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Diabetes-Mediated Exacerbation of Neuronal Damage and Inflammation After Cerebral Ischemia in Rat: Protective Effects of Water-Soluble Extract from Culture Medium of *Ganoderma lucidum* Mycelia

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1. Introduction

Diabetes mellitus is a metabolic disorder, suffered by hundreds of millions of people throughout the world, which is characterized by hyperglycemia resulting from insufficiency of insulin secretion and/or action (Wild et al., 2004). Complications associated with diabetes affecting vessels, eyes, kidney, and peripheral nerves reduce the QOL of diabetic patients. Also, diabetes is widely recognized as a major risk factor for atherosclerotic disease such as acute brain ischemia. Indeed, diabetic patients have a higher risk of stroke compared with non-diabetic patients (Baynes 1991; Stephens et al., 2009). Additionally, they are more likely to have a poor prognosis and increased mortality after stroke (Biller et al., 1993; Vinik et al., 2002). Previous studies have demonstrated that the diabetic state increases oxidative stress in the brain and aggravates cerebral ischemic injury in both type I (Li et al., 2004; Saito et al., 2005; Rizk et al., 2005) and type II diabetic animal models (Anabela et al., 2006; Tureyen et al., 2011). In addition to neuronal damage attributed to hypoxia and ATP depletion caused by vascular obstruction in ischemic core region, cerebral injury caused by subsequent reperfusion is also involved in the pathophysiology of transient ischemia (Doyle et al., 2008; Nakka et al., 2008; Wang et al., 2010). During reperfusion, the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is enhanced by abrupt re-oxygenation (Cuzzocrea et al., 2001; Saito et al., 2005). Besides direct injurious effects to the cell membrane, proteins and DNA by

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oxidation, ROS and RNS activate the pro-apoptotic pathway including the activation of caspase family proteases leading to DNA fragmentation in the neuronal cells of the ischemic penumbral region (Sugawara et al., 2007; Nakka et al., 2008). Hyperglycemia is assumed to be a major factor responsible for excessive generation of ROS. In the diabetic state, "glucose toxicity" caused by augmentation of the intracellular glucose oxidation process and non-enzymatic glycation of protein molecules leads to over production of ROS (Baynes, 1991; Giacco et al., 2010). Moreover, experimental transient hyperglycemia caused by intravenous infusion of glucose has been shown to increase ROS production and exacerbate brain injury after ischemia and subsequent reperfusion in rats (Tsuruta et al., 2010).

In addition to apoptotic cell death, inflammatory neurodegeneration is a crucial process contributing to cerebral damage after ischemia and reperfusion (Brown et al., 2010). ROS has been shown to activate nuclear factor-κB (NF-κB), which enhances transcription of the genes encoding pro-inflammatory cytokines and cell adhesion molecules, leading to neuroinflammatory responses (Saeed et al., 2007). Activation of the transcription factor NF-κB by ROS in microglia and astrocytes leads to an increase in the expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines including TNF-α, IL-1β and IL-6, which accelerate inflammatory responses and promote neuronal cell death in the ischemic region (Brown et al., 2010). Gene expression of IL-1β and IL-6 is much higher in type2-diabetic mice as compared with normoglycemic control mice (Tureyen et al., 2011). These pro-inflammatory cytokines and inflammatory mediators have been indicated to induce further inflammatory responses involving extravasation of neutrophils, macrophages/microglia through the expression of intracellular adhesion molecule 1 (ICAM-1) in endothelial cells (Wang et al., 2002; Chrissobolis et al., 2011). Myeloperoxidase (MPO) expressing in neutrophils and macrophages/ microglia, which has often been used as a histopathological marker for inflammation, generates ROS such as hypochloride and super oxide anion radical ($O_2^- \cdot$) and leads to further tissue damage (Breckwoldt et al., 2008). Thus, enhanced oxidative stress and inflammatory responses induced by hyperglycemia may substantially contribute to the exacerbation of cerebral injury caused by transient ischemia and subsequent reperfusion in the diabetic state.

Ganoderma lucidum (*G. lucidum*), a very popular medicinal fungus, has long been known for its beneficial effects on human health and longevity in Asian countries. Its fruiting bodies and cultured mycelia are used to treat chronic hepatopathy (Shi et al., 2008), hypertension (Kabir et al., 1988), hyperglycemia (Zhang et al., 2004), and tumor (Lu et al., 2003; Kubo et al., 2005). The pharmacological activities of *G. lucidum* constituents responsible for many of its health benefits, such as antioxidant (Zhu et al., 1999), anticancer (Lu et al., 2003; Kubo et al., 2005), anti-inflammatory (Akihisa et al., 2007), and immunomodulatory activities (Lai et al., 2010) have been elucidated. The water-soluble extract from culture medium of *G. lucidum* mycelia (MAK), a commercially available nutritional supplement, is a freeze-dried powder of a hot-water extract prepared from a solid culture medium composed of bagasse and defatted-rice bran overgrown with *G. lucidum* mycelia by cultivation for about 3.5 months. In a previous study, we demonstrated that the orally administered MAK attenuated oxidative stress and relieved exacerbation of cerebral injury induced by middle cerebral artery occlusion and

reperfusion (MCAO/Re) in streptozotocin (STZ)-induced diabetic rats (Iwata et al., 2008). However, the mechanism of its cerebroprotective effect still remains unclear. Thus, we evaluated the effects of chronic oral pretreatment of MAK on the production of $O_2^{-}\cdot$ and apoptosis in STZ-rat brain after MCAO/Re. Furthermore, to clarify whether MAK suppresses inflammatory responses induced by the transient ischemia and reperfusion, we examined the effects of MAK on the expression profile of TNF-α, IL-1β, iNOS, COX-2, ICAM-1 and MPO in the brain during reperfusion.

2. Materials and methods

2.1 Experimental diabetic animals

Male Sprague Dawley rats (4-week old, weight 120-140 g; Tokyo Exp. Animal Co., Ltd., Tokyo, Japan) were purchased and housed in a temperature-controlled environment (23 ± 0.5°C) with a cycle of 12 hrs light and 12 hrs dark. The rats were given a standard rodent chow and water *ad libitum*. Animal care and the surgical procedure were performed in accordance with guidelines approved by the National Institutes of Health and the Josai University Animal Investigation Committee. A diabetic state was induced in the rats (diabetic group) by a single injection of STZ (60 mg/kg, i.p.) dissolved in 0.1 mM sodium citrate (pH 4.5), while the rats of the non-diabetic group were injected with buffer only (Iwata et al., 2008). Seven days after the STZ-injection, the plasma glucose level was determined using a glucose analyzer (Ascensia, Bayer Medical Co., Ltd., Land Nordrhein-Westfalen, Germany). Diabetes was defined by a blood glucose level greater than 300 mg/dl. Then, the diabetic and non-diabetic groups were divided into two groups respectively and were housed for an additional 6 weeks until stroke was induced by MCAO. MAK (1 g/kg; MAK group) or distilled water (control group) was administrated orally once daily for the last 2 weeks.

2.2 Middle cerebral artery occlusion and reperfusion

Focal cerebral ischemia was induced by MCAO with a standard intraluminal filament technique as previously described (Iwata et al., 2010). The animals were anesthetized with 4% halothane and maintained with 1.5% halothane and 30% oxygen under spontaneous respiration. After a midline incision at the neck, the right common carotid artery was exfoliated under an operating microscope. All branches of the external carotid artery were isolated and ligated. The tip of the 4-0 surgical nylon monofilament rounded by flame heating was inserted up through the internal carotid artery. When a small resistance was felt, insertion was stopped. The distance from bifurcation of the common carotid artery to the tip of the suture was approximately 20 mm in all rats. Cerebral blood flow was detected by laser Doppler Flowmetry (ATBF-LC1, Unique Medical Co., LTD., Tokyo) and about 50% reduction of its baseline associated with MCAO was ascertained in the rats. Rectal temperature was maintained at 37°C with a heat lamp and a heating pad during the operation. After 2 hrs of occlusion, the filament was withdrawn to allow for reperfusion. Then, the animals were permitted to recover from the anesthesia at room temperature. The rats were reperfused for 3 or 24 hrs before they were killed. The sham operation with the same manipulation without introduction of the monofilament was also performed in the 4 rats of the non-diabetic and diabetic groups, respectively.

2.3 Neurological evaluation

Post-ischemic neurological deficits were evaluated after 3 or 24 hrs of reperfusion on a 5-point scale as described as follows: grade 0:no deficit, grade 1:failure to extend right forepaw fully, grade 2:spontaneous circling or walking to contralateral side, grade 3:walking only when stimulated, grade 4:unresponsive to stimulation and a depressed level of consciousness, grade 5:death (Iwata et al., 2008; Vinik et al., 2002). Animals that did not show neurological deficits were excluded from the study.

2.4 Infarct and edema assessment

After 3 or 24 hrs of reperfusion, the animals were deeply anesthetized with diethyl ether and decapitated. The brain was removed and cut into four 2-mm coronal sections using a rat brain matrix, and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Wako Pure Chemicals) at 37°C for 15 min. The coronal slices were fixed in 10% formaldehyde for photography (Iwata et al., 2008; Vinik et al., 2002). Then, infarct areas were determined by using the image analysis system (Scion Image 1.62), and were added to obtain the infarct volumes per brain. Corrected infarct volume (%) = [left hemisphere volume - (right hemisphere volume - the infarct volume)] / left hemisphere volume × 100. Edema in the ischemic hemisphere was also calculated: edema (%) = (right hemisphere volume - the infarct volume) / left hemisphere volume.

2.5 Detection of $O_2^{-}\cdot$ generation in brain

Detection of intracellular $O_2^{-}\cdot$ generation in the penumbral region of the cortex following MCAO/Re was performed by staining freshly frozen brain sections (8 μm thick) with the fluorescent probe dihydroethidium (DHE). The brain sections were immediately incubated with DHE (10 μmol/l, Sigma) in phosphate-buffered saline for 30 min at 37°C (Muranyi et al., 2006). To determine the fluorescent intensity of oxidized DHE, three microscopic fields at the penumbral cortex regions of each hemisphere were captured using a confocal laser-scanning microscope at excitation of 510 nm and emission of 580 nm. Fluorescence intensity of the oxidized DHE was quantified using imaging software (FV10-ASW 1.7, OLYMPUS Co. Ltd., Tokyo, Japan).

2.6 TUNEL staining

Apoptosis in the brain tissues was measured using the Apoptosis in situ Detection Kit Wako (Wako, Laboratories, Osaka, Japan), which is based on the TUNEL (Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP nick end labeling) procedure, that is the addition of fluorescein-dUTP to 3'-terminals of apoptotically fragmented DNA with TdT followed by immunochemical detection using anti-fluorescein antibody conjugated with horseradish peroxidase (POD) and DAB (3-3'-diaminobenzidine tetrachloride) as a substrate. Coronal brain sections (8 μm thick) were used for the assay. The slides were lightly counterstained with hematoxylin and observed under a microscope (BX51W1, OLYMPUS). Quantification of TUNEL positive cells was achieved by cell counting in areas of the penumbral cortex affected by ischemia. Three randomly chosen visual fields were counted in each region by an investigator without knowledge of the experimental conditions. The percentage of apoptotic cells was calculated by the apoptotic index, i.e. dividing the number of positive-staining nuclei by the total number of nuclei (Li et al., 2004).

2.7 Real-time PCR analysis

The temporal gene expression patterns of pro-inflammatory cytokines (IL-1β and TNF-α) and inflammatory mediators (COX-2, iNOS and ICAM-1) were evaluated by quantitative real-time PCR analysis as described earlier (Liu et al. 2007). The rats subjected to MCAO were killed at 3 or 24 hrs of reperfusion, and the total RNA sample was obtained from the ischemic penumbral cortex of each rat. Total RNA was extracted with RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Total RNA (0.5 μg) from each sample was reverse-transcribed with oligo dT and random hexamer primers using reverse transcriptase (PrimeScript™ RT Enzyme Mix I, Takara RNA PCR Kit, Takara Biomedicals, Shiga, Japan). Real-time PCR was performed with 10 ng of cDNA and a pair of gene specific primers (Takara Biomedicals) added to the SYBR Premix EX *Taq* (Takara Biomedicals) and subjected to PCR amplification in the iCycler iQ Real-Time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (1 cycle at 95°C for 10 sec, and 50 cycles at 95°C for 5 sec and 60°C for 34 sec). The expression of β-actin was used to normalize cDNA levels. The PCR products were analyzed by a melting curve to ascertain the specificity of amplification. Data were normalized to β-actin and were expressed as mean ± SD relative to the sham-operated non-diabetic group.

2.8 Immunohistochemistry

Immunostaining was performed as previously described (Faraco et al., 2007). Briefly, rats were sacrificed at the indicated time points and transcardially perfused with cold saline. Brains were fixed with 4% phosphate-buffered paraformaldehyde. Coronal brain sections (8 μm thick) were incubated sequentially with 3% hydrogen peroxide for 40 min at room temperature to inhibit endogenous peroxidase, followed by incubation with blocking buffer (100% block ace; Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) for 2 hrs. Slides were incubated with polyclonal rabbit anti-IL-1β antibody (1:300, Santa Cruz Biotechnology, CA, USA), polyclonal rabbit anti-TNF-α antibody (1:200; Rabbit mAb, Hycult Biotech, PB Uden, The Netherlands), polyclonal rabbit anti-COX-2 antibody (1:200, Cayman Chemical, Michigan, USA), polyclonal rabbit anti-iNOS antibody (1:200, Santa Cruz), monoclonal mouse anti-RECA-1 (1:200, Abcam Biotechnology, Cambridge, UK), polyclonal rabbit anti-Cleaved caspase-3 (1:100, Cell Signalling Technology, Danvers, MA, USA), and monoclonal mouse anti-MPO (1:100, Hycult Biotech) in 0.01 mol/l phosphate-buffered saline overnight at 4°C. After washing with PBS, these were correspondingly incubated with Cy3- and FITC-conjugated secondary antibodies (1:200, Chemicon, California, USA) for 2 hrs at room temperature. Finally, sections were incubated with the nuclear stain TO-PRO-3 (1:10000, Invitrogen, California, USA) in phosphate-buffered saline for 10 min at room temperature with gentle agitation, washed and mounted using 70% glycerol mounting medium. Immunofluorescence was visualized using a Laser Scanning Confocal Microscope (FluoView FV1000, OLYMPUS). Fluorescence intensity was quantified using imaging software (FV10-ASW 1.7, OLYMPUS).

2.9 Western blotting

The cytosolic and nuclear extracts were prepared by the method of Meldrum (Meldrum et al., 1997; Aragno et al., 2005). Briefly, the penumbral cortex was homogenized at 10% (w/v) in a polytron homogenizer (Kinematica AG, Switzerland) using a homogenization buffer (20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.5 mM EDTA, 1% Nonidet P-40, 1 mM

EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg/ml pepstatin A, and 4 μg/ml leupeptin). Homogenates were centrifuged at 1,000×g for 5 min at 4°C. Supernatants were removed and centrifuged at 105,000×g at 4°C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer (20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg/ml pepstatin A, and 4 μg/ml leupeptin). The suspensions were incubated on ice for 30 min for high-salt extraction followed by centrifugation at 15,000×g for 20 min at 4°C. The samples were stored at -80℃ until used. Brain tissues were homogenized in the SDS sample buffer (125 mM Tris (pH 6.8), 4% SDS, 10% sucrose, 0.01% bromophenol blue, and 10% 2-mercaptoethanol) and boiled for 1 min. Protein concentration was quantified by using the Bradford method (Protein Assay Reagent Kit, Bio-Rad Laboratories). The samples (40 μg) were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, England) through a semidry-type blotting (Bio-Rad) method, blocked by 5% nonfat dry milk in PBS with Tween-20 (PBS-T) (137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, 0.1% Tween-20), and incubated with appropriate antibodies as described below. The filters were incubated with each primary antibody overnight at 4°C, with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hr at room temperature in 5% nonfat dry milk/PBS-T. Finally, the target molecules were visualized through an enhanced chemiluminescence western blotting detection system (Amersham Biosciences) on the X-ray film (Amersham Biosciences). The following primary and secondary antibodies used in this study were NF-κB (rabbit, 1:2,000 Santa Cruz) and β-actin (mouse, 1:10,000, Sigma), anti-rabbit IgG HRP antibody (1:5,000, Amersham Biosciences), and anti-mouse IgG HRP antibody (1:10,000, Amersham Biosciences).

2.10 Statistical analysis

Statistical analysis was performed with a two-way ANOVA followed by post hoc Tukey's multiple-comparison test. Neurological deficit scores were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney *U* test. In all cases a *P*-value of <0.05 was regarded as statistically significant.

3. Results

3.1 Physiological characteristic parameter

The control diabetic group had typical characteristics of type 1 diabetes such as a decrease in the body weight (296.6 ± 33.3 g) and hyperglycemia (535.4 ± 104.0 mg/dl) compared with the control non-diabetic group (340.0 ± 22.4 g, 119.8 ± 17.1 mg/dl), which were similar to previous reports (Iwata et al., 2008; 2010). Chronic treatment of MAK for 2 weeks showed a slight but significant decrease in blood glucose of the diabetic rats (404.4 ± 109.2 mg/dl, $P<0.01$), whereas MAK had little effect on the body weight (266.6 ± 33.3 g).

3.2 Infarct volume and neurological deficits after transient MCAO with reperfusion

Figure 1 shows the effects of MAK on the brain infarction by MCAO/Re in the non-diabetic and diabetic groups. Representative coronal brain sections stained by TTC after MCAO/Re

showing viable (red) and dead (white) tissues are in (A). In the sham operated animals, there was no apparent damage in any brain region. The infarct area in the control non-diabetic rats after 24 hrs of reperfusion extended to the corpus striatum and cortex, whereas only a small striatal infarct was observed after 3 hrs of reperfusion. In the control diabetic group, brain injury induced by MCAO/Re was remarkably exacerbated. The cerebral infarct was produced within 3 hrs of reperfusion and the infarct region was extended to a large part of the left striatum and cortex in the diabetic rats. In contrast, the ischemic damage in the MAK-pretreated non-diabetic and diabetic animal brain was smaller as compared to those of the respective controls. Quantitative determination of infarct volume (B) indicated that the cerebral infarct volume was increased and associated with reperfusion time, which was markedly accelerated in the control diabetic group. The infarct volume evaluated at 3 hrs after reperfusion in the control diabetic group was significantly increased about 5-fold as compared with the non-diabetic group. Brain edema also tended to be exacerbated by diabetes, whereas no significant difference was detected between the non-diabetic and diabetic groups (data not shown). Consistent with the result of brain infarct volume, neurological deficits were exacerbated by reperfusion and diabetes (Fig. 2). MAK-pretreated diabetic rats showed significant alleviation in the neurological deficits compared to the control diabetic rats.

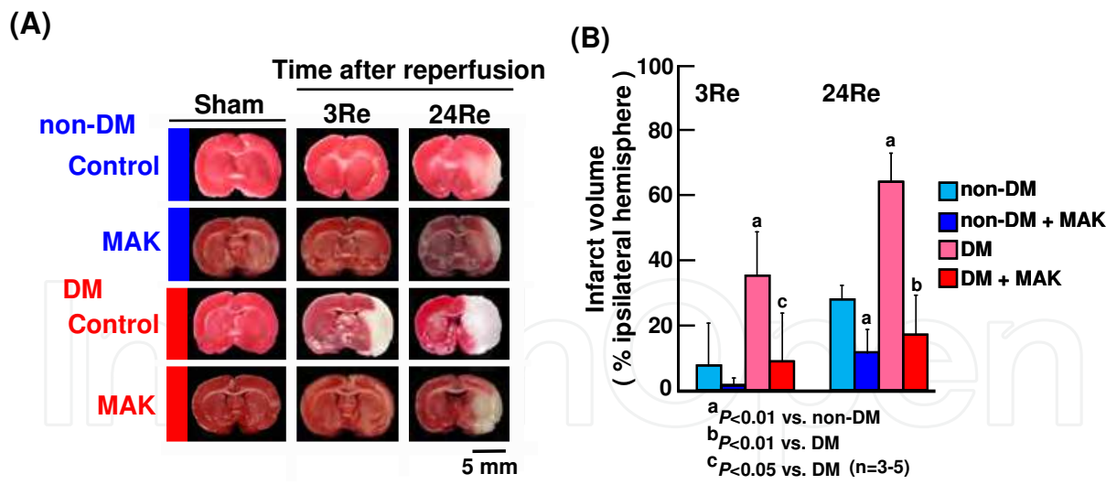


Fig. 1. Effects of MAK on infarction induced by MCAO/Re in non-diabetic (DM) and DM rat brains.

Representative coronal brain section photographs of the DM and non-DM rats stained by TTC at 3 or 24 hrs of reperfusion after 2 hrs MCAO (A). Infarct volume in ischemic hemispheres of the DM and non-DM groups after MCAO/Re by TTC staining (B).

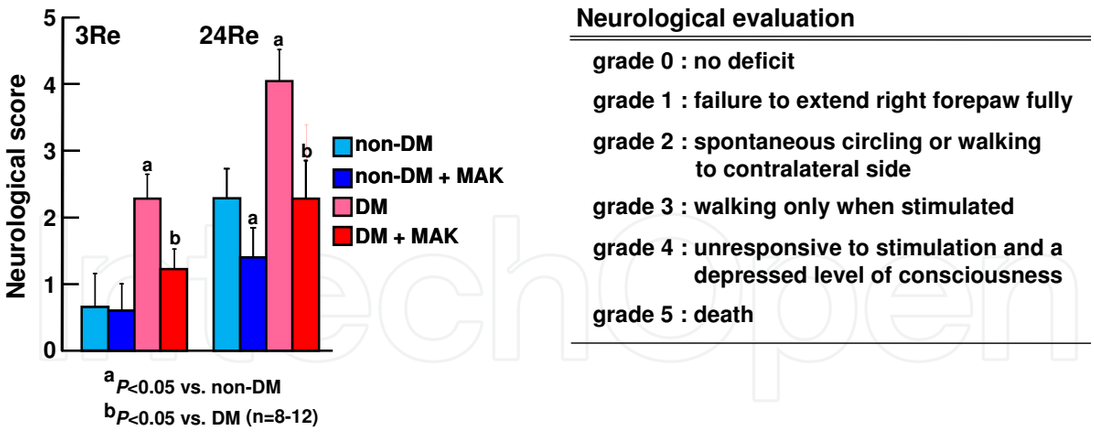


Fig. 2. Effects of MAK on neurological deficits induced by MCAO/Re in non-DM and DM rats.

Post-ischemic neurological deficits were evaluated at 3 or 24 hrs of reperfusion after 2 hrs of MCAO by a 5-point scale as described in the table.

3.3 $O_2^{-}\cdot$ generation after transient MCAO with reperfusion

Intracellular $O_2^{-}\cdot$ generation in the ischemic penumbral region of the cortex induced by MCAO/Re was detected using the fluorescent probe DHE (Fig. 3). $O_2^{-}\cdot$ generation was increased by diabetes and MCAO/Re. No fluorescence of DHE was detected in the cortex of sham operated non-diabetic rats, whereas DHE fluorescence was present in the cortex of sham-operated diabetic rats. MCAO and subsequent following 24 hrs of reperfusion induced DHE fluorescence in the ischemic region of both the non-diabetic and diabetic cortex, which was remarkably augmented in the diabetic brain. Pretreatment of MAK reduced the $O_2^{-}\cdot$ generation in the cortex enhanced by MCAO/Re and diabetes.

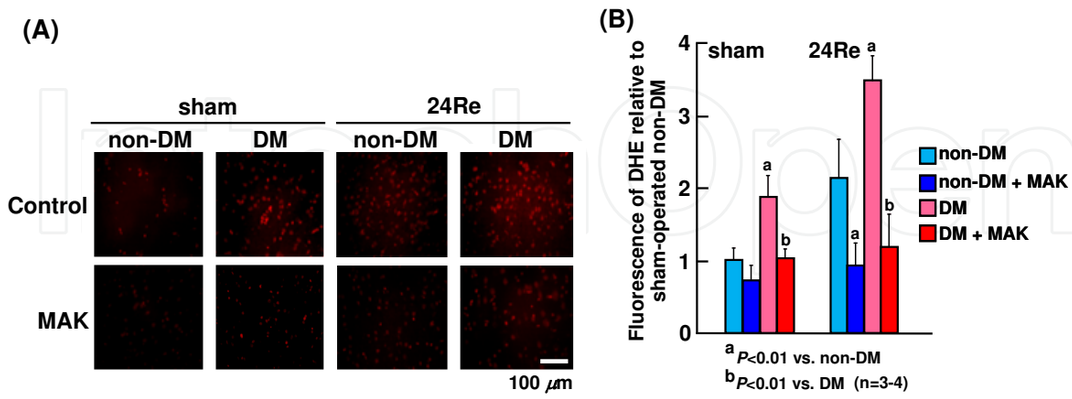


Fig. 3. Effects of MAK on production of superoxide after MCAO/Re in non-DM and DM rat brains.

Representative photographs of superoxide production detected by DHE staining in the cortex coronal sections of the non-DM and DM rats (A). Quantitative analysis of DHE fluorescence intensity in the cortex (B).

3.4 Apoptosis after transient MCAO with reperfusion

Representative histological images of TUNEL staining and cleaved caspase-3 activity in the control non-diabetic, MAK-pretreated non-diabetic, control diabetic and MAK-pretreated diabetic groups subjected to MCAO and 3 or 24 of hrs reperfusion are observed in Figs. 4 and 5. TUNEL staining of the ischemic penumbral region of the cortex was performed to determine nucleosomal DNA fragmentation accompanied by apoptotic cell death. In sham-operated non-diabetic and diabetic rats without MCAO/Re, no TUNEL positive cells were detected in the brain sections. TUNEL positive cells were increased in both the control non-diabetic and diabetic rats especially by MCAO and 3 hrs of reperfusion, which was remarkably suppressed by the pretreatment of MAK. The activation level of caspase-3, which directly activates DNase in the apoptotic final process was determined by immunostaining for cleaved caspase-3 in the ischemic penumbral cortex of non-diabetic and diabetic rats. Similar to the result of TUNEL staining, the number of cells expressing cleaved caspase-3, an activated form of this enzyme, was remarkably increased by MCAO/Re in the control diabetic group as compared with the control non-diabetic group. Pretreatment of MAK significantly inhibited the caspase-3 activation induced by MCAO/Re in these groups.

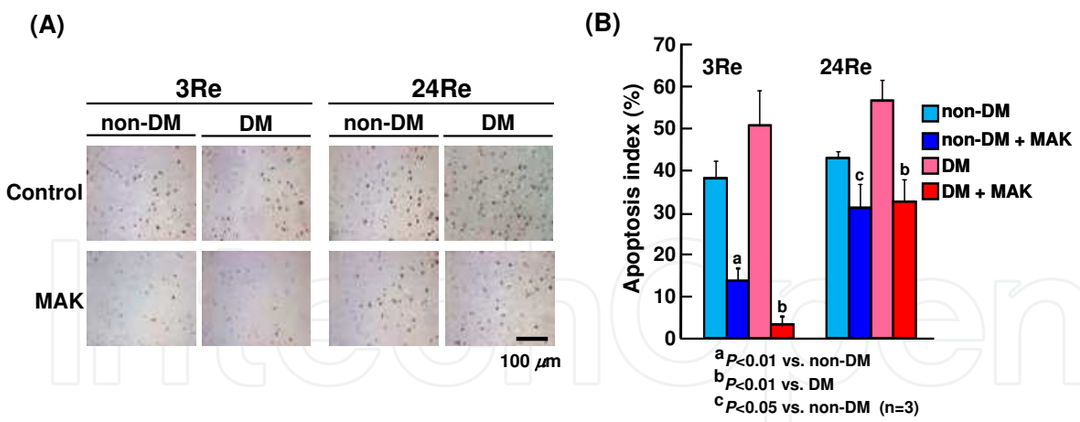


Fig. 4. Effects of MAK on neuronal apoptosis induced by MCAO/Re in non-DM and DM rat brains.
Representative photographs of apoptotic cells detected by TUNEL staining in the cortex coronal sections of non-DM and DM rats (A). Quantitative analysis of TUNEL-positive cells index in the cortex (B).

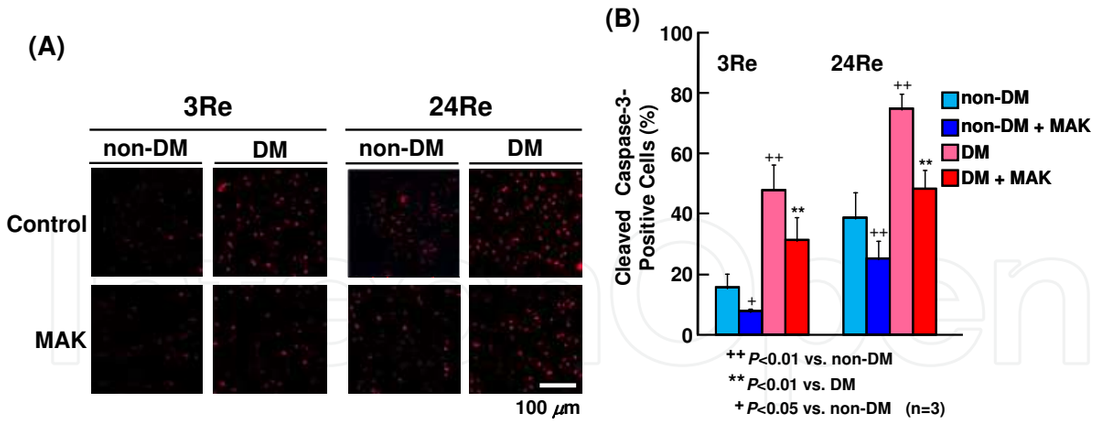


Fig. 5. Effects of MAK on cleaved caspase-3 after MCAO/Re in non-DM and DM rat brains. Representative photographs of cleaved caspase-3 staining in the cortex coronal sections of non-DM and DM rats (A). Quantitative analysis of cleaved caspase-3 positive cells fluorescence intensity in the cortex (B).

3.5 Expression of IL1-β and TNF-α in the cortex

Figure 6 shows the effects of MAK on the expression of mRNA of IL-1β (A) and TNF-α (B) in the non-diabetic and diabetic rat penumbral cortex after MCAO/Re. The control diabetic group had a 5.6-fold increase in the level of basal expression of IL-1β mRNA as compared with the control non-diabetic group. MCAO/Re increased the expression level of IL-1β mRNA in the ischemic cortex of both groups, which was remarkably accelerated and augmented by diabetes. The expression level of IL-1β mRNA in the control diabetic group reached maximum in an early period of reperfusion and at 3 hrs of reperfusion was about 10-fold higher than that of the control non-diabetic rats, which was significantly suppressed by pretreatment of MAK. Also, the control diabetic group had an increased level of the basal

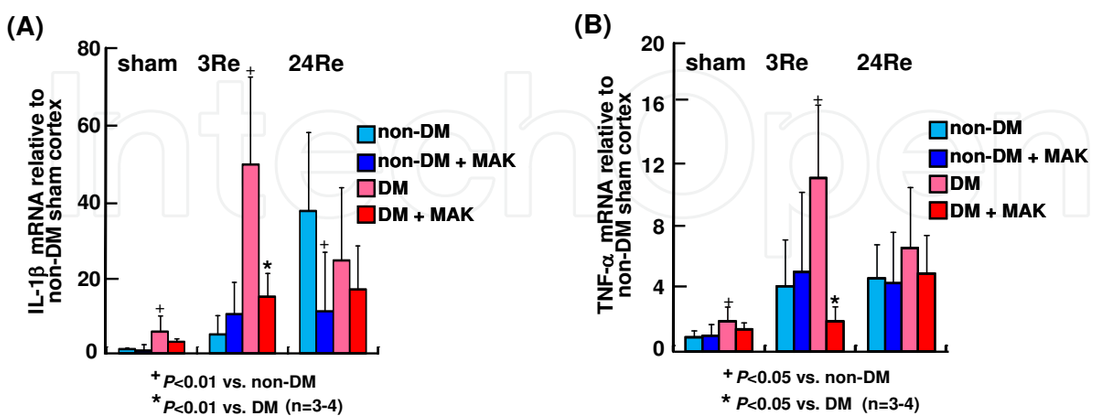


Fig. 6. Effects of MAK on expression of pro-inflammatory cytokines mRNA in the penumbral cortex after MCAO/Re in non-DM and DM rat brains.
The expression levels of IL-1β (A) and TNF-α mRNA (B) in the non-DM and DM rat penumbral cortex after MCAO/Re were determined by real-time PCR analysis.

expression of TNF-α mRNA as compared with the control non-diabetic rats. MCAO/Re increased the expression of TNF-α mRNA, which was remarkably potentiated in the diabetic group. Immunohistochemistry for these cytokines confirmed an up-regulation in biosynthesis of IL-1β and TNF-α by MCAO/Re and diabetes (Figs. 7 and 8). The expression of these pro-inflammatory cytokines was markedly accelerated in the ischemic diabetic rat cortex, whereas pretreatment of MAK significantly suppressed the augmented expression of both these cytokines.

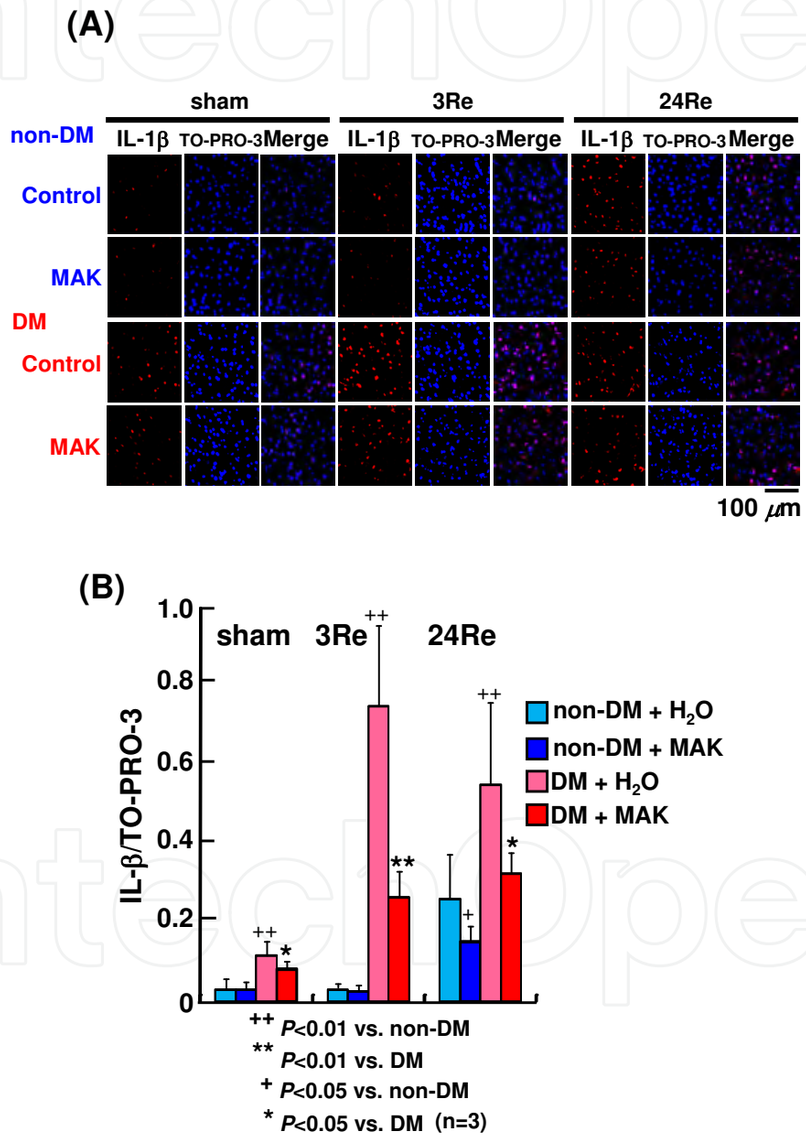


Fig. 7. Effects of MAK on expression of IL-1β in the penumbral cortex after MCAO/Re in non-DM and DM rat brains.
Representative photographs of IL-1β immunostaining (red fluorescence) and nuclei by TO-PRO-3 (blue fluorescence) in the cortex coronal sections of non-DM and DM rats. (A). Quantitative analysis of IL-1β fluorescence intensity in the cortex (B).

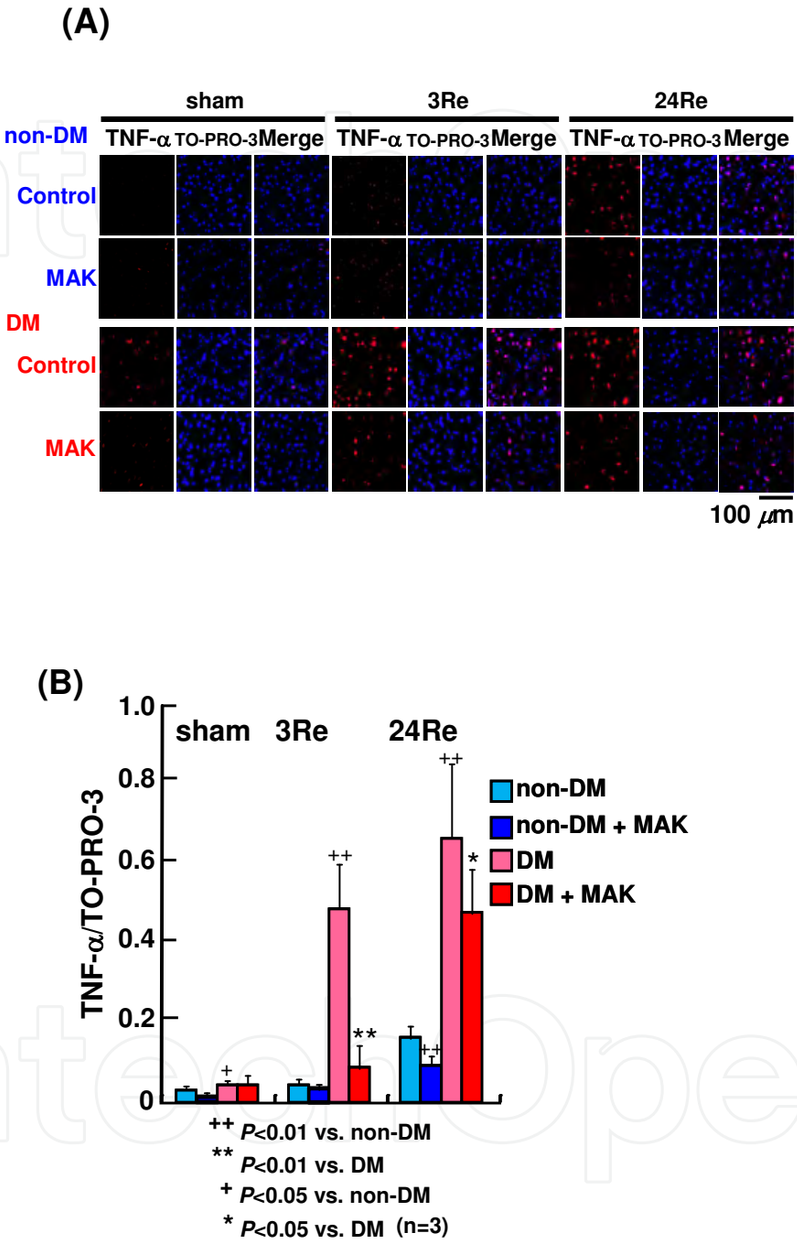


Fig. 8. Effects of MAK on expression of TNF-α in the penumbral cortex after MCAO/Re in non-DM and DM rat brains.
Representative photographs of TNF-α immunostaining (red fluorescence) and nuclei by TO-PRO-3 (blue fluorescence) in the cortex coronal sections of non-DM and DM rats. (A). Quantitative analysis of TNF-α fluorescence intensity in the cortex (B).

3.6 Activation of NF-κB in the cortex

The expression of pro-inflammatory cytokines and inflammatory mediators such as COX-2, iNOS and ICAM-1 in the cells is controlled by the transcription factor NF-κB. Thus, the activation level of NF-κB in the cortex tissue was estimated by western blotting (Fig. 9). Translocation of NF-κB from cytosol to nucleus was not detected in the cortex tissue of sham-operated non-diabetic rats. In the control non-diabetic rats submitted to MCAO/Re, there was a significant increase in NF-κB translocation. In contrast, the translocation of NF-κB was observed even in the sham-operated diabetic rat cortex and was significantly enhanced by MCAO and subsequent reperfusion. Pretreatment of MAK markedly inhibited the activation of NF-κB both in the non-diabetic and diabetic groups.

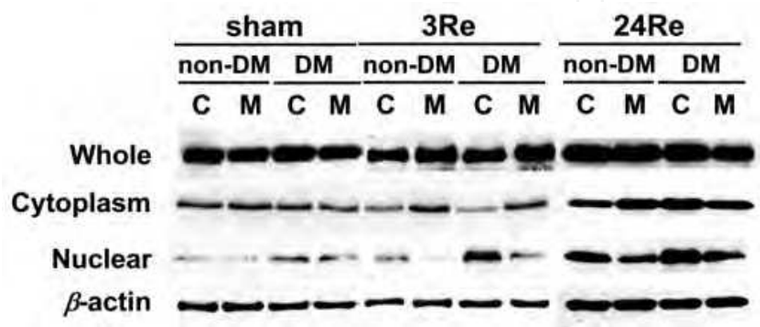


Fig. 9. Effects of MAK on NF-κB expression after MCAO/Re in non-DM and DM rat brains. Representative western blot of NF-κB in cytoplasm and nucleus extracts from the non-DM and DM rat cortex (C: control, M: MAK).

3.7 Expression of inflammatory mediators in the cortex

Figure 10 shows the effects of MAK on MCAO/Re-induced gene expression of COX-2, iNOS and ICAM-1 in the non-diabetic and diabetic rat cortex. After MCAO/Re, no distinct increase in the expression level of COX-2, iNOS and ICAM-1 mRNA was observed in the control non-diabetic group, whereas the gene expression of these inflammatory mediators was strongly enhanced in the control diabetic group. Immunohistochemistry revealed that the control diabetic rats had an increased basal expression level of COX-2 and iNOS as compared with the control non-diabetic rats (Figs. 11 and 12). The expression of ICAM-1 in endothelial cells, which indicates increased extravasation of neutrophils and macrophages/microglia into brain parenchyma, was determined using double immunostaining for ICAM-1 and endothelial cell antibody (RECA-1) (Fig. 13). The cortex of sham-operated diabetic rats had a significant up-regulated level of ICAM-1 in endothelial cells as compared with the sham-operated non-diabetic rats, which had no ICAM-1-like immunoreactive cells. MCAO/Re increased the expression of ICAM-1 both in the non-diabetic and diabetic groups, which was remarkably enhanced in the diabetic rats during 24 hrs of reperfusion. Pretreatment of MAK significantly reduced the expression of ICAM-1 both in the non-diabetic and diabetic rat cortex. Similarly, the MPO activity in the cortex was progressively increased during reperfusion, which was enhanced by diabetes and was significantly suppressed by pretreatment of MAK (Fig. 14).

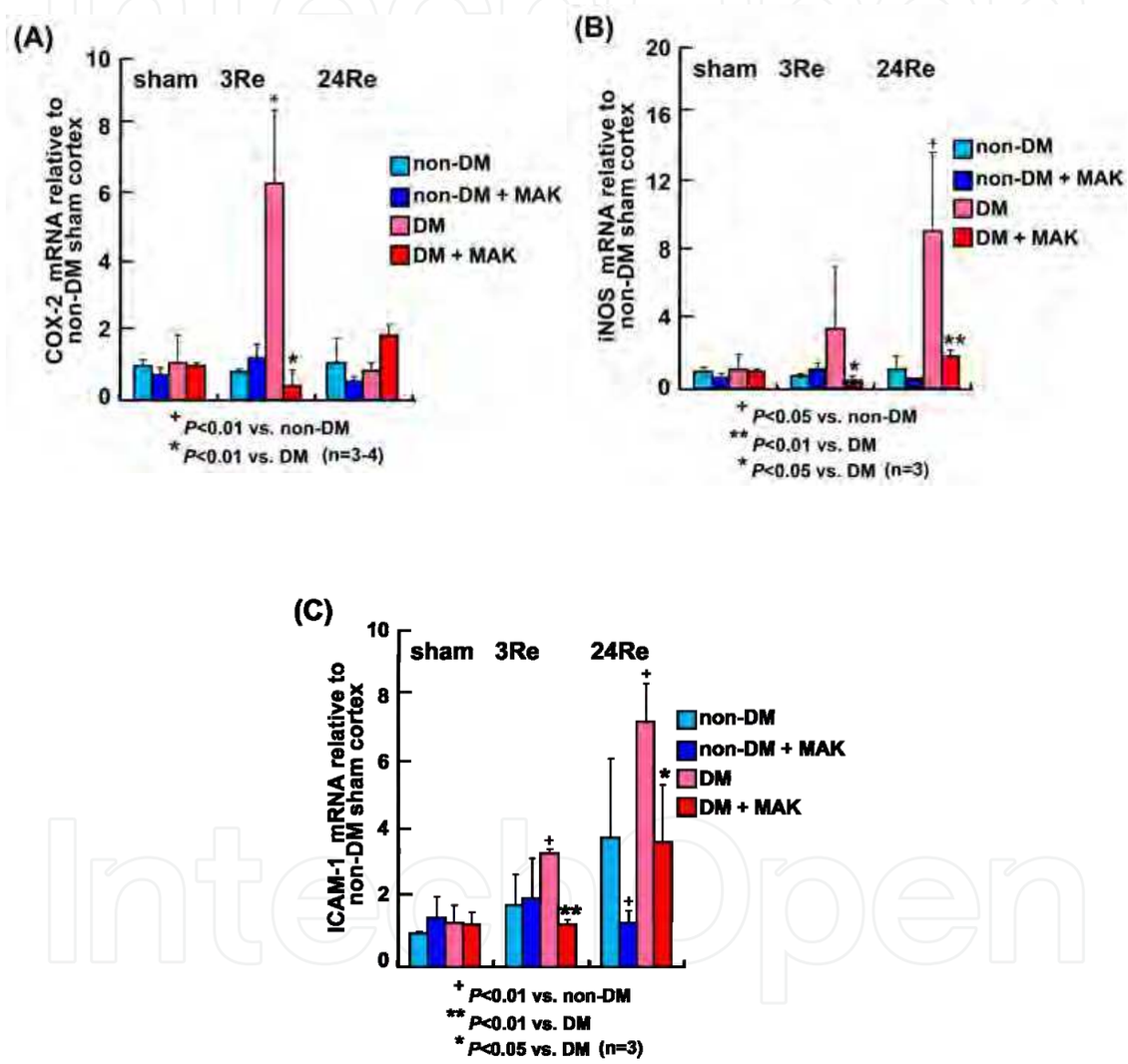


Fig. 10. Effects of MAK on expression of mRNA of inflammatory mediators in the penumbral cortex after MCAO/Re in non-DM and DM rat brains.
Expression levels of COX-2 (A), iNOS (B) and ICAM-1 mRNA (C) were determined by real-time PCR analysis in the non-DM and DM rat penumbral cortex after MCAO/Re.

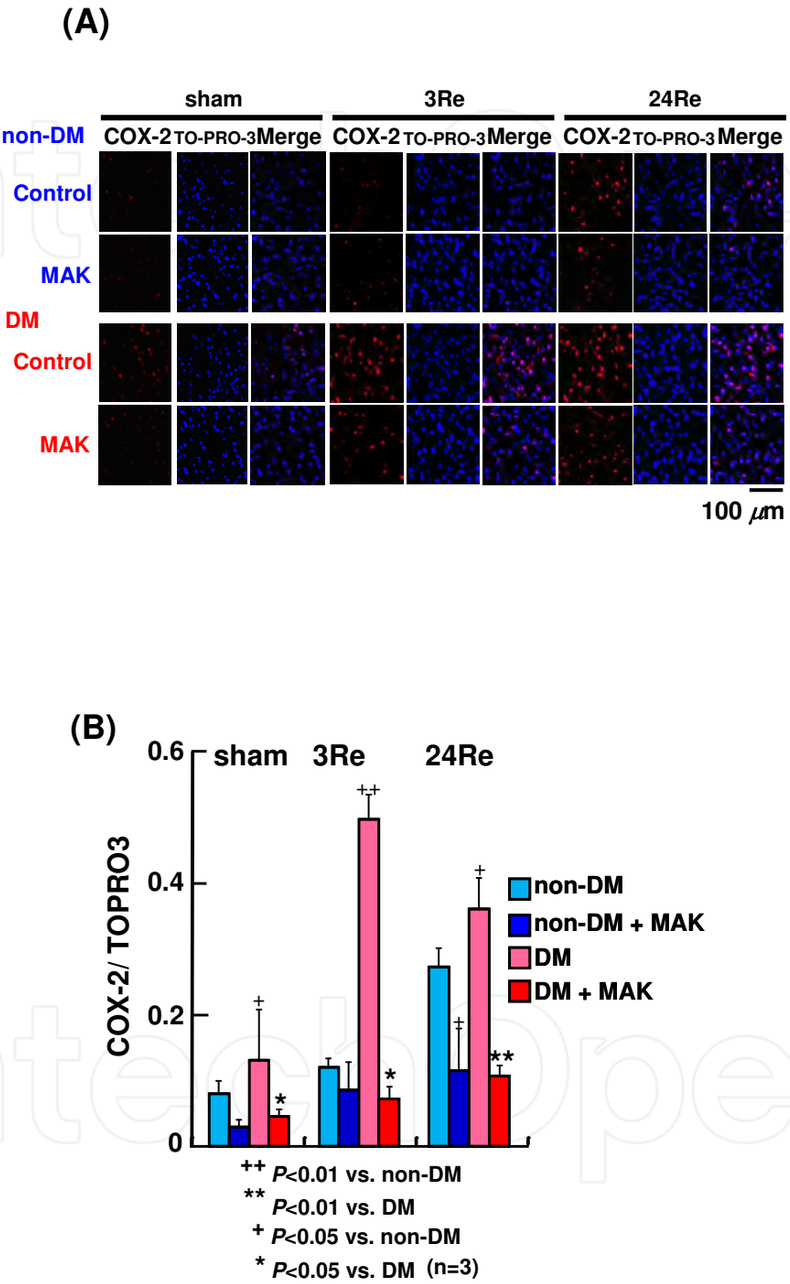


Fig. 11. Effects of MAK on expression of COX-2 in the penumbral cortex after MCAO/Re in non-DM and DM rat brains.
Representative photographs of COX-2 immunostaining (red fluorescence) and nuclei by TO-PRO-3 (blue fluorescence) in the cortex coronal sections of non-DM and DM rats. (A). Quantitative analysis of COX-2 fluorescence intensity in the cortex (B).

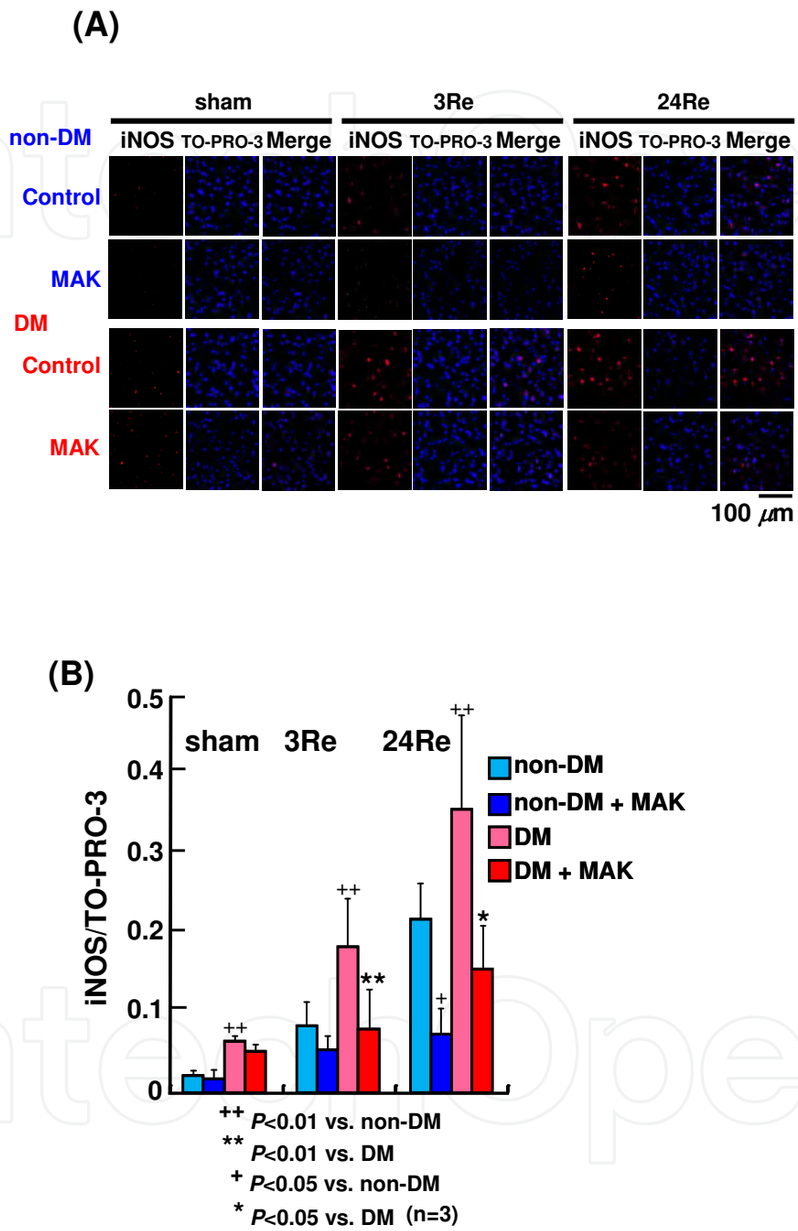


Fig. 12. Effects of MAK on expression of iNOS in the penumbral cortex after MCAO/Re in non-DM and DM rat brains.
Representative photographs of iNOS immunostaining (red fluorescence) and nuclei by TO-PRO-3 (blue fluorescence) in the cortex coronal sections of non-DM and DM rats. (A). Quantitative analysis of iNOS fluorescence intensity in the cortex (B).

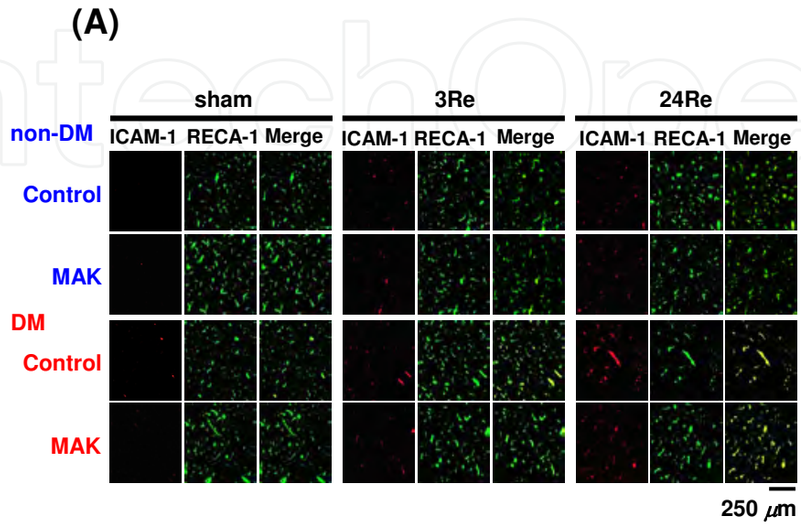
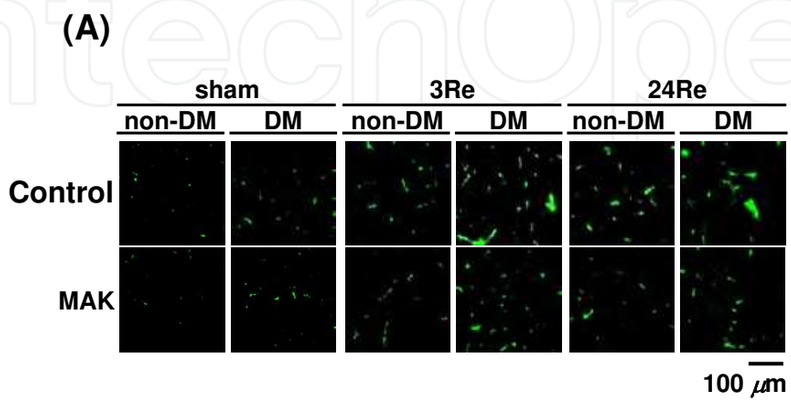


Fig. 13. Effects of MAK on expression of ICAM-1 in the penumbral cortex after MCAO/Re in non-DM and DM rat brains.
Representative photographs of ICAM-1 immunostaining (red fluorescence) and RECA-1 (green fluorescence) in the cortex coronal sections of non-DM and DM rats. (A). Quantitative analysis of ICAM-1 fluorescence intensity in the cortex (B).



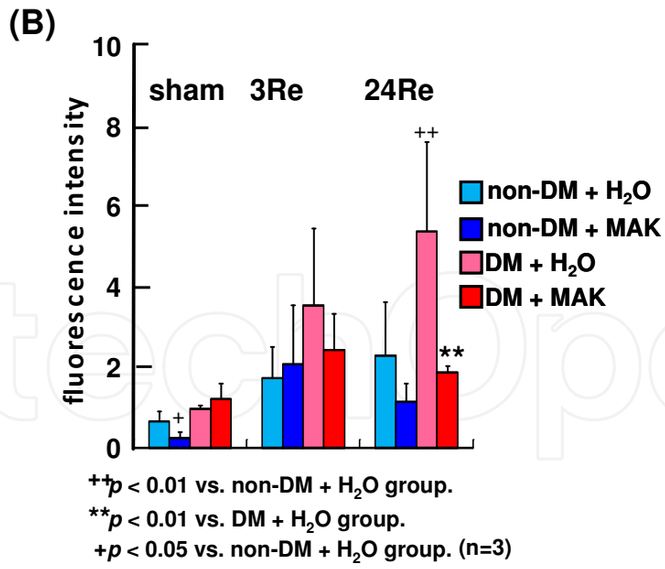


Fig. 14. Effects of MAK on MPO expression after MCAO/Re in non-DM and DM rat brains. Representative photographs of leukocytic infiltrate by MPO staining in the cortex coronal sections of non-DM and DM rats (A). Quantitative analysis of MPO fluorescence intensity in the cortex (B).

4. Discussion

The present study shows that the STZ-induced diabetic state enhanced brain infarction and neurological dysfunction caused by transient focal ischemia and subsequent reperfusion in rats. Diabetes spontaneously enhanced ROS generation and expression of pro-inflammatory cytokines and inflammatory mediators via NF-κB activation in the brain, which is accelerated after cerebral ischemia and subsequent reperfusion leading to neuronal apoptosis and inflammatory neurodegeneration. Chronic oral treatment of MAK alleviated the exacerbation of cerebral injury and neurological deficits in the diabetic state, which could be attributed to its antioxidant activity and anti-inflammatory effects.

ROS-induced oxidative stress is considered to be involved in the pathogenesis of transient cerebral ischemic injury (Fiskum et al., 2004; Saito et al., 2005; Niizuma et al., 2009). In particular, reperfusion after a long period of vessel occlusion triggers explosive generation of ROS, which causes cell death by peroxidative damage of lipids, proteins and nucleic acids (Warner et al., 2004; Anabela et al., 2006). In addition to the early necrotic cell death in the ischemic core region, ROS triggers apoptosis, a delayed death of cells, in the ischemic penumbra (Nakka et al., 2008; Niizuma et al., 2010). Furthermore, ROS induces rupture of blood brain barrier through transcriptional activation of matrix metalloproteinase and pro-inflammatory cytokines resulting in the extension of cerebral infarction and exacerbation of brain edema (Cunningham et al., 2005; Zhao et al., 2006). In this study, we observed that exacerbation of damage in the brain of diabetic rats was accelerated in a time-dependent manner during the reperfusion phase. MCAO and 3 hrs of reperfusion in the control non-diabetic rats showed little infarction in the brain and moderate neurological deficits, whereas the diabetic rats subjected to MCAO and 3 hrs of reperfusion had a large infarction that was similar in size to that in the non-diabetic rats after 24 hrs of reperfusion and severe neurological deficits. Additionally, a histochemical study revealed that generation of $O_2^{-}\cdot$ and the occurrence of apoptosis in the ischemic penumbra were markedly increased in the brain of diabetic rats. A large amount of ROS locally generated by cerebral ischemia/reperfusion induces free radical chain reactions (Saito et al., 2005), which may be enhanced by increased oxidative stress in the diabetic state. Evidence is being accumulated that oxidative stress is enhanced by hyperglycemia in the diabetic state (Kusaka et al., 2004; Rizk et al., 2005; Tsuruta et al., 2010). In the diabetic state, "glucose toxicity" caused by augmentation of intracellular glucose oxidation process and nonenzymatic glycation of protein molecules leads to over production of ROS and damage of neurons and endothelial cells (Baynes 1991). Previous studies have demonstrated that the hyperglycemic condition in cerebral ischemia without diabetes exacerbates brain injury due to enhanced production of ROS in the brain (Anabela et al., 2006; Tsuruta et al., 2010). Augmented oxidative stress involving increased ROS generation, augmented lipid peroxidation and reduction of antioxidants has been indicated in the brain, kidney, pancreas and liver of STZ-induced diabetic rats (Muralikrishna Adibhatla et al., 2006). Actually, the occurrence of apoptosis in the ischemic penumbral region has been shown to be enhanced by diabetes and correlate with serum glucose (Li et al., 1999). Antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), which scavenge ROS, are considered to contribute to the neuronal protection against ischemia/reperfusion (Warner et al.,

2004). Indeed, overexpression of SOD in transgenic mice has been shown to have a reduced infarction volume and edema after transient ischemia and reperfusion (Tsubokawa et al., 2007). Impairment of these antioxidant enzymes caused by nonenzymatic glycation is responsible for the increase of lipid peroxidation in the brain of diabetic animals. In a previous study, we also observed that the level of lipid peroxidation marker TBARS (thiobarbituric acid reactive substances) was elevated and activity of SOD, CAT and GPx was decreased in STZ-induced diabetic rat brain, which were reversed by chronic treatment of ascorbic acid (Iwata et al., 2010). Thus, the enhanced oxidative stress in diabetes is considered to cause functional impairment of antioxidant enzymes and the resulting diminution in antioxidative defense leads to further enhancement of radical reactions and other dysfunctions in the brain.

Inflammatory mechanisms that are activated after cerebral ischemia play an important role in the pathogenesis of brain injury (Caso et al., 2007; Ridder et al., 2009). The transcription factor NF-κB assumes a key role in many biological processes including cellular stress responses and regulates apoptosis and inflammation (Ridder et al., 2009). Activation of NF-κB is crucial for the inflammatory responses leading to gene expression of pro-inflammatory cytokines and mediators in immunocytes (Hu et al., 2005; Ridder et al., 2009). In this study, we demonstrated that the diabetic rats had an increased basal level of the gene expression of pro-inflammatory cytokines IL-1β and TNF-α, and inflammatory mediators COX-2 and iNOS as compared to that of non-diabetic rats. MCAO/Re increased the gene expression of these cytokines and enzymes, which was remarkably accelerated and augmented by diabetes. The result from immunoblot analysis of NF-κB in the diabetic rat cortex confirmed that the increased production of these cytokines and enzymes was mediated by the enhanced activation of NF-κB. Furthermore, we observed that the post-ischemic induction of MPO and ICAM-1, which are hallmarks of neutrophil and macrophage/microglia activation and extravasation, was significantly increased in the diabetic rat brain indicating exaggeration of inflammatory responses in ischemic injury. Our present data are in agreement with other studies describing the exacerbated inflammation in diabetic model animals (Li et al., 2004; Tsuruta et al. 2010; Tureyen et al., 2011). For example, a model of type-2 diabetes *db/db* mouse that has a point mutation of the leptin receptor showed increased brain damage and higher expression levels of IL-1β, IL-6, ICAM-1, MPO and other inflammatory markers as compared with the *db/+* control after MCAO/Re (Tureyen et al., 2011). A recent study using an electrochemical $O_2^{-}\cdot$ sensor has shown that experimental transient hyperglycemia induced by intravenous infusion of glucose increased local $O_2^{-}\cdot$ generation and exacerbated brain injury after ischemia and subsequent reperfusion in the rats (Tsuruta et al., 2010). Interestingly, high-mobility group box-1 and ICAM-1 in brain and plasma, which are induced in early inflammation and enhance inflammatory responses, were correlated with total $O_2^{-}\cdot$ generation during ischemia and reperfusion. Nevertheless, the hyperglycemia-induced overproduction of ROS may be mainly attributed to exacerbated inflammatory responses and cerebral damage after ischemia and reperfusion in diabetes.

MAK, a nutritional supplement, is a water-soluble extract from a solid culture medium composed of bagasse and defatted-rice bran overgrown with *G. lucidum* mycelia. MAK is used as a health supplement and revitalizer, and a number of studies have demonstrated its anti-tumor (Kubo et al., 2005) and immunomodulating activities (Nakagawa et al., 1999) in

animals. However, the mechanistic basis and active ingredients responsible for its pharmacological effects have not been well defined. Previously, we showed that MAK inhibits the generation of $O_2^{-}\cdot$ and lipid peroxidation in a concentration dependent manner *in vitro* (Okazaki et al., 2008). Furthermore, oral administration of MAK to STZ-induced diabetic animals significantly reduced the blood glucose level and lipid peroxidation, and suppressed impairment of SOD, CAT and GPx in the brain (Iwata et al., 2008), liver and kidney (Okazaki et al., 2008). Collectively, MAK can act as an antioxidant *in vivo* and shows anti-diabetic effects by relieving diabetic-induced oxidative stress. We observed that the oral pretreatment of MAK with diabetic rats decreased the cerebral $O_2^{-}\cdot$ generation, apoptosis and subsequent inflammatory responses induced by MCAO/Re, which could be as a result of improved antioxidant status in the diabetic state. MAK had a slight effect on the elevated blood glucose level in the diabetic rats, confirming that the cerebroprotective effect of MAK could be due to its antioxidant activity. Recently, polysaccharides (Lin et al., 2010) and triterpenes (Dudhgaonkar et al., 2009), which are two major active constituents of *G. lucidum*, have been reported to suppress lipopolysaccharide-induced expression of inflammatory mediators via down-regulation of MAP kinase signaling cascade and NF-κB activity both *in vitro* and *in vivo*, whereas the mechanism of their anti-inflammatory effects remains unclear. As MAK is assumed to contain similar polysaccharides and triterpenes, it might inhibit inflammatory responses directly via its immunomodulatory effects. There are a number of factors that may explain the severe symptom of brain ischemia in the diabetes. For example, the ischemic cerebral injury in diabetic state may be aggravated by acidosis, activation of aldose reductase and NAD(P)H oxidase, enhanced production of advanced glycation end-products, protein kinase C activation induced by excessive Ca^{2+} influx, etc. The effects of MAK on these factors and identification of its active ingredients need to be further investigated.

5. Conclusion

In this study, we demonstrated that the STZ-induced diabetic state markedly aggravated MCAO/reperfusion-induced neurological deficits, infarction and apoptosis in the rat brain. Furthermore, we elucidated that the levels of $O_2^{-}\cdot$ generation and pro-inflammatory cytokines (IL-1β and TNF-α) and inflammatory mediators (COX-2 and iNOS) expression via NF-κB activation were up-regulated in the diabetic cortex, which were remarkably enhanced during reperfusion after ischemia. Post-ischemic activation of neutrophil and macrophage/microglia and extravasation estimated by ICAM-1 and MPO expression were also enhanced by diabetes. Chronic pretreatment of MAK protected the diabetic rats against the exacerbation in cerebral ischemic injury and inflammatory responses. These results suggest that daily intake of MAK relieves the exacerbation of cerebral ischemic injury in the diabetic state, which may be mainly attributed to the improvement of augmented oxidative stress by its anti-oxidant effects.

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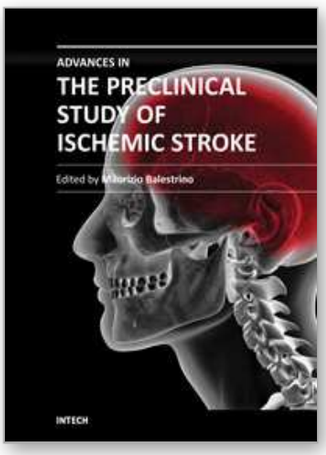

Advances in the Preclinical Study of Ischemic Stroke
Edited by Dr. Maurizio Balestrino

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This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

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