Acta Neurol. Belg., 2009, **109**, 32-37

Genistein, a soybean isoflavone, reduces the production of pro-inflammatory and adhesion molecules induced by hemolysate in brain microvascular endothelial cells

Hua LU[1], Ji-Xin SHI[1], Dong-Mei ZHANG[2], Hui-Lin CHEN[1], Meng QI[1] and Hong-Xia YIN[1]

[1]Department of Neurosurgery, Jinling Hospital, Clinical School of Nanjing University, Nanjing, China ;
[2]The State Key Laboratory of Pharmaceutical Biotechnology and Department of Biochemistry, College of Life Sciences, Nanjing University, Nanjing, China

Abstract

Genistein (4',5,7-trihydroxyisoflavone) is the most abundant isoflavone found in the soybean that exhibits an anti-inflammatory effect. The present study was designed to examine the effects of genistein on expression levels of hemolysate-induced proinflammatory and adhesion molecules in SD rat brain microvascular endothelial cells (BMECs). Genistein treatment attenuated hemolysate-induced nuclear factor-kappa B (NF-κB) p65 translocation in BMECs. In addition, genistein suppressed the expression levels of tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein 1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1), but not vascular cell adhesion molecule-1 (VCAM-1). The inhibitory rate of 50 μM genistein for TNF-α, MCP-1 and ICAM-1 was 65.4%, 60.5% and 54.9% respectively. These inhibitory effects of genistein on proinflammatory and adhesion molecules were not due to decreased BMEC viability as assessed by MTT test. Taken together, the present study suggests that genistein suppresses expression levels of hemolysate-induced pro-inflammatory and adhesion molecules in cerebral endothelial cells.

Key words : Genistein ; inflammation ; brain microvascular endothelial cells ; hemolysate.

Introduction

The inflammatory response plays a crucial role in tissue repair after various forms of injury, but it can also be detrimental to the surrounding tissue. Accumulating evidence suggests that inflammation occurs after subarachnoid hemorrhage (SAH) and intracerebral hemorrhage (ICH), thus, is likely to contribute to the brain damage caused by this injury (Xue 2003 ; Provencio 2005 ; Kim 2007). Recently, the vascular endothelium has no longer been regarded as an inert vascular lining that can be injured and morphologically changed. It has many different functions which are susceptible to change or dysfunction and can contribute to cell and tissue injury. Microvascular endothelial cells at a site of inflammation are both active participants in and regulators of inflammatory processes (Pober 2007). Endothelial dysfunctions closely related with vascular inflammatory process may represent an early stage of vasculopathy which can lead to inflammation disorders. Vascular inflammation is caused by increase in leukocyte- endothelium interaction via an up-regulation of endothelial cell adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and proinflammatory factors like tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1), which are induced by activation of nuclear factor-kappa B (NF-κB) (Saeed 2004 ; Ichiki 2008).

Genistein, a major soy isoflavone, has revealed the anti-inflammatory effects both *in vivo* and *in vitro* (Ruetten 1997 ; Middleton 2000 ; Havsteen 2002). Genistein was reported to inhibit the production of proinflammatory molecules in human chondrocytes (Hooshmand 2007). These anti-inflammatory properties of genistein provide the rationale for investigating the role of genistein in conditions such as SAH and ICH. However, the molecular mechanisms explaining how genistein suppresses the inflammatory response are not known in detail.

Red blood cell hemolysate has been considered to be a spasminogen in subarachnoid hemorrhage and to contribute to brain edema formation after intracerebral hemorrhage (Xi 2001 ; Sasaki 2004). Therefore, the aim of the present study was to investigate the effects of genistein on the expression levels of

hemolysate-induced proinflammatory and adhesion molecules, which could account for its beneficial effect in the hemorrhagic stroke.

Materials and methods

Materials

Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco. Fetal calf serum, and penicillin/streptomycin were purchased from Sunshine biocompany (China). Genistein was purchased from Sigma and dissolved in DMSO at 10 mM stock solutions. Rabbit anti- NF-κB (p65) polyclonal antibody and second antibody were provided by Santa Cruz Biotechnology Inc.

Isolation, culture and treatment of brain microvascular endothelial cells

Primary cultures of brain microvascular endothelial cells from newborn Sprague-Dawley (SD) rats were established as described previously (Yakubu 1999, 2005). In brief, rats were decapitated and the meninges removed. Under sterile conditions, cerebral cortices were isolated and minced in a medium containing DMEM, 100 U/ml penicillin and 100 mg/ml streptomycin. After a rinse with medium, the tissue was homogenized and serially passed through nylon meshes of 149, and 74 mm. The tissue retained by the 74 mm meshes was digested at collagenase-dispase solution (1 mg/ml) for 2 h at 37°C. At the end of the incubation, the dispersed microvascular endothelial cells were separated using percoll density gradient centrifugation. Endothelial cells were resuspended in culture medium consisting of 20% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Twenty-four hours after plating, adherent cells were washed and fed fresh medium. The culture medium was changed every 2 to 3 days until cells attained confluence and maintained in a 5% CO2-95% air incubator at 37°C. Cultures were verified to be 95% endothelial-specific factor VIII-related antigen positive by immunostaining and used during the first two passages for experiments.

For experiments investigating the stimulatory effects of hemolysate on TNF-α, MCP-1, ICAM-1 and VCAM-1mRNA expression and the inhibitory effects of genistein on hemolysate-induced proinflammatory molecules expression in BMECs, cells were seeded in 35-mm dishes (1×10^5 cells/ml) and were starved overnight with 1% FBS-conditioned media before treatment. Cells were treated with genistein for 30 min in a concentration range of 10-100 μM and thereafter stimulated with 10% hemolysate for 12 h. The genistein was dissolved and stocked in DMSO (100 mg/ml) and added to indicated final concentrations to cell cultures grown in DMEM (final DMSO concentration was 0.05%.

For experiments evaluating the effects of genistein on the cell viability of BMECs, cells were seeded in 96-well plates at a density 5×10^4 cell/well for MTT assay.

Preparation of hemolysate

Hemolysate was prepared as previously described (Iwabuchi 1999). Briefly, heparinized rats arterial blood was centrifuged at 2500 × g for 15 minutes at 4°C, and the supernatant was aspirated. The erythrocyte-rich precipitates were washed with cold saline solution, lysed by a freeze-thaw procedure. The membrane debris was pelleted by centrifugation at 31,000 × g for 15 min and the erythrocyte lysate was stored at -80°C. The concentration of hemolysate was determined by measuring hemoglobin spectrophotometrically. Two absorbance peaks at 540 and 576 nm were observed in the hemolysate sample, confirming the presence of oxyhemoglobin (OxyHb). The concentration of OxyHb in the preparation of 100% hemolysate was 10.92 mM. In this study, treatment with hemolysate at a concentration of 10% by volume was added to cell media.

Cell viability assay

The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure cell viability. Briefly, the cells were seeded onto 96-well culture plate at a density 5×10^4 cells per well. They were treated with genistein for 24h. Four hours before the end of incubation, 20 μL MTT solution (5.0 mg/L) was added to each well. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 nm with a reference setting of 630 nm on an ELISA reader (model FL600, Bio-Tek Instruments, Inc).Optical density was measured by MTT assay using a plate microreader. The absorbance was used as a measurement of cell viability, normalized to cells incubated in control medium, which were considered 100% viable.

RNA isolation and real-time PCR of proinflammatory cytokines mRNA

At the end of the specific treatment cells were harvested and total cellular RNA was immediately isolated by TRIzol reagent (Invitrogen Life Technologies Inc). Reverse transcription of 0.5 μg of

RNA for each sample was carried out in a 20 μl reaction volume using 4 μM of p(DT)15 oligo(dt) primer (Roche) and 200 U of MMLV reverse transcriptase at 37°C for 50 min. TNF-α, MCP-1, ICAM-1,VCAM-1 and β-actin mRNA levels were quantified by real-time PCR (RT-PCR) using a SYBR Green approach (Molecular Probes) in Rotor-Gene 3000 realtime sequence detection system (Corbett Research) as outlined in Fig. 1. Fluorescence data, collected in the extension step, were analyzed, and normalized using β-actin as the housekeeping gene. Melting curve analysis of each PCR product was performed at the end of each real-time amplification to verify their specificity.

Western blot analysis

The nuclei extracts were separated by 12% SDSPAGE and transferred to nitrocellulose membranes. The membrane was blocked with 3% nonfat dry milk and subsequently incubated for 2 h with 1:500 dilution primary antibodies that detect p65 component of NF-κB. Membranes were washed and the proteins were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibodies. The blots were visualized with an enhanced chemiluminescent method kit (Wuhan Boster Biological Technology Ltd, Wuhan, China).

Statistics

All data are presented in the statistics of three independent experiments in the form of mean ± S.D. The significance of the difference was analyzed by Student–Newman–Keuls test. P-values less than 0.05 were considered statistically significant.

Results

Effects of hemolysate on proinflammatory molecules mRNA expression in BMECs

Our initial experiments were carried out to establish whether hemolysate exposure could induce the mRNA expression of proinflammatory molecules on BMECs. As shown in Fig. 1, incubation of BMECs with hemolysate from 2 to 24 h significantly increased the TNF-α, MCP-1, ICAM-1 and VCAM-1 mRNA expression as compared to control.

Effects of genistein on hemolysate-induced NF-κB activation in BMECs

To study the effect of genistein on hemolysate - induced NF-κB activation in BMECs, Western blot analysis of the nuclear fraction of cell lysates was performed. As shown in Fig. 2, the translocation of NF-κB in the nuclear fractions of BMECs was increased by treatment with 10% hemolysate. However, pretreatment of BMECs with genistein attenuated hemolysate-induced nuclear NF-κB activation in a dose-dependent manner (Fig. 2).

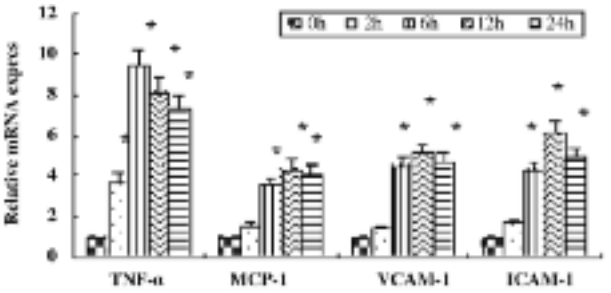

Fig. 1. — Effects of hemolysate on TNF-α, MCP-1, ICAM-1 and VCAM-1mRNA expression in BMECs. Cell confluent monolayers were grown in 35-mm dishes and stimulated with hemolysate for different periods of time (0-24 h). mRNA expression for TNF-α, MCP-1, VCAM-1, ICAM-1 and β-actin genes in BMECs was assessed by real time PCR as described in Section 2. In order to quantify TNF-α, MCP-1, ICAM-1 and VCAM-1 gene expression, the TNF-α, MCP-1, VCAM-1 and ICAM-1 mRNA level was normalized by the total RNA content (μg/μl) and β-actin mRNA level. Results were expressed as fold induction with respect to time 0. Means and S.D. of at least three experiments are shown. *$p < 0.05$ vs. 0 h.

Effect of genistein on hemolysate-induced proinflammatory molecules expression in BMECs

The expression level of TNF-α, MCP-1, VCAM-1 and ICAM-1 in BMECs, determined by RT-PCR, was also augmented by treatment with hemolysate (Fig. 1). Pretreatment of BMECs with genistein blocked a hemolysate-induced increase of TNF-α, MCP-1and ICAM-1 expression, but not VCAM-1 (Fig. 3). The inhibitory rate of 50 μM genistein for TNF-α, MCP-1and ICAM-1 was 65.4%, 60.5% and 54.9% respectively.

Effects of genistein on BMEC Cell Viability

To evaluate whether the inhibitory actions of genistein on NF-κB activation, expressions of TNF-α, MCP-1, ICAM-1, and VCAM-1 were due to decreased BMECs viability, effect of genistein on cells viability was assessed using MTT assay. As shown in Fig. 4, there is no significant change in cell viability by treatment with various concentrations of genistein.

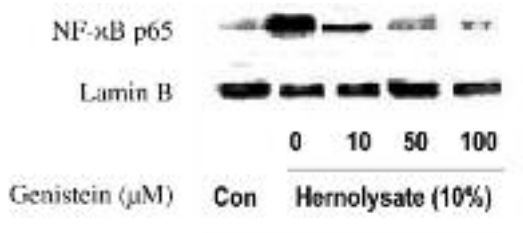

FIG. 2. — Representing western blot analysis demonstrating the effect of concentration-dependent genistein on the hemolysate -induced NF-κB p65 translocation in the nuclear fraction of BMECs. Con ; hemolysate-nontreated control.

Discussion

In the present study, we demonstrated that genistein treatment blocks NF-κB p65 translocation in BMECs in a dose-dependent manner. NF-κB is an essential transcription factor that regulates the gene expression of various cytokines, chemokines, growth factors, and cell-adhesion molecules (Chen 1999). The most predominantly characterized NF-κB complex is a p50-p65 heterodimer, which at rest is associated with an inhibitor protein, IκB, and is retained in the cytoplasm. Most importantly, phosphorylation regulates the IκB subunit and its dissociation from the inactive cytoplasmic complex, followed by translocation of the active dimmer, p50 and p65, to the nucleus, where it binds to the NF-κB motif of a gene promoter and functions as a transcriptional regulator (Schmid 2008).

We demonstrated the importance of hemolysate exposure on its ability to increase the expression of TNF-α, MCP-1, ICAM-1 and VCAM-1 in BMECs. The findings of the present study also indicate that genistein suppresses the mRNA expression of proinflammatory molecules such as TNF-α, MCP-1 and

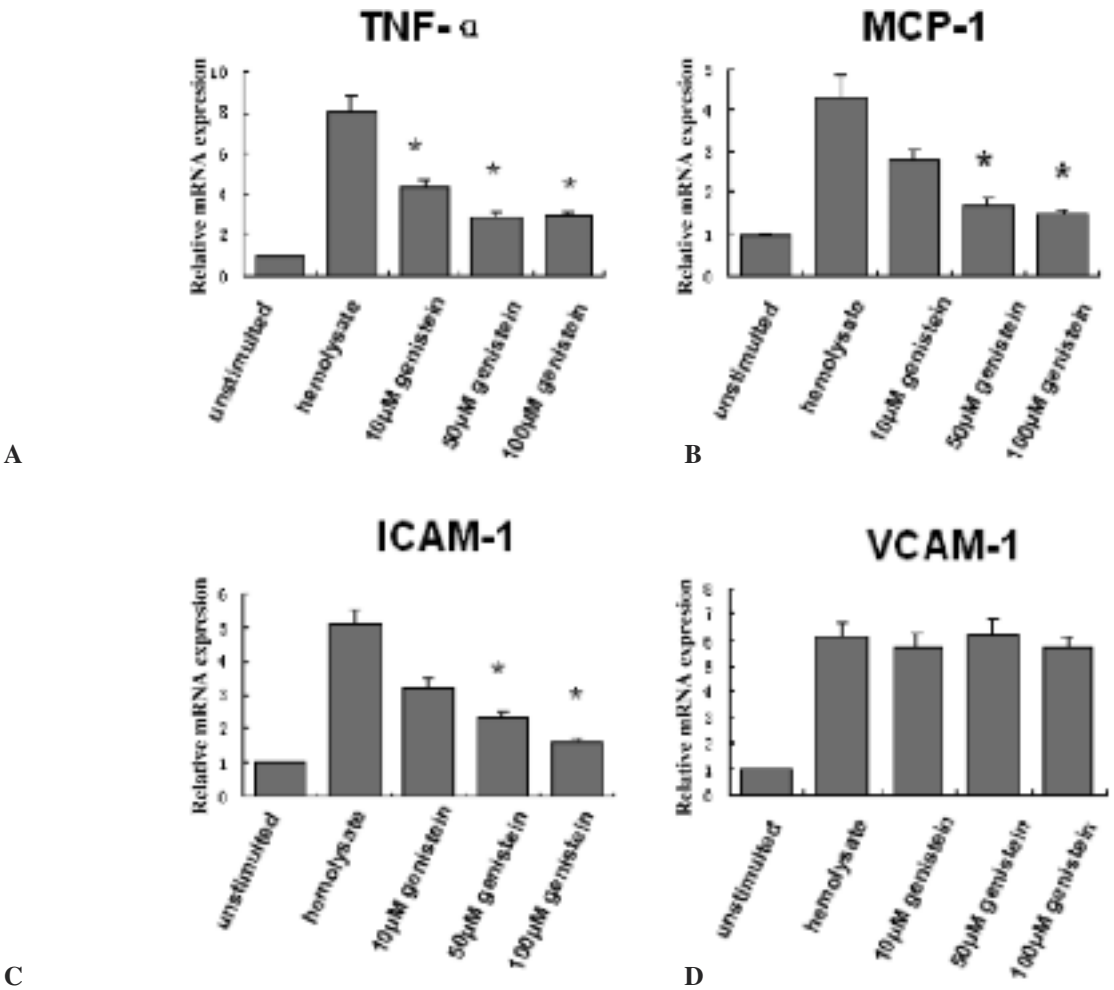

FIG. 3. — Effect of genistein on hemolysate-induced TNF-α(A), MCP-1(B), ICAM-1(C) and VCAM-1(D) expression in BMECs. Cells were treated for 30 min with genistein in a concentration range of 10-100 μM and thereafter stimulated for 12 h with 10% hemolysate. *$p < 0.05$ vs. hemolysate.

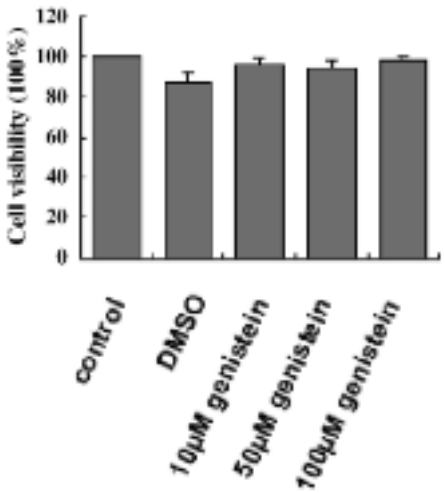

FIG. 4. — Effect of genistein on the cell viability of BMECs for 24 h. DMSO ; dimethylsulfoxide.

ICAM-1 in hemolysated-induced BMECs, while it has no effect on VCAM-1 expression. TNF-α shows a wide spectrum of biological activities. Among them, *in vivo*, TNF-α in combination with IL-1 is responsible for many alterations of the endothelium. It inhibits anticoagulatory mechanisms and promotes thrombotic processes and therefore plays an important role in circulatory stasis. TNF-α is also a potent chemoattractant for neutrophils and also increases their adherence to the endothelium (Bijuklic 2007). In SAH, TNF-α probably mediates some of the brain damage (Fassbender 2001). In this study, increase of TNF-α mRNA induced by hemolysate in BMECs could be reversed by pretreatment with genistein.

We also demonstrated that hemolysate significantly induces ICAM-1 and VCAM-1 mRNA expression in BMECs and genistein inhibits this ICAM-1 expression, but not VCAM-1. These adhesion molecules are considered as a marker of the activation of the inflammation process in central nervous system and play a critical role in adhesion and recruitment of mononuclear leukocytes during times of vascular inflammation (Berti 2002). Regulation of ICAM-1 expression occurs at the transcriptional level and is mediated via the transcription factor NF-ϰB (Coimbra 2006). From this point of view, inhibition of ICAM-1 expression by genistein might due to inhibition of NF-ϰB activation in BMECs. Our data suggest VCAM-1 may have other regulative mechanisms in transcriptional level.

Monocyte chemoattractant protein 1 (MCP-1), a member of the C-C chemokine family, attracts blood monocytes and triggers their adhesiveness and transmigration through the endothelial layer. Because MCP-1 plays a key role in the subendothelial recruitment of monocytes (Geissmann 2003), as awell as B and activated T cells (Mantovani 2004), and plays an essential pathogenic role in the initiation and development of inflammation diseases (Ferreira 2005 ; Tucci 2006), we tested whether genistein modulates the expression level of MCP-1 in cultured BMECs. We found that treatment of BMECs with genistein blocked a hemolysate-induced increase in MCP-1 expression.

Cell viability was quantified by measuring the reduction MTT assay. MTT is reduced to a coloured compound (formazin) by mitochondria. The amount of formazin production is proportional to the number of cells present. Therefore, the MTT assay indirectly measures the live cell number and proliferation over time. In this study, we examined whether the inhibitory actions of genistein on NF-ϰB activation, and expression of TNF-α, MCP-1, and ICAM-1 were due to decreased BMEC viability. The effect of genistein cell viability was assessed using MTT assay. As a result, there is no significant change in cell viability by treatment with different concentration of genistein used in this study.

In summary, hemolysate can induce the expression of proinflammatory and adhesion molecules in BMECs, and genistein treatment suppresses the hemolysate–induced increases of proinflammatory and adhesion molecules in BMECs, suggesting that genistein could suppress the vascular inflammatory process in hemorrhage stroke.

Acknowledgments

This work was supported by a grant from the National Natural Science Foundation of China (no. 30700866).

REFERENCES

BERTI R., WILLIAMS A. J., MOFFETT J. R., HALE S. L., VELARDE L. C. *et al.* Quantitative real-time RT-PCR analysis of inflammatory gene expression associated with ischemia-reperfusion brain injury. *J. Cereb. Blood. Flow. Metab.*, 2002, **22** : 1068-1079.

BIJUKLIC K., JENNINGS P., KOUNTCHEV J., HASSLACHER J., AYDIN S. *et al.* Migration of leukocytes across an endothelium-epithelium bilayer as a model of renal interstitial inflammation. *Am. J. Physiol. Cell. Physiol.*, 2007, **293** : 486-492.

CHEN F., DEMERS L. M., VALLYATHAN V., DING M., LU Y. *et al.* Vanadate induction of NF-kappa B involves I kappa B kinase beta and SAPK/ERK kinase 1 in macrophages. *J. Biol. Chem.*, 1999, **274** : 20307-20312.

COIMBRA R., MELBOSTAD H., LOOMIS W., PORCIDES R. D., WOLF P. *et al.* LPS-induced acute lung injury is

attenuated by phosphodiesterase inhibition : effects on proinflammatory mediators, metalloproteinases, NF-kappaB, and ICAM-1 expression. *J. Trauma*, 2006, **60** : 115-125.

Fassbender K., Hodapp B., Rossol S., Bertsch T., Schmeck J. *et al.* Inflammatory cytokines in subarachnoid haemorrhage : association with abnormal blood flow velocities in basal cerebral arteries. *J. Neurol. Neurosurg. Psychiatry*, 2001, **70** : 534-537.

Ferreira A. M., Rollins B. J., Faunce D. E., Burns A. L., Zhu X. *et al.* The effect of MCP-1 depletion on chemokine and chemokine-related gene expression : evidence for a complex network in acute inflammation. *Cytokine*, 2005, **30** : 64-71.

Geissmann F., Jung S., Littman D. R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*, 2003, **19** : 71-82.

Havsteen B. H. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.*, 2002, **96** : 67-202.

Hooshmand S., Soung Do Y., Lucas E. A., Madihally S. V., Levenson C. W. Genistein reduces the production of proinflammatory molecules in human chondrocytes. *J. Nutr. Biochem.*, 2007, **18** : 609-614.

Ichiki T., Jougasaki M., Setoguchi M., Imamura J., Nakashima H. *et al.* Cardiotrophin-1 stimulates intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 in human aortic endothelial cells. *Am. J. Physiol. Heart. Circ. Physiol.*, 2008, **294** : 750-763.

Iwabuchi S., Marton L. S., Zhang J. H. Role of protein tyrosine phosphorylation in erythrocyte lysate-induced intracellular free calcium concentration elevation in cerebral smooth-muscle cells. *J. Neurosurg.*, 1999, **90** : 127-135.

Kim J. M., Lee S. T., Chu K., Jung K. H., Song E. C. *et al.* Systemic transplantation of human adipose stem cells attenuated cerebral inflammation and degeneration in a hemorrhagic stroke model. *Brain Res.*, 2007, **1183** : 43-50.

Mantovani A., Sica A., Sozzani S., Allavena P., Vecchi A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends. Immunol.*, 2004, **25** : 677-686.

Middleton E. Jr., Kandaswami C., Theoharides T. C. The effects of plant flavonoids on mammalian cells : implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.*, 2000, **52** : 673-751.

Pober J. S., Sessa W. C. Evolving functions of endothelial cells in inflammation. *Nat. Rev. Immunol.*, 2007, **7** : 803-815.

Provencio J. J., Vora N. Subarachnoid hemorrhage and inflammation : bench to bedside and back. *Semin. Neurol.*, 2005, **25** : 435-444.

Ruetten H., Thiemermann C. Effects of tyrphostins and genistein on the circulatory failure and organ dysfunction caused by endotoxin in the rat : a possible role for protein tyrosine kinase. *Br. J. Pharmacol.*, 1997, **122** : 59-70.

Saeed R. W., Varma S., Peng T., Tracey K. J., Sherry B. *et al.* Ethanol blocks leukocyte recruitment and endothelial cell activation in vivo and in vitro. *J. Immunol.*, 2004, **173** : 6376-6383.

Sasaki T., Kasuya H., Onda H., Sasahara A., Goto S. *et al.* Role of p38 mitogen-activated protein kinase on cerebral vasospasm after subarachnoid hemorrhage. *Stroke*, 2004, **35** : 1466-1470.

Schmid J. A., Birbach A. IkappaB kinase beta (IKKbeta/IKK2/IKBKB)-A key molecule in signaling to the transcription factor NF-kappaB. *Cytokine Growth Factor Rev.*, 2008, **19** : 157-165.

Tucci M., Quatraro C., Frassanito M. A., Silvestris F. Deregulated expression of monocyte chemoattractant protein-1 (MCP-1) in arterial hypertension : role in endothelial inflammation and atheromasia. *J. Hypertens.*, 2006, **24** : 1307-1318.

Xi G., Hua Y., Bhasin R. R., Ennis S. R., Keep R. F. *et al.* Mechanisms of edema formation after intracerebral hemorrhage : effects of extravasated red blood cells on blood flow and blood-brain barrier integrity. *Stroke*, 2001, **32** : 2932-2938.

Xue M., Del Bigio M. R. Comparison of brain cell death and inflammatory reaction in three models of intracerebral hemorrhage in adult rats. *J. Stroke Cerebrovasc. Dis.*, 2003, **12** : 152-159.

Yakubu M. A., Leffler C. W. Regulation of ET-1 biosynthesis in cerebral microvascular endothelial cells by vasoactive agents and PKC. *Am. J. Physiol.*, 1999, **276** : 300-305.

Yakubu M. A., Leffler C. W. Regulation of cerebral microvascular endothelial cell cyclooxygenase-2 message and activity by blood derived vasoactive agents. *Brain Res. Bull.*, 2005, **68** : 150-156.

Ji-Xin Shi,
Department of Neurosurgery,
Jinling Hospital,
Clinical School of Nanjing University,
305 East Zhongshan Road,
Nanjing 210002 (China).
E-mail : jxshi@yahoo.cn