitus (non-DM) rats, which was suppressed by orally administered MAK. Although apoptosis ascribed to oxidative stress and inflammatory processes is believed to play a major role, MAK has been shown to suppress necrosis as well as apoptosis, indicating that further studies are needed concerning the mechanism of activity because the brain-protective effect of MAK cannot be solely explained by common autoxidation. Therefore, we summarized the mechanism of activity of brain-protective MAK in addition to the already evaluated deteriorating processes in brain injuries.

2. Difference of Damage between Non-Diabetic and Diabetic Condition

2.1. Involvement of Oxidative Stress

In the cerebral cortex at the superacute phase of cerebral infarction, there is an ischemic core with severe damage, and a peri-infarction region with reversible mild-to-moderate injury (penumbra). In the ischemic core, electrotransport-system dysfunction, arachidonic acid

release associated with phospholipase A_2 activation, and conversion from xanthine dehydrogenase to xanthine oxidase due to an unexplained cause occur, producing ROS [13].

By contrast, in endothelial cells in the penumbra, ischemia-activated neutrophilic reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase produces superoxides, which are associated with secondary deterioration due to endothelial dysfunction. Subsequently, in reperfusion, a large volume of blood enters the neuronal region in the state of ischemia-associated ROS production. Therefore, oxygen delivery is increased; ROS are explosively produced, damaging the mitochondrial membrane. Simultaneously, in ischemic neuronal cell injury, release of glutamate, an excitatory amino acid, and elevation of intracellular calcium concentration occurred, leading to calmodulin activation and ROS production *via* neuronal nitric oxide synthase (nNOS). In addition, activated phospholipase A_2 advances the arachidonic acid-cascade and increases ROS production, which are believed to be involved in various pathological conditions associated with reperfusion (Figure 1), glutamate-calcium hypothesis [14-16]).

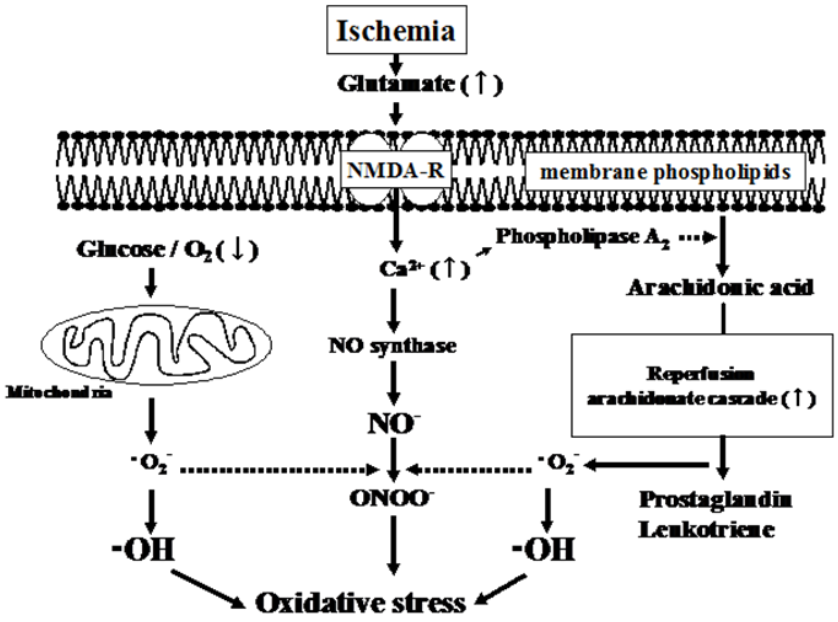

Figure 1. ROS generation after cerebral ischemia.

Although the development of ischemia/reperfusion-driven oxidative stress and subsequent signal transduction has already been addressed, a diabetic state is reported to be associated with profound oxidative stress [17]. The production of oxidative stress in the diabetic state is divided into six steps: (1) Reducing sugar such as glucose nonenzymatically forms a Schiff base with an amino group followed by the production of stable Amadori compound through Amadori rearrangement, resulting in ROS production in an autooxidative process of the compound. (2) Irreversible dehydration and condensation are repeated, forming advanced glycation end products (AGEs), which binds to AGE receptors to stimulate ROS production, activate nuclear factor-kappa B (NF-κB) to stimulate various cytokines and growth factor, and induce an inflammatory response. Chronic hyperglycemic state including diabetes stimulates AGE production, the accumulation of which increases oxidative stress

[17-19]. (3) In hyperglycemia, the polyol pathway is activated, and glucose is converted to fructose. Fructose has a potent glycation effect compared to glucose. ROS is further produced, leading to increased intracellular oxidative stress and cellular dysfunction in association with NADPH consumption associated with polyol metabolism and the resultant decreased level of glutathione reductase (4). Activation of protein kinase C (PKC) increases endothelial NADPH oxidase activity, stimulating the production of oxidative stress. (5) AGEs reduce the activity of antioxidative enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GPx) and reduce the levels of low-molecular-weight antioxidants including vitamins C and E. (6) The interaction between superoxide anion radical ($\cdot O_2^-$) and nitric oxide (NO) leads to the production of peroxynitrite ($ONOO^-$) resulting in protein nitration. In a diabetic state, those phenomena are enhanced to induce oxidative stress-driven atherosclerosis and micro- and macro-angiopathy. ROS including $\cdot O_2^-$, hydrogen superoxide (H_2O_2) and hydroxyl radical (OH) induces DNA damage, lipid peroxidation, and protein degeneration associated with adverse effects to the organism. As evidence suggests that ROS is involved in diabetes and many other disorders [12, 20], the decrement of oxidative stress in order to prevent and alleviate diseases has gained attention.

Iwata et al. assessed the level of *in vivo* oxidative stress with the use of circulating levels of hydroxyperoxide as the marker in SD rats as a model of type 1 diabetes mellitus (DM) with a blood sugar level of 543.2 ± 42.6 mg/dL in which a single administration of streptozotocin had been performed [8]. The levels of derivatives of reactive oxygen metabolites (dROMs) were expressed in CARR units (1U-CARR corresponds to 0.08 mg hydrogen peroxide/100mL). Oxidative stress was significantly elevated at the level of 252.8 ± 32.9 U·CARR in the diabetic group as compared with 131.0 ± 7.0 U·CARR in the non-diabetic group (blood sugar level of 123.5 ± 13.5 mg/dL), for a roughly two-fold increase. Moreover, antioxidative potential was significantly lowered at a level of 1475.7 ± 67.1 μmol/L in the DM group as compared with in the non-DM group (1875.4 ± 156.1 μmol/L). Lipid peroxide-level in the brain tissue was significantly elevated in the DM group by a factor of 1.3 to 2.0 as compared with that in the non-DM group, indicating the brain tissue under oxidative stress in DM, reflecting the oxidative stress level and antioxidative potential.

To maintain biological homeostasis, intrinsic and extrinsic removal mechanisms are essential against various toxicities of ROS. In terms of intrinsic ROS removal mechanisms, antioxidant enzymes including SOD, catalase (CAT), and GPx are involved. Therefore, the activity of antioxidative enzymes was measured: the activities of SOD, CAT, and GPx were significantly lowered in almost all brain regions (cortex, striatum plus hippocampus, cerebellum, and brain stem) [21].

By contrast, an extrinsic ROS removal system such as ingestion of antioxidative compounds including vitamin C is believed to be crucial [22-25]. Evidence suggests that, in DM, reduced circulating levels of vitamins C and E lowers *in vivo* antioxidative potential, and induces the production of $\cdot O_2^-$ and H_2O_2, augmenting nonenzymatic glycation of proteins [26]. Furthermore, as glycation leads to ROS production, reduced activity of antioxidative enzymes may be ascribed to increased production of ROS including $\cdot O_2^-$ and H_2O_2. These results suggest that oxidative stress is always present in a diabetic state even if otherwise normal, and that oxidative stress is present locally in the brain and systemically because of reduced activity of antioxidative enzymes and elevated levels of peroxidized lipids.

2.2. Altered Gene Expression in Ischemic Brain Injury

In ischemic brain injury, infarctional lesions are induced immediately after the occlusion of brain vessels, and extends from the ischemic core to the penumbra. Activated microglia induce the production of inflammatory cytokines such as interleukin-1 beta (IL-1β) and chemokines such as monocyte chemoattractant protein (MCP-1) in the progression of infarction [27]. In addition, astrocytes are activated, leading to increased production and secretion of S100B, which interacts with neurons, microglias, and astrocytes, resulting in increased expression of inflammatory factors and exacerbation [28]. As recent evidence suggests that inflammatory processes including C-reactive protein (CRP), an inflammatory marker, and interleukin-6 (IL-6) may be closely associated with atherosclerosis and arteriolosclerosis, which may cause cerebral infarction, clarification and control of the progression of inflammation are believed to be effective to protect the brain. Moreover, after cerebral ischemia, various reactions including apoptosis, stress against endoplasmic reticulum (ER), and protective factor production develop in addition to inflammation. Especially in a diabetic state, the above-mentioned and other factors are involved, complicating the deterioration. Iwata et al. collected reperfusion (Re)-treated brain specimens over time after the middle cerebral artery occlusion (MCAO) to evaluate brain injury and inferred the mechanism of progression and exacerbation by means of genome-wide expression analyses of brain tissue in nondiabetic and diabetic conditions. Coronal sections of 2 mm-thickness were obtained from removed brains after 1, 3, 6, 12, 24, 48, and 72 hr in the non-DM group, and after 0.5, 1, 3, 6, 12, and 24 hr, and stained with 2,3,5-triphenyltetrazolium chloride (TTC) to measure infarctional volume and cerebral edema. In the non-DM group, infarctional lesions started to develop 12 hr after ischemia/reperfusion, whose volume gradually expanded and reached approximately 50% of ipsilateral hemisphere at 48 hr and later (Figure 2). By contrast, edema progression was profound in the DM group as compared with in the non-DM group [29]. Thus, brain injury associated with ischemia/reperfusion developed earlier in a diabetic- than in a non-diabetic condition, suggesting that the progression may hamper survival 24 hr after reperfusion or later. Moreover, we exploited a DNA microarray method for genome wide expression analyses against a total of 27342 genes to determine the mechanism of brain-injury progression, and identified genes whose expression increased or decreased by a factor of 2 as compared with that of the non-DM sham surgery group. In the non-DM group, the expression changed in 64 genes at 1 hr after ischemia/reperfusion, 28 at 3 hr, and 145 at 12 hr. In the DM group, the change occurred in 82 genes at 1 hr, 215 at 3 hr, and 368 at 12 hr: in the brain tissue of the DM group, as compared with those of the non-DM group, the greater number of genes altered the expression time-dependently. In particular, gene expression associated with inflammation, oxidative stress, and apoptotic pathway increased over time, which occurred profoundly early 1 hr after reperfusion in the diabetic state (data not shown). Subsequently, the time course of inflammation-related gene expression was investigated up to 72 hr by means of real-time reverse transcription-polymerase chain reaction (RT-PCR) on the basis of results of DNA microarray analyses. The penumbra of the cerebral cortex increased the expression of tumor necrosis factor-alpha (TNF-α) and IL-1β by a factor of 2.0 and 5.6, respectively, which was already profound in the early phase after ischemia/reperfusion. By contrast, the expression of IL-6, cyclooxygenase-2 (COX-2) and matrix metalloproteinase-9 (MMP-9) increased later over time following

ischemia/reperfusion (MCAO/Re) and the gene expression of the upstream mediators including TNF-α and IL-1β in the DM rat group as compared with in the non-DM rat group.

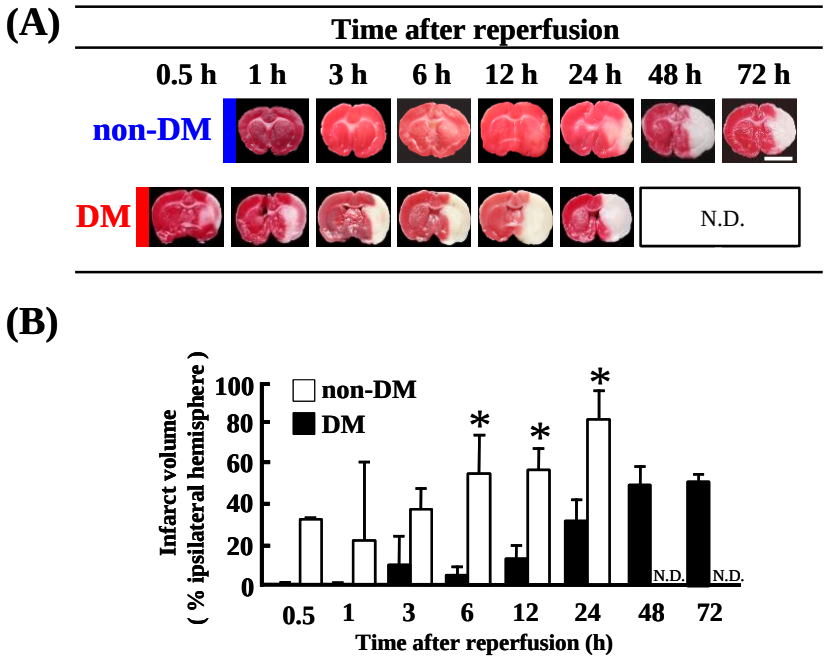

Figure 2. Brain infarct and edema in non-DM and DM ischemic rats detected by TTC staining.

(A) Images of representative coronal brain sections from non-DM (non-type 1 diabetes mellitus) rats and DM (type 1 diabetes mellitus) rats stained by TTC (2,3,5-triphenyltetrazolium chloride) at various time points after MCAO (middle cerebral artery occlusion)/Re (reperfusion) showing viable (red) and dead (pale pink) tissues. Scale bar = 5 mm. (B) Infarct volume in the ischemic hemispheres of non-DM and DM rats after MCAO/Re, assessed by TTC staining. The infarct volume was calculated as follows: infarct volume (%) = [LV - (RV-MV)]/LV×100 (MV: infarct volume, RV: right hemisphere volume, LV: left hemisphere volume). Scale bar = 5 mm. N.D.: not determined. Data are means ± S.D. (n = 3-4 per time point). *$P < 0.01$ *vs.* corresponding values for the non-DM.

The brain is a visceral organ where the blood-brain barrier (BBB) strictly controls molecular transition from circulating blood to cerebrospinal fluid. Transient brain ischemia is associated with an increased level of cytokines such as IL-6 in circulating blood and activated MMP-9 and subsequent BBB disruption, leading to cytokine-influx to brain tissue and more extensive damage [30]. Cerebral edema is divided into cytotoxic edema characterized by intracellular water retention and vasogenic edema characterized by BBB disruption. However, the distinction is equivocal in the clinical setting. Edema is also involved in the exacerbation of cerebral infarction associated with ischemia/reperfusion. It is believed in the rat model of cerebral edema that at an early stage, foot-process edema of astrocytes is followed by detachment from endothelial cells with transition to vasogenic edema due to BBB disruption [31]. As mentioned above, on the basis of expression analysis of MMP-9 gene over time, BBB disruption is considered to be ascribed to additionally increased level of MMP-9, which

was significantly increased in the sham group of DM, with the ischemic procedure. Therefore, permeability was assessed as the function of BBB with the use of Evans blue staining. The Evans blue binds circulating proteins and become high-molecular compounds, which normally cannot cross the BBB. While the leakage into cerebral tissue was observed 24 hr after ischemia/reperfusion in the non-DM group, it is earlier observed 1 hr after reperfusion in the diabetic status, indicating that BBB is disrupted in the earlier stage (Figure 3). The evidence indicates that ischemic brain injury may develop earlier in the DM group than in the non-DM group and that the injury pattern reflects the altered gene-expression of inflammatory mediators: i.e., in a diabetic state, excessive oxidative stress is constantly present even when otherwise normal; and in brain tissue, peroxidized lipid accumulates as antioxidative enzymes are inactivated. Moreover, in the condition of various oxidative stress-induced inflammatory processes, further ischemia/reperfusion-stimulated excessive inflammation and cell death may develop in a short time-frame, which causes BBB disruption and further deterioration.

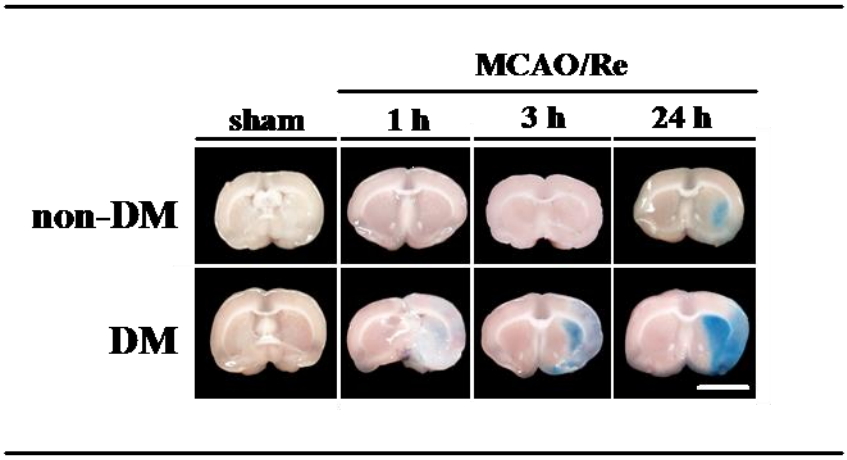

Figure 3. Blood-brain barrier permeability by Evans blue stain.

Representative coronal brain sections of DM and non-DM rats stained by Evans blue at 1, 3 and 24 hr after MCAO/Re. Scale bar: 5 mm. sham: sham-operated rats which are meaning normal rats operated as a control.

2.3. Involvement of HMGB1 Pathway in Ischemic Brain Disorder

The expression of many genes and proteins controls cytoprotection and cell death in brain cells of ischemic disorder. Heat shock protein 70 (Hsp70), which is a stress protein and a penumbra marker, is expressed in neurons, inducing modification and ubiquitination of denatured proteins to protect the brain. Moreover, evidence suggests that Hsp70 [32] and αB-crystalline of an effector of unfolded protein response [33] is expressed in astrocytes along with hemeoxygenase-1 (HO-1) [34] in microglias and some neurons, protecting brain. By contrast, apoptosis-related factors including Bcl-2-associated X protein (Bax) and caspase, and endoplasmic reticulum-stress factors including X-box binding protein (XBP), and C/EBP

homologous protein (CHOP) are expressed. The brain is an organ composed of various cells including neurons and glial cells such as astrocytes, microglias and oligodendrocytes, where each cell has specific expression of genes and proteins. This fact hampers clarification of the mechanism in brain injury. We focused on inflammatory processes after ischemic brain injury among a plethora of pathways associated the transient cerebral ischemia; and compared the changes of high mobility group box 1 (HMGB1), which have recently gained attention as an inducer of this pathway, between non-diabetic and diabetic groups [29].

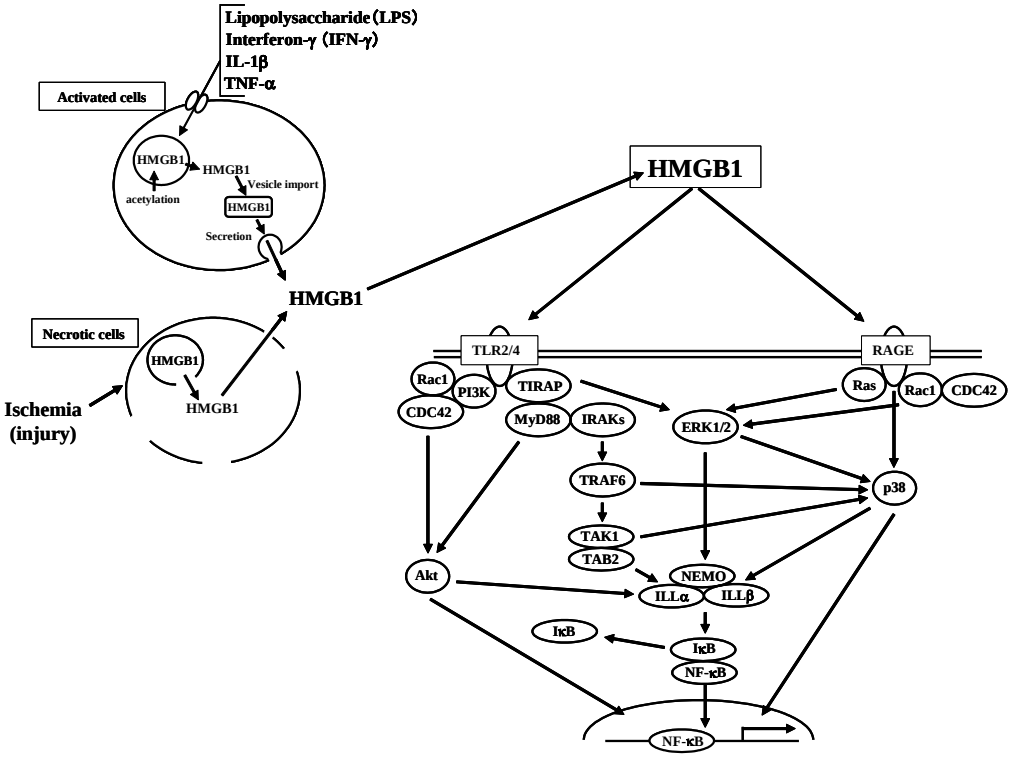

Figure 4. Pathways of HMGB1 secretion.

HMGB1 was found as a DNA-binding non-histone protein that plays a crucial role in maintaining DNA conformation. Initially, many studies focused on the involvement in transcriptional regulation, repair, and inflammation [35]. However, in 1999, the utility was intensively studied as a severity marker of sepsis reflecting life-threatening possibilities and as a management marker [36]. The amino acid-sequence is conserved among biological species with a difference of only two residues among 215 amino acid residues between rodents and human beings [37]. All of HMGB1 are usually present within the nucleus. However, it is released passively from the nucleus *via* cytoplasmic cytosol to the extracellular space in activated monocytes and macrophages [36, 38], maturated dendritic cells [39], endothelial cells [40], and necrotic cells [41]. Figure 4 shows that extracellular released HMGB1 activates NF-κB by interaction with receptors for advanced glycation end products (RAGE), toll-like receptor-2 and TLR4 on the cytoplasmic membrane of various cells [37, 41]. An experiment using glial cells from mice revealed that administered HMGB1 induced

the expression of inflammatory factors including TNF-α, inducible nitrite synthase (iNOS), and COX-2 [42], indicating that HMGB1 itself is proinflammatory and cytotoxic factor: *i.e.*, extracellular released HMGB1 affects directly adjacent cells. It is currently believed that HMGB1 is a key mediator in acute inflammation including sepsis, acute lung injury, trauma, postsurgical condition, disseminated intravascular coagulation, and ischemia/reperfusion as well as in a plethora of conditions including chronic inflammation such as rheumatoid arthritis, atherosclerosis, and the growth and invasion of malignant tumors [43-45]. Evidence suggests that HMGB1 is closely associated with the inflammatory process after cerebral ischemia. However, the role of HMGB1 receptors and the mechanism of brain-damage development remains unclear.

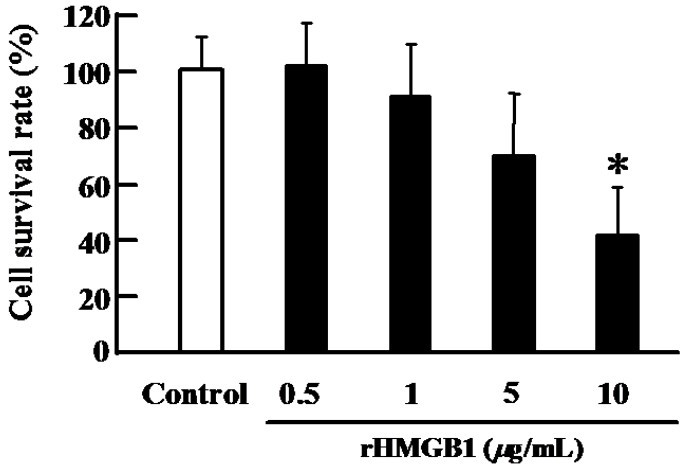

Figure 5. Cell death induced by HMGB1 in PC12 cells.

Therefore, Iwata et al. assessed the effect of HMGB1 on cellular injury after transient cerebral ischemia [29]. As mentioned above, HMGB1, a nuclear protein, is released into the extracellular space, and has itself a deleterious effect. For the replication of that phenomenon in cultured cells, a nerve growth factor was administered to rat adrenal pheochromocytoma-derived PC12 cells in order to differentiate to neuron-like cells. HMGB1 was administered to those cells, and cell survival was determined at 24 hr by means of MTT assay. The survival was decreased as increasing HMGB1 concentration (Figure 5). Moreover all of H_2O_2 (oxidative stressor), TNF-α, and exogenous HMGB1 released intracellular HMGB1 from nucleus *via* cytoplasm into the extracellular space, which was compatible with previous trials (Figure 6). Intranuclear HMGB1 is released actively from activated macrophages and monocytes, and passively from necrotic cells by the stimulation of TNF-α, IL-1β, and lipopolysaccharide (LPS), which are produced in the early phase of inflammation. Acetylation [46] and phosphorylation [47] control the translocation to the nucleus, while secretory lysosome controls the secretion to extracellular space. In addition, it has been reported that HMGB1 is not released from apoptotic cells, but from necrotic cells [48]. In recent years, HMGB1 has been believed to be a mediator in ischemic brain injury [49] as well as in intrapulmonary bleeding of acute lung injury [50], and liver injury after hepatic ischemia/reperfusion [51].

PC12 cell (1×10^5 cells/well) were incubated with rHMGB1 (recombinant HMGB1) for 24 hr and the cell survival was determined by MTT assay. The open column shows the non-DM group and the closed column shows the DM group. Data are means ± S.D. (n = 6). *P < 0.01 for statistical significance compared to the control.

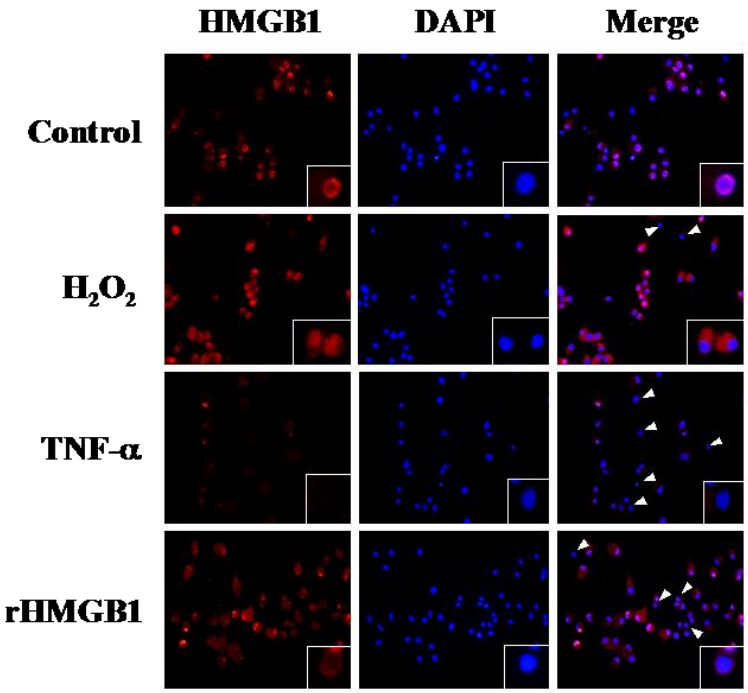

Figure 6. Immunohistochemical staining of HMGB1 in PC12 cells.

PC12 cells were treated with H_2O_2, TNF-α and rHMGB1, and then HMGB1 was stained with anti-HMGB1 antibody (red) and DAPI (blue). Arrowhead indicates, HMGB1 in cytoplasm.

As ischemia/reperfusion precipitates cell death in the diabetic rat brain, the association between locoregional variation of necrotic cell-released HMGB1 and ischemic brain injury over time was evaluated. Initially, expression of the HMGB1 gene in the cortical penumbra after the brain ischemic procedure was compared between both groups. There was no significant difference, suggesting that diabetic state and ischemia/reperfusion do not affect HMGB1-gene expression. Thereafter, coronary-section specimens of frozen brain tissue were stained for HMGB1 and nucleus with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). In addition, triple immunostaining was performed against neurons (neuron specific nuclear protein: NeuN), astrocytes (glial fibrillary acidic protein: GFAP), and microglias (ionized calcium-binding adaptor molecule 1: Iba1). HMGB1 was gradually translocated from the nucleus to the cytoplasm 6 to 12 hr after ischemia/reperfusion in the cerebrocortical HMGB1-enriched cells of the non-DM rat sham group, which is in accordance with findings of Qui's study [49] indicating that neurons are the major source of HMGB1 in early ischemic brain injury. By contrast, the translocation of HGMB1 to cytoplasm had already occurred before

ischemia/reperfusion and been excreted extracellularly from all cells of the ischemic core after 1 hr in the diabetic rat group [29].

Evidence suggests that the serum level of HMGB1 is elevated in patients with cerebral infarction as a mediator of ischemic brain injury [52]. Therefore, Iwata et al. assessed the variation over time in the cerebrospinal fluid (CSF) and in the plasma. There was no difference in HMGB1 level between the non-DM sham group and the DM sham group of rats. While the HMGB1 level markedly increased 1 hr after reperfusion in the DM rat, the level gradually increased up to 12 hr after reperfusion. The variation of HMGB1 in plasma level over time is similar to that in CSF. The evidence suggests that after transient cerebral ischemia HMGB1 in cerebral neuron translocates from the nucleus to the cytoplasm and is immediately excreted into the extracellular space, contributing to early inflammation as an upstream initiator locally in the brain and systemically [29].

Previous studies have suggested that the proinflammatory effect of HMGB1 is mild alone, but is enhanced markedly in the concomitant presence of other factors including IL-1 and LPS [53, 54]. The HMGB1 releases due to TNF-α and IL-1β, in addition to the release from necrotic cells, are considered to constitute a major pathway. Therefore, we assessed the expression of proinflammatory cytokines after ischemia/reperfusion. The gene expression of IL-1β and TNF-α was significantly increased in the sham group of diabetic rats by a factor of 5.6 and 2.0, respectively as compared with in the non-diabetic rat group. The expression of MMP-9 was increased by a factor of 2.6 in the sham (control) rat group in the diabetic rat, while the incremental pattern did not differ between the non-DM and DM groups after cerebral ischemia/reperfusion. Other studies have suggested that induced MMP-9 is involved in the disruption of the BBB as a deleterious factor in cerebral infarction [55, 56]. Taking these findings together, we conclude that the expression of TNF-α and IL-1β stimulates HMGB1 release in the diabetic state, which itself induces the production TNF-α and IL-1β, forming an inflammation-amplifying vicious cycle.

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily expressed on neurons, glial cells and endothelia in the brain [57-60]. RAGE-ligands include AGEs, β-amyloids, and S-100 proteins in addition to HMGB1 [61]. In particular, HMGB1 has a seven-fold affinity as compared with AGE, which was initially found as a ligand to HMGB1 in an integral study of rat-derived cultured neurons with the use of [^{125}I]-HMGB1 [62]. Moreover, interaction of HMGB1 with RAGE on cell membranes leads to NF-κB activation [63] *via* phosphorylation of mitogen-activated protein kinase concerning intracellular signal transduction in macrophages [64] and neutrophils [65]. In terms of ischemia/reperfusion-associated injury, the presence of receptors is crucial for evaluation of the inflammatory process in which HMGB1, as a receptor, activates intracellular signal transduction. Intriguingly, the volume of ischemia/reperfusion-caused infarcts has been shown to decline in RAGE-knock-out mice and with soluble RAGE [66], indicating that increased RAGE expression adversely affects ischemic cerebral injury. Therefore, the magnitude of RAGE expression is determined, which gradually increased up to 12 hr after reperfusion in the non-DM rats, while the expression is increased before reperfusion in the DM rats of the sham group, which persisted stably up to 12 hr after reperfusion. RAGE expression is induced by hypoxia-inducible factor-1 (HIF-1) [67, 68]. TNF is also known as a RAGE inducer. Mitochondrial electron transport system-derived ROS augments RAGE expression in hyperglycemia [69]. RAGE expression is increased, which could be mitigated with antioxidants in the brain of diabetic animal models [70], suggesting

that lowered oxidative stress suppressed the expression. In our study, an increased level of ROS and TNF was observed, suggesting that those effects influence increased RAGE expression, and that the further expression caused by ischemia/reperfusion contributes to worsening ischemic cerebral injury.

Toll-like receptor (TLR), which is considered another receptor of HMGB1, is a one-transmembrane receptor; plays an important role in the innate immune system; and recognizes components of many pathogens including bacteria, viruses and fungi and conducts signal transduction. Evidence suggests that the signal transduction into cells is involved in NF-κB-induced inflammatory process through MAPK activation along with RAGE. Recent studies have demonstrated that HMGB1 increases MMP-9 expression through toll-like receptor 4 (TLR4) after cerebral ischemia in neurons and astrocyte, resulting in BBB disruption and cerebral edema [55, 71]. An elevated level of MMP-9 is caused by HMGB1 independently of TNF-α [71], and HMGB1-TLR signal transduction pathway is closely associated with the inflammatory process [49, 72]. Our unpublished data reveal that TLR4 expression does not change up to 12 hr after reperfusion in non-DM rats, while the expression peaks after 3 hr in a diabetic state, followed by a decrease after 12 hr. The evidence suggests that extracellular release of HMGB1 and increased number of receptors may stimulate intracellular signal transduction. Furthermore, phosphorylation of ERK1/2 and p38, and translocation of NF-κB into the nucleus were slightly enhanced up to 12 hr after reperfusion in cerebrocortical penumbra of the non-DM group, whereas the phosphorylation of ERK1/2 was prominent 3 hr after reperfusion, and present at 12 hr. Phosphorylation of p38 and intranuclear translocation of NF-κB were already present in the sham group, and ischemic procedure resulted in its augmentation. Akt phosphorylation peaked 3 hr after reperfusion in both groups, although the phosphorylation was increased in the sham group of DM. The expression of COX, which is downstream of signal transduction, was markedly increased 3 to 12 hr after reperfusion only in the DM group.

Until now, HMGB1-targeted treatment strategies have been advanced, one of which is ethyl pyruvate. Treatment with this compound downregulates expression of HMGB1 in cardiac ischemia/reperfusion, and suppresses activation of HMGB1/TLR4/NF-κB pathway, exerting a cerebroprotective effect in traumatic brain injury [73, 74]. Intravenous administration of nafamostat mesilate suppresses TNF-α expression, which indirectly suppresses HMGB1 expression and mitigates lung injury in an endotoxic shock model [75]. In addition, anti-HMGB1 antibody has a BBB-protective effect and decreases infarct volume [76, 77]. HMGB1-target therapies are expected to be established.

Recent studies using human endothelia have suggested that hyperglycemia-induced ROS increases the expression of HMGB1 in rats [69, 78]. We observed increase of *in vivo* oxidative stress and decreased antioxidative capacity; and increased level of lipid peroxide associated with decreased level of antioxidative enzymatic activity in brain tissue, confirming that that oxidative stress is augmented in the brain [8]. One possible explanation is that augmented oxidative stress in the diabetic state markedly affects the HMGB1/RAGE pathway, resulting in worsened ischemic brain injury. Taking these findings together, as shown in Figure 7, ROS production is increased in the diabetic state as compared with in the non-diabetic state; ischemia/reperfusion early alters intracellular distribution of HMGB1; excreted abundant HMGB1 interacted with receptors of RAGE and TLR2/4, activates intracellular signal transduction, and inducing inflammation and cell death. In conclusion, increased

extracellular HMGB1 is a major deleterious factor of ischemic cerebral injury, and induces severe inflammation and cell death in the penumbra as well as in the ischemic core.

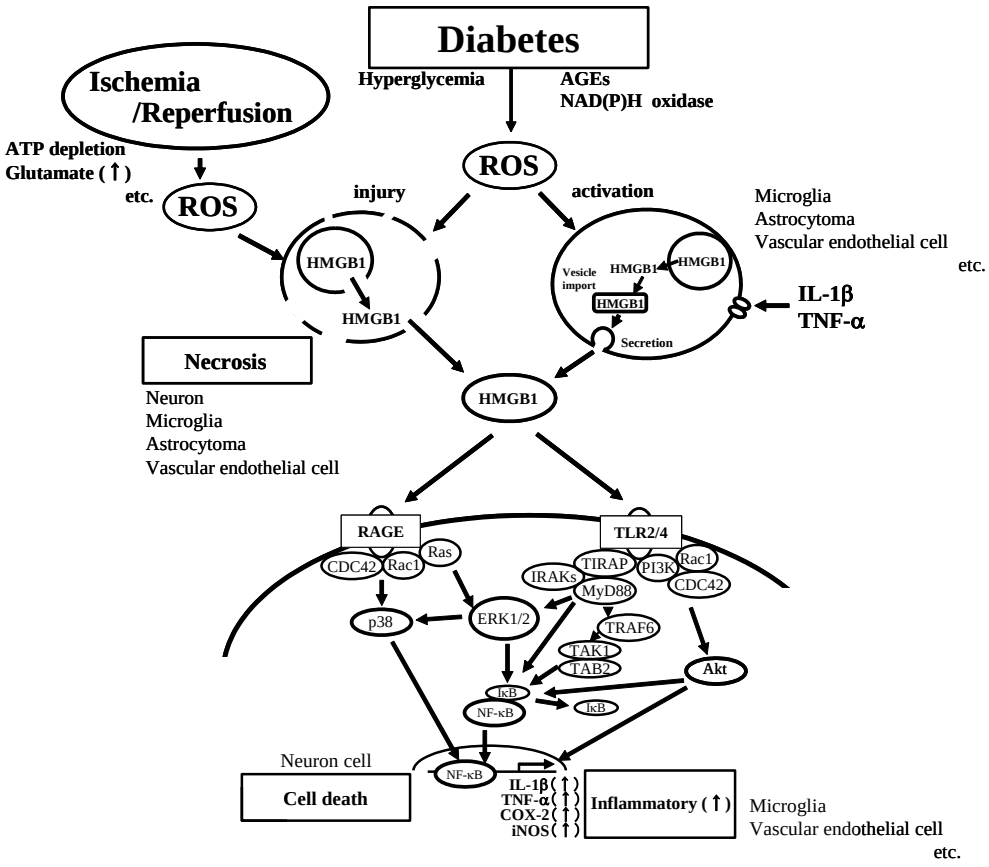

Figure 7. HMGB1 secretion and signal transduction pathways.

HMGB1 can be released into the extracellular space actively from macrophages and monocytes or passively from necrotic cells. Extracellular HMGB1 interacts with different receptors such as RAGE or TLR2/4 and promotes inflammatory responses leading to mitogen-activated protein kinase (MAPK) and NF-κB activation. A diabetic state induces pro-inflammatory cytokines in the brain, conceivably *via* hyperglycemia and/or oxidative stress, accelerating intracellular translocation and the release of HMGB1 from neuronal cells after ischemic injury. Increase in extracellular HMGB1 may further induce the inflammatory response and cellular necrosis in the ischemic penumbra, leading to aggravation of ischemic injury in a diabetic state.

2.4. Involvement of Cytokines

In gene expression analyses with the use of the above-mentioned DNA microarray methods, inflammation-associated gene expression was elevated early in ischemia, strongly suggesting the involvement of inflammation in acute cerebral ischemia. With regard to

intracellular localization of HMGB1, which is an intracellular protein and an inflammatory mediator, intracellular distribution of HMGB1 profoundly changed in the DM group, which suggests activation of downstream signal transduction. As, until now, previous studies have suggested that cytokines including TNF-α and IL-1β stimulate the release of HMGB1 from activated cells and propel inflammation, an in-depth understanding concerning cytokine-expression in ischemic brain injury is crucial.

Therefore, the expression of IL-1β was determined in cerebrocortical penumbra over time after ischemia/reperfusion. The expression was increased 24 hr after the ischemic procedure. By contrast, the expression was significantly increased in the DM sham group by a factor of 3.5 as compared with in the non-DM sham group; and profoundly increased 3 hr after reperfusion by a factor of 25 (Figure 8), revised from reference [79]. The TNF-α expression was increased 24 hr after the ischemic procedure by a factor of 5 as compared with the non-DM sham rat group. By contrast, the expression was increased by a factor of 1.4 in the DM sham rat group as compared with in the non-DM sham rat group; and increased profoundly by a factor of 11 after reperfusion as compared with in the non-DM rat group, whose level remained elevated 24 hr after reperfusion. Moreover, the expression of proinflammatory factors including COX-2 and iNOS was determined over time: COX-2 is a relatively downstream inflammatory mediator and the expression is controlled by transcription factors such as NF-κB. COX-2 expression was markedly increased in each DM rat group: i.e., in the sham rat groups (non-DM; 0.08 ± 0.02, DM; 0.13 ± 0.08); and at 3 hr (non-DM; 0.12 ± 0.01, DM; 0.50 ± 0.04) and at 24 hr (non-DM; 0.27 ± 0.03, DM; 0.36 ± 0.05) after reperfusion. Similarly, the expression of iNOS was increased over time with MCAO/Re procedure in the non-DM and DM groups, and the increment was greater in the sham rat group, and 3 and 24 hr after reperfusion in the DM rats [79]. Leukocyte infiltration to inflammatory sites is controlled by cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1). The expression is rapidly enhanced *via* proinflammatory cytokines in inflammation, leading to recruitment to endothelium and extraversion of leukocytes. Therefore, the expression of ICAM-1 and a leukocyte infiltration marker, myeloperoxidase (MPO) was determined. The ICAM-1 expression increased over time after the ischemia/reperfusion procedure in both of nondiabetic and diabetic states; and the increment was also greater in the DM sham rat group (non-DM; 0.02 ± 0.03, DM; 0.12 ± 0.02) and at 24 hr after reperfusion (non-DM; 0.29 ± 0.07, DM; 0.94 ± 0.24). In addition, the MPO expression gradually increased, whose activity was greater 24 hr after reperfusion in the diabetic state than in the nondiabetic state [79]. The evidence suggests that because cerebral cortex is more likely to provoke inflammation in the diabetic state than in the nondiabetic state, ischemia/reperfusion precipitates dramatic expression of proinflammatory mediators in the penumbra: i.e., the constellation of reactions is involved in enlargement of cerebral infarct lesions, strongly suggesting the importance of inflammation-control for cerebral protection.

As inflammation is involved in the early stage of ischemic cerebral injury and diabetes, the therapeutic effect of the biological product etanercept (ETN), which captures and neutralizes free TNF-α, was assessed in the ischemia/reperfusion procedure [80]. ETN administered after ischemia/reperfusion was ineffective at any dose in both non-DM and DM rats. However, the single dosage after ischemia or before the ischemic procedure had a cerebroprotective effect in the non-DM group; and ETN was effective when the administration occurred repetitively immediately after the development of diabetes, suggesting that suppression of sole cytokines cannot lead to a meaningful therapeutic effect

because the inflammatory process develops abruptly in diabetes and after ischemia. A drug is not ordinarily administered as therapeutic intervention before the occurrence of cerebral ischemia. However, diabetes is a risk factor for cerebral infarction. Preceding inflammation may markedly worsen disability caused by cerebral ischemia. Therefore, reduction of early inflammation as a prophylaxis may lessen disability in cerebral infarction. Whether disability caused by cerebral infarction can be alleviated with daily ingested food or its constituents as a pharmaceutical agent was investigated.

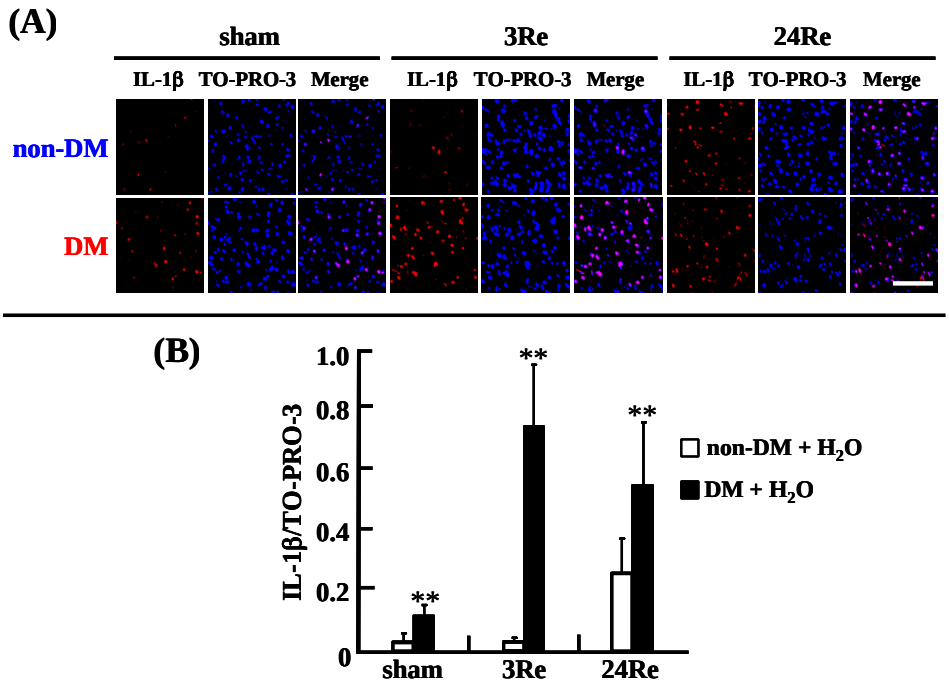

Figure 8. Expression of IL-1β in the penumbral cortex after MCAO/Re in non-DM and DM rat brains.

Representative photographs of IL-1β immunostaining (red fluorescence) and nuclei by TO-PRO-3 (blue fluorescence) in the cortex coronal sections of the non-DM and DM rats (A). Quantitative analysis of IL-1β fluorescence intensity in the cortex (B). Scale bar: 100 μm. Data are means ± S.D. (n = 3). $^{**}P < 0.01$ for statistical significance compared to the non-DM + H_2O rat group.

3. Improvement of Ischemic Cerebral Injury with Antioxidative Food or Constituents

3.1. Evaluation of Cerebroprotective Effect of Ascorbic Acid

There are two strategies of improving blood flow therapy and brain protective therapy toward cerebral infarction acute treatment. The latter strategy focuses on the protection of both brain cells and endothelium. Oxidative stress plays a critical role in the acute phase. Its

modulation has been considered important, and many agents have been developed. Among others, edaravone (1-phenyl-3-methyl-5-pyrazolone), which suppresses oxidative stresses such as ・OH radicals, and the damage of free radical-susceptible membrane lipids has been shown to be effective in the clinical setting in Japan. As edaravone reduces hemorrhage size after the administration of a thrombolytic agent, edaravone, tissue-type plasminogen activator (tPA), and a combination of those agents are believed to be beneficial. Moreover, in the Japanese guidelines for the management of stroke in 2009, edaravone is classified into grade B (recommended to perform), and suppression of oxidative stress is shown to be effective to protect the brain [81]. As above-mentioned, substantial oxidative stress occurred after ischemia/reperfusion, which is greater in DM rats than in non-DM rats; and the each cerebral region is under prominent oxidative stress. Therefore, a typical antioxidative food or its constituent, L-ascorbic acid (AA, vitamin C) was investigated concerning cerebroprotective effect after reperfusion.

AA is a major antioxidative vitamin. Through the transporter, ingested AA is transferred and stored in body tissues, especially in the adrenal grands, ocular lenses, and brain in higher concentrations, exerting physiological functions. Although AA and other antioxidants are present in the blood circulation, AA especially promptly captures and eliminates excessive ROS [82]. Rats and mice can synthesize AA from D-glucose, and rats synthesize in the liver at a level of 150 mg per kilogram of body weight per day. By contrast, primates such as human beings cannot synthesize AA, and need to ingest it by means of meals and others. Therefore, antioxidative capacity was assessed in terms of elimination of superoxide anions and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. As compared with edaravone, AA is effective in elimination of superoxide and a stable synthetic radical, DPPH by a factor of 40 and 1.8, respectively (Table 1). Thereafter, the cerebroprotective effect of oral two-week AA administration against ischemia/reperfusion was assessed. Infarcts were observed in cerebral striate and cortical matter in the non-DM rats group with ischemia/reperfusion receiving water alone (27.8% ± 4.2), but considerably reduced in the AA group at a dose of 100 mg/kg (16.5% ± 7.7). Infarcts were greater in the DM rats receiving water than in the non-DM counterparts by a factor of approximately 2.4 (65.6 ± 7.5 vs. 27.8% ± 4.2), affecting thalamus and whole gray matter. By contrast, in the AA-administered group, infarcts were present in confined regions of gray matter and thalamus (23.0% ± 9.9). The two-week AA administration mitigate considerably brain injury in both non-DM and DM rats (Figure 9, revised from reference [21]).

Table 1. Scavenging effect of the free radical

Sample	Superoxide anion radical scavenger IC_{50} (mg/mL)	DPPH radical scavenger EC_{50} (mg/mL)
MAK	1.059	0.327
edaravone	1.246	0.007
ascorbic acid (AA)	0.039	0.004
vitamin E	47.616	0.114

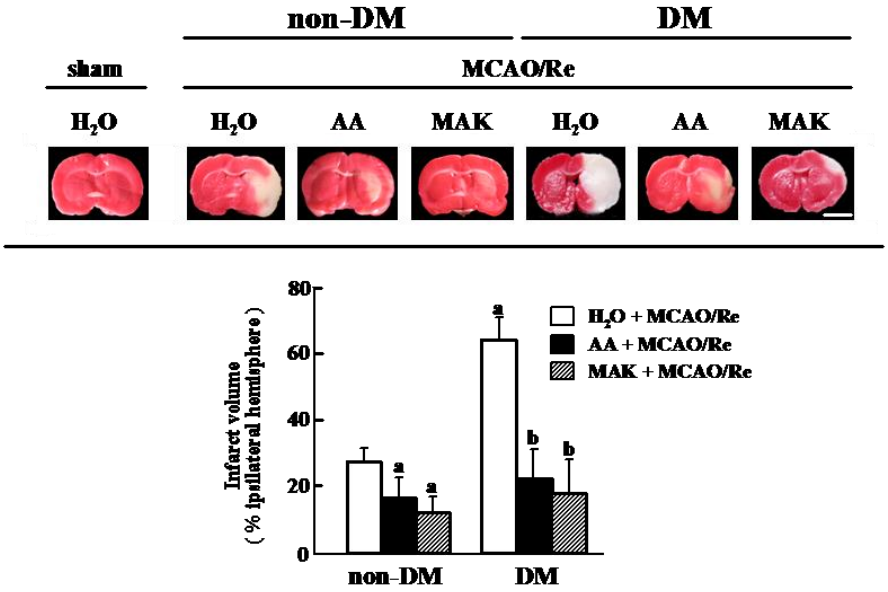

Figure 9. Effects of antioxidant on infarction induced by cerebral ischemia in rats.

Representative data of TTC staining from the rats of the non-DM with sham operation group, distilled water administered (H_2O) or AA or MAK supplemented non-DM or DM group with MCAO with reperfusion (MCAO/Re). AA (100 mg/kg body weight) or MAK (1 g/kg body weight) or water was administrated once daily for 2 weeks by per os (p.o.). Scale bar = 5 mm. The data are presented as mean ± S.D. (n = 6-10). $^{a}P < 0.05$ *vs.* non-DM (H_2O + MCAO/Re), $^{b}P < 0.01$ *vs.* DM (H_2O + MCAO/Re).

In addition, in order to determine the protective efficacy of AA, the magnitude of oxidative stress and activity of antioxidative enzymes in those rats were assessed. The magnitude of oxidative stress in the DM rats was twice as high as in the non-DM rats (252.8 ± 32.9 U.CARR vs. 131.0 ± 7.0 U.CARR), whereas the magnitude was reduced in the DM rats receiving oral AA to the level of the non-DM rat group (137.3 ± 32.1 U.CARR). Antioxidative capacity was reduced in the DM rat group as compared with that in the non-DM rat group receiving water by a factor of approximately 1.3 (1475.7 ± 67.1 U.CARR *vs.* 1875.4 ± 156.1 U.CARR).

The evidence indicates that the DM group was exposed to increased oxidative stress, while the antioxidative capacity was reduced. By contrast, in the AA-administered rat group, there was no significant variation of antioxidative capacity (1454.7 ± 101.9 U.CARR) [21]. In addition, the lipid peroxide-content of cerebral tissue was significantly elevated in the DM rat group as compared with in the non-DM rat group by a factor of 1.3 to 2.0, while the content was reduced significantly in the AA-administered DM rat group. The activities of cerebral SOD, CAT, and GPx were significantly reduced in the brain. Those results suggest that antioxidative enzymatic activity is reduced, leading to reduced antioxidative capacity with increased oxidative stress in the DM brain tissue. However, when AA was administered, enzymatic activity was not reduced, without difference with that of the non-DM rat group. Taking these findings together, antioxidative enzymatic activity is often reduced but AA retains the activity similar to that of nondiabetic level, inhibiting significantly lipid peroxide

formation in the brain. In conclusion, the maintenance of antioxidative enzymatic activity may play a role in eliminating excessively induced oxidative stress in ischemic brain injury among diabetic rats.

As AA-administration had cerebroprotective effect in both non-DM and DM rat groups, its mechanism was assessed histopathologically. Superoxide production was increased in cortical penumbra in the DM rat group as compared with in the non-DM rat group by a factor of approximately 1.8 at baseline, which was suppressed to the level of the non-DM rat group. Cerebral superoxide production was increased with ischemia/reperfusion in the both groups, though greater in the DM rat group, which was suppressed with AA-administration. As inflammation plays an important role in the development of ischemic cerebral injury, the expression of related cytokines was assessed. TNF-α and IL-1β were intensively expressed at baseline, and the AA-administration significantly inhibited that expression. In addition, AA reduced considerably both of those increasingly expressed cytokines after ischemia/reperfusion.

AA crosses BBB through two sorts of transporters, which is controlled by two mechanisms. One is sodium-coupled ascorbic acid transporter 2 (SVCT2) expressed in the choroid plexus and neurons, which transports reduced form of AA. Evidence suggests that the SVCT2 expression is intensified with ischemia/reperfusion, stimulating transfer of AA into the brain [83]. AA transfer is believed to be conducted by SVCT1. The other is glucose transporter 1 (GLUT1) expressed in BBB and neurons, which is involved in transport of dehydroascorbic acid (DHA), i.e., oxidized form of AA, in addition to glucose. GLUT1 expression is reduced by a hypoxia-induced transcription factor, hypoxia inducible factor (HIF) [84]. Thus, both transporters are closely associated with ischemia, but the expression in a diabetic state is poorly understood. Therefore, genetic expression of SVCT2 and GLUT1 was assessed. There was no difference in the SVCT2 expression at baseline between the non-DM and DM groups even after AA-administration. However, after the ischemia/reperfusion procedure, the SVCT1 expression was increased despite the AA-administration in the non-DM rat group, while not increased in the DM rat group and increased only at the AA-administration. The GLUT1 expression was marginally reduced at baseline in the DM rat group as compared with in the non-DM rat group, but was increased with AA by a factor of 2 in the both rat groups. The GLUT1 expression was profoundly intensified immediately after ischemia/reperfusion in both of the non-DM and DM rat group, on which AA had no effect [85].

Other studies reported that AA and glutathione (GSH) is depleted in the cerebral tissue early in ischemia [86, 87], indicating the importance of AA-supply for the protection against excessive ROS caused by mitochondrial dysfunction. The evidence is supported by previous studies that diabetic hyperglycemia inhibited the GLUT1 expression in BBB through competitive inhibition by D-glucose [88]. Moreover, because Seno et al. used human umbilical vein-endothelium to reveal that INF-α and IL-1β suppress SCVT2 and affect transport of AA [89], inflammation may be closely involved in decreased expression of SVCT2. With regard to the levels of AA in plasma and cerebral tissue, the plasma level decreased after the sham-procure and the ischemia/reperfusion procedure in the DM rat group; and was similar to that in the non-diabetic state in the AA-administered rat group. In the cerebral tissue, the AA level was increased with the sham procedure in the non-DM AA-administered rat group and reduced with the ischemia/reperfusion in both of non-DM and DM rat groups, whereas the AA level was similar to that in the sham group, in the AA-

administered rat group. Given together, in the diabetic state, the SVCT expression is not elevated after ischemia/reperfusion, suggesting the AA depletion in the circulation because of oxidative stress; and the expression of GLUT1 which is involved in the transfer of oxidative AA is reduced at baseline. Those two factors may be associated with worsened cerebral injury. By contrast, the AA-administration in DM-rats improved the transporter expression to similar level of that of the non-DM rat group, suggesting increased AA-transfer to the brain.

In conclusion, increased AA-transfer suppressed excessive ROS production after cerebral ischemia/reperfusion and the inhibition of lipid peroxide production may lead to cerebroprotection.

3.2. Cerebroprotective Effect of Water-soluble Extract from Culture Medium of *Ganoderma lucidum* Mycelia (MAK)

MAK is produced according to the below-mentioned procedures: hyphal bodies of Reishi mushroom of *Ganoderma lucidum* are inoculated to *solid medium* including bagasse and rice bran; and the whole medium is hot-water-extracted, sprayed, and dried with whole medium immediately before fruit body formation (Figure 10).

The mushroom spores were germinated and cultured in liquid medium. The resulting *Ganoderma lucidum* mycelia pellet was injected into solid medium which was composed of bagasse and defatted rice bran (8:1, w/w). The medium was disrupted before the formation of fruit bodies (4 months). The disrupted material was incubated in water at 40-60°C for 60 hr to promote autolysis of the mycelia and partial digestion of the culture medium with mycelial enzymes. The digest was then extracted with water at 60°C. The extract was aseptically filtered and lyophilized. The resulting pale-brownish powder was designated MAK.

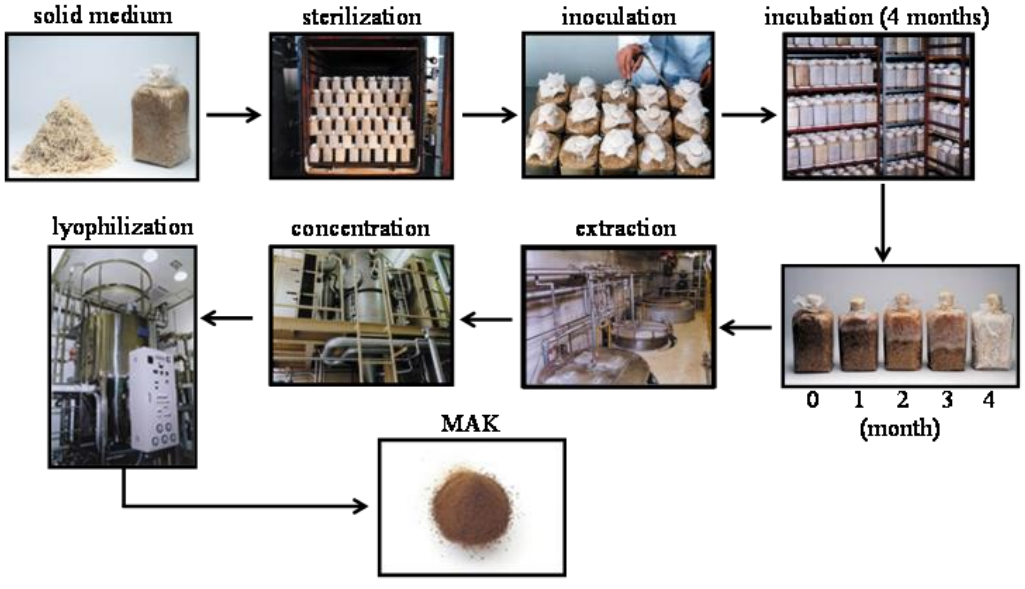

Figure 10. Preparation of a water-soluble extract from culture medium of *Ganoderma lucidum* mycelia (MAK).

MAK has been used to maintain health among consumers for roughly two decades as a revitalizing healthfood product and nutrition supplement. Reishi mushroom also has long been used as a traditional medicine for revitalization and longevity, whose hyphal body or the constituents is reported to have various physiological activities including antihyperglycemic [90], immunomodulating [91], antineoplastic [92], antiviral [93], cholesterol-lowering [94], and antioxidative effects [95]. As MAK also contains solid medium-degradation products including water-soluble lignin caused by the hyphal bodies and hyphal autodigestion in addition to hyphal body components, its activity may differ from that of Reishi mushroom. The activities that have been reported include antihyperglycemic [96-99], immunostimulating [100], antineoplastic [101], antidepressive-like [102], and antihypertensive effects [103]. Moreover we revealed that oral MAK could alleviate ischemic cerebral injury [79, 103], and address, in this chapter, how MAK protects the brain.

Initially, antioxidative capacity was assessed in terms of the elimination of superoxide anions and DPPH radicals. Although the DPPH radical elimination of MAK was inferior to that of AA, the superoxide anion elimination was similar to that of edaravone (Table 1). The cerebroprotective effect of MAK after two-week oral administration was also assessed. The infarct size was reduced (12.5% ± 4.9) in the MAK group at a dose of 1 g/kg body weight, which is greater than effect expected from AA, as compared with that of the water-administered non-DM rat group (27.8% ± 4.2) after the ischemia/reperfusion. By contrast, although the infarct size was 65.6 ± 7.5% in the water-administered DM rat group, the size was profoundly reduced at 17.8% ± 11.2 (Figure 9, revised from reference [8]). Therefore, the mechanism of cerebroprotective effect of MAK was assessed. The magnitude of oxidative stress in the DM rats was approximately twice as high as in the non-DM rat group (252.8 ± 32.9 U.CARR vs. 131.0 ± 7.0 U.CARR). By contrast, MAK had superior effect to AA at 109.3 ± 15.1 U.CARR in the DM rat group. Moreover, as compared with no treatment, MAK administration reduced the stress at 84.3 ± 2.6 U.CARR. The evidence suggests that MAK has an intensive *in vivo* antioxidative property. By contrast, when water was administered, antioxidative capacity was significantly lower in the DM rat group at 1649.0 ± 101.2 μmol/L than in the non-DM rat group at 1475.7 ± 67.1 μmol/L, whereas MAK marginally reversed antioxidative capacity at 1649.0 ± 101.2 μmol/L in the DM rat group. In the MAK-administered group, the intracerebral content of lipid peroxide was reduced, and the activities of SOD, CAT and GPx, which were low in the brain of the DM rat group, were enhanced/suppressed whereas, in the non-DM MAK rat group, the CAT activity was enhanced in the cerebral gray matter and cerebellum. Then, intracerebral superoxide production was evaluated with the use of the dihydroethidium (DHE) stain after ischemia/reperfusion: although the sham DM rat group showed a two-fold increase of superoxide, MAK administration reduced the level to that of the non-DM rat group. It was revealed that MAK considerably reduces the increase of superoxide after ischemia/reperfusion, indicating that chronic oxidative stress in the diabetic state was mitigated and involved in the elimination of excessive oxidative stress in ischemic brain injury. As discussed here, oxidative stress-associated inflammation is closely involved in the deterioration of ischemic brain injury. Thus, the management of inflammation may be important in inhibiting cell death. We evaluated the effect of MAK on ischemia/reperfusion-induced cerebral apoptosis with the use of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, and the expression of proinflammatory factors with the use of immunohistological method [79]. The number of TUNEL-positive cells in

cerebral penumbra in the DM rat group, as compared with in the non-DM rat group, significantly started to increase 3 hr after reperfusion, whereas the positive cell-number was significantly reduced in the non-DM and DM MAK-administered rat groups. These results indicate that MAK mitigates ischemia/reperfusion-induced inflammatory processes, and inhibits cell death, exerting a cerebroprotective effect [79].

As discussed above, an explanation about deterioration of ischemic brain injury in DM rats is that variation in intracellular localization of HMGB1 occurs substantially, i.e., a lot of intranuclear HMGB1 in neurons is translocated into the cytoplasm and subsequently released into the extracellular space, inducing profound inflammation and cell death in surrounding tissue (Figure 7). In addition, MAK is believed to inhibit necrosis as well as apoptosis of cerebral neurons. Therefore, in order to determine the association of the cerebroprotective effect of MAK with the intracellular behavior of HMGB1, the effect of MAK on HMGB1 behavior induced by various stimulations in cultured neuron-like pheochromocytoma cell line (PC12 cells) was assessed. PC12 cells were incubated with MAK of 1 μg/mL and 100 μg/mL, and exposed to biosimilar oxidative stress of H_2O_2 for 6 hr. Thereafter, intracellular ROS production was measured. ROS production was suppressed dependent of the MAK concentration. Thus, the finding that MAK inhibits oxidation of cerebral tissue in DM rats and lowers lipid peroxide content indicates that MAK may inhibit intraocular ROS production. Then, the effect of MAK in intracellular HMGB1 behavior was assessed. The translocation of intranuclear HMGB1 into cytoplasm and its extracellular release occurred in biosimilar oxidative stress H_2O_2-exposed cells, but the release was suppressed in the MAK-pretreated cells. Furthermore, the translocation and release of HMGB1 similarly occurred in cells pretreated with TNF-α and recombinant HMGB1 (rHMGB1), which MAK suppressed (unpublished data). These results suggest that MAK could affect the initiation of inflammation due to oxidative stress efficiently, but not in TNF-α and HMGB1, which are precedingly released in the advanced inflammatory process after ischemic injury: i.e., MAK may inhibit intracellular ROS production and HMGB1 release in response to oxidative stress.

MAK may exert antioxidative activity in the brain after crossing the BBB through *Ganoderma lucidum* hyphal body-derived constituents including proteoglycans and terpenoids as well as hyphal body-degraded solid medium constituents including soluble lignin and hyphal autodigestion products, but further studies are required to investigate that activity. Thus, the cerebroprotective effect of MAK that the present study revealed could be alleviated *via* HMGB1, but other factors may be involved. A recent study reported that, in an experimental hypoxic cerebral ischemia-animal model of mice with type 2 diabetes (KK-A^y), MAK ingestion inhibited programmed cell death, necroptosis, extorting a cerebroprotective effect [104]. Therefore, in-depth investigation is required to elucidate the mechanisms.

CONCLUSION

Diabetes raises the oxidative stress of the whole body, increases the lipid peroxides of the brain. In addition, a diabetic state induces pro-inflammatory cytokines in the brain, conceivably *via* hyperglycemia and/or oxidative stress, accelerating intracellular translocation and the release of HMGB1 from neuronal cells after ischemic injury. The early release of HMGB1 and the promoting of inflammatory response may be involved in the aggravation of

neuronal damage caused by transient cerebral ischemia in diabetes. Therefore, it is important to inhibit the cytokines and HMGB1 released in response to ischemia during the treatment of post-ischemic injury in diabetic patients. In conclusion, MAK treatment inhibits the apoptotic and release of HMGB1 and attenuates the exacerbation of cerebral injury and neurological deficits in the diabetic state. These phenomena could be attributed to the antioxidant activity and anti-inflammatory effects of MAK.

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