The Effect of Jeongshin-tang on Interleukin-1β and β-Amyloid-Induced Cytokine Production in Human Brain Astrocytes

Bo Kyung Kim*, Soon Shik Shin[1], Seon Tae Kang[2]

Department of Neuropsychiatry, College of Oriental Medicine, Dongeui University
1:Department of oriental medicine, College of Oriental Medicine, Dongeui University, 2:Daesung oriental medical clinic.

Jeongshin-tang (JST) is a Korean herbal prescription, which has been successfully applied for the various neuronal diseases. However, it's effect remains unknown in experimental models. To investigate the biological effect of JST in Alzheimer's disease (AD) in vitro model, we analized the production of interleukin (IL)-6 and IL-8, and expression of cyclooxygenase (COX)-2 in IL-1β plus β-amyloid [25-35] fragment (A)-stimulated human astrocytoma cell line U373MG. JST alone had no effect on the cell viability. The production of IL-6 and IL-8 was significantly inhibited by pretreatment with JST (1mg/ml) on IL-1β plus A-stimulated U373MG cells. Maximal inhibition rate of IL-6 and IL-8 production by JST was about 41.22% ($P < 0.01$) and 34.45% ($P < 0.05$), respectively. The expression level of COX-2 protein was up-regulated by IL-1β plus A but the increased level of COX-2 was inhibited by pretreatment with JST (1 mg/ml). These data indicate that JST has a regulatory effect on cytokine production and COX-2 expression, which might explain it's beneficial effect in the treatment of AD.

Key words : Jeongshin-tang(精神湯), alzheimer's disease β-amyloid interleukin-6, interleukin-8, cyclooxygenase-2

Introduction

Jeongshin-tang has been used as a Korean genuine medicine for the treatment of various brain diseases in Korea. However scientific investigations have been carried out very little. Alzheimer's disease (AD) is a degenerative disease of the brain, which causes dementia. The disease is characterised by three main pathogenic factors: senile plaques, neurofibrillary tangles and inflammation[1]. The participation of the local inflammatory reaction is confirmed especially by the results of studies dealing with activated microglia, reactive astrocytes, complement system, cytokines, reactive mediators of oxygen and nitrogen (free radicals), all of which participate significantly in inflammatory processes[1]. These inflammatory markers are locally produced by brain cells, and occur in close proximity of β-amyloid (A) deposits[2,3]. In AD pathogenesis, the chronic inflammation might be an essential co-factor involving reactive microglia, astrocytes and pro-inflammatory cytokines. The astrocytes are the predominant neuroglial cells of the central nervous system (CNS) and play an important role in regulation of immune and inflammatory responses by producing cytokines, chemokines, and effector molecules[4,5]. A peptide induce the pro-inflammatory cytokine and chemokine secretion in rat astrocytes and human astrocytoma cells[6,7].

The pro-inflammatory cytokines produced by activated microglia, especially in interleukin (IL)-1β, can trigger enhanced amyloid precursor protein (APP) systhesis and A production and set up a vicious circle whereby A deposits stimulate further cytokine production by activated micorglia, leading to even higher production of APP and its A fragment[8]. A peptide can induce inflammatory reaction through the production of TNF- or IL-6 in brain[9,10]. IL-6 is over-expressed in AD brains[11], and an elevated production of IL-6 has been linked to early stages of amyloid deposition and plaque formation[12]. These findings have suggested a role of IL-6 in neurodegenerative processes leading to AD. IL-6 appears to play multiple roles in a variety of brain functions, since this cytokine may affect cell to cell signaling, coordination of neuroimmune responses and neuronal differentiation, growth and survival[12]. IL-6 is also synthesized and released from human neuronal cells and may modulate the protection of neurons from insults[13]. IL-6 affects neurodegenrative lesions in the AD brain and it has been

* To whom correspondence should be addressed at : Bo Kyung Kim, Department of Neuropsychiatry, College of Oriental Medicine, Dongeui University, 100 Jilli (1)lo, Busanjin-gu Busan, 614-052, Republic of Korea.
· E-mail : npjolie@demc.or.kr, · Tel : 051-867-8809
· Received : 2003/11/17 · Revised :2003/12/22 · Accepted : 2004/01/15

implicated in the early stages of plaque formation.

A recent report suggests that IL-1β, a critical neurotoxic component in AD, is produced by activated microglia, which in turn can participate in the activation of astrocytes[14]. Chemokine, IL-8 is low-molecular-weight proinflammatory proteins produced by a wide range of cells, including microglia, and astrocytes. IL-8 was expressed locally in response to inflammatory stimuli and may amplify subsequent tissue reactions through their activating and chemotactic activities[15,16].

It was also reported that A peptide could induce the production of Cyclooxygenase (COX)-2 in neuroglial cells. Increased levels of COX-2 expression in astrocytes surrounding A contribute to neurotoxicity and damage in AD brain. In AD brains, it has been also reported that the expression of COX-2 mRNA and protein was elevated[17,18]. COX-2 upregulation is thought to mediate neuronal damage presumably by producing excessive amounts of harmful prostanoids and free radicals[19].

In this study, we investigated the inhibitory effect of JST on IL-1β and A-induced cytokines (IL-6, IL-8) production and COX-2 expression in U373MG cells.

Materials and Methods

1. Reagents

Cell culture medium, RPMI 1640, ampicillin, and streptomycin were purchased from Gibco BRL (Grand Island, NY). Avidin-peroxides, A [25-35] fragment(A), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), and other reagents were obtained from Sigma (St. Louis, MO). Recombinant human IL-1β was obtained from R&D Systems (Minneapolis, MN). Anti-human IL-6 and IL-8, biotinylated anti-human IL-6 and IL-8, and recombinant human IL-6 and IL-8 were purchased from Pharmingen (Sandiego, CA).

2. U373 MG cell culture

The Astrocytoma cell line, U373MG, was used in this study. The cells were maintained in RPMI-1670 medium (Gibco RBL) with 10% fetal bovine serum (FBS, JH Bioscience) at 37°C under 5 % CO_2 in air.

3. Drugs and Preparation of JST

Extract of JST was prepared by decocting the dried prescription of herbs with boiling distilled water (102 g / 1 L). The extraction decocted for approximately 3 h has been filtered, lyophilized, and kept at 4°C . The yield of extraction was about 16%. The ingredients of JST were shown in Table 1.

Table 1. The amount and composition of Jeongshin-tang

韓藥名	herbal name	usage(g)
玄 參	*Scrophularia buergeriana*	12
丹 參	*Salvia miltiorrhiza*	12
貝 母	*Fritillaria ussuriensis*	12
牡 蠣	*Ostrea talienwhanensis*	6
龍 骨	*Ossia mastoid Fossilia*	6
茯 神	*Poria cocos*	6
釣鉤藤	*Uncaria rhynchophylla*	6
連 翹	*Forsythia koreana*	4
南 星	*Arisaema japonica*	4
陳 皮	*Citrus unshiu*	4
石菖蒲	*Acorus gramineus*	4
酸棗仁	*Zizyphus spinosa*	4
柏子仁	*Biota orientalis*	4
天 麻	*Gastrodia elata*	4
天門冬	*Asparagus cochinchinensis*	4
麥門冬	*Ophiopogon japonicus*	4
竹 茹	*Phyllostachys nigra*	4
黃 蓮	*Coptis japonica*	2
	Total amount	102

4. MTT assay

Cell viability was determined using MTT assay. Briefly, 500 l of astrocyte cells suspension (3 x 10^5 cells) was cultured in 4-well plates for 24 h after treatment by each concentration of JST. 50㎕ of MTT solution (5 mg/ml) was added and then cells were incubated for 4 h at 37°C. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, optical density of 96-well culture plates was measured using enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

5. Cytokines assay

Cytokine production was measured by a modified ELISA as described previously[20]. ELISA for IL-6 and IL-8 was carried out in duplicate in 96 well ELISA plates (Nunc, Denmark) coated with each of 100 l aliquots of anti-human IL-6 and IL-8 monoclonal antibodies at 1.0 g /ml in PBS at pH 7.4 and was incubated overnight at 4°C. The plates were washed in PBS containing 0.05% Tween 20 (Sigma) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN3 for 1 h. After additional washes, sample or IL-6 and IL-8 standards were added and incubated at 37°C for 2 h. After 2 h incubation at 37°C, the wells were washed and then each of 0.2 g/mlof biotinylated anti-human IL-6 and IL-8 were added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 20 min at 37°C. Wells were again washed and 2,2-azio-bis (3-ethylbenzthiazoline-6-sulfonic acidsubstrate (ABTS, Sigma) was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was

run on each assay plate using recombinant IL-6 and IL-8 in serial dilutions.

6. Western blot analysis

Cell extracts were prepared by detergent lysis procedure. Cells (5 x 10^6 cells) were scraped, washed once with PBS, resuspended in lysis buffer. Samples were vortexed for a few seconds every 15 min at 4°C for 1 h and centrifuged at 15000 g for 5 min at 4°C. Supernatants were assayed. Samples were heated at 95°C for 5 min, and briefly cooled on ice. Following the centrifugation at 15000 g for 5 min, 50 l aliquots were resolved by 10% SDS-PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris (pH 8.5), 0.2 M glycerin, 20% methanol at 25V. Blots were blocked for at least 2 h with PBS-tween-20 (PBST) containing 5% nonfat dry milk and then incubated with anti-COX-2 antibody (1:1000 dilutions in blocking solution). After washing in PBST three times, the blot was incubated with secondary antibody(1:1000 dilution in blocking solution) for 30 min and the antibody-specific proteins were visualized by the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp).

7. Statistical analysis

Each datum represents the mean ± SEM of the different experiments under the same conditions. The Student's t-test was used to make a statistical comparison between the groups. Results with $P < 0.05$ were considered statistically significant.

Results

1. Effect of JST on the Cell Viability in U373MG Cells

We examined the effect of JST on the viability of U373MG cells using MTT assay. Cells were treated with various concentrations of JST (0.01 - 1 mg/ml) and then stimulated with IL-1β plus A for 24 h. In the cells treated with IL-1β plus A, cell viability decreased to 94.91 ±3.5% compared with the control value (100.0 ± 3.2%). However, JST (0.01 - 1 mg/ml) did not significantly affect cell viability in each condition and had no toxicity on U373MG cells (Fig. 1).

2. Production of Cytokines in IL-1β plus A -Stimulated on U373MG Cells

We observed to ascertain the optimal time condition for production of IL-6 and IL-8 by IL-1β plus A stimulations. U373MG cells were treated with IL-1β plus A for various time intervals. The constitutive and inducible production of IL-6, IL-8 was demonstrated by the ELISA method. IL-1β plus A significantly enhanced IL-6 and IL-8 production compared with unstimulated cells. Maximal level of IL-6 Production was appeared at 24 h (by a 4.1 ± 0.18 -fold increase over unstimulated cells ($P < 0.05$) and then declined. IL-8 production increased in a time-dependent manner, but only at 24 h, there was significant difference (by a 2.1 ± 0.25-fold increase over control, $P < 0.05$). We used 24 h incubation for optimal cell stimulation in subsequent experiments.

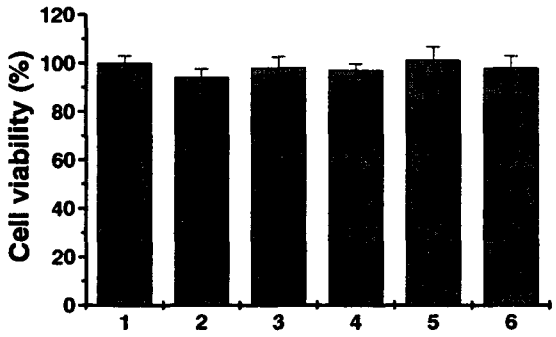

Fig. 1. Effect of JST on the Cell Viability in U373MG Cells. Cell viability was evaluated by MTT colorimetric assay for 24 h incubation after stimulation of IL-1β (10 ng/ml) plus A (30 μ M), in the absence or presence of JST (0.01-1 mg/ml). The percentage of viable cells was over 94%. Data represent mean SEM of six independent experiments. 1, Unstimulated cells; 2, IL-1β + A; 3, JST (1 mg/ml); 4, JST (0.01 mg/ml) + IL-1β + A; 5, JST (0.1 mg/ml) + IL-1 β + A; 6, JST (1 mg/ml) + IL-1β + A.

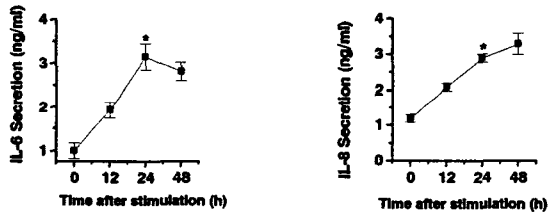

Fig. 2. Effect of IL-1β Plus A on the Cytokine Production. U373MG cells (3 x 10^5) were treated with IL-1 (10 ng/ml) plus A (30 μ M) for various times. Cell-free supernatants were tested at time courses for up to 48 h. The amount of IL-6 and IL-8 production was measured using ELISA method. Data represent mean ± SEM of four independent experiments. *P < 0.05; significantly different from the unstimulated cells.

3. Effect of JST on Concentrations of IL-6 Production on U373MG cells

The influence of JST at various concentrations on production of IL-6 was tested after 24 h of activation with IL-1β plus A. As shown in Fig 3, IL-6 production was synergistically enhanced with co-stimulation of IL-1β plus A. IL-6 production in response to IL-1β plus A was inhibited by pretreatment with 0.01 - 1 mg/ml JST in a dose-dependent manner (about 13.45% at 0.01 mg/ml JST; 21.05% at 0.1 mg/ml JST 41.22% at 1 mg/ml JST, $P < 0.01$), but at 0.01-0.1 mg/ml JST, there was no significant difference.

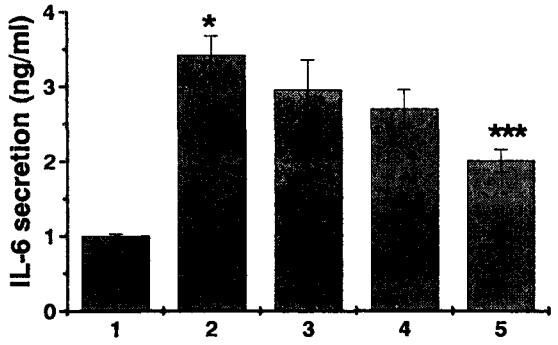

Fig. 3. Effect of JST on IL-6 Production in U373MG Cells at Various Concentrations. JST (0.01-1 mg/ml) was tested after 24 h of activation with IL-1β (10 ng/ml) plus A (30 μ M). Data represent mean SEM of six independent experiments. 1, Unstimulated cells; 2, IL-1β + A; 3, JST (0.01 mg/ml) + IL-1β + A; 4, JST (0.1 mg/ml) + IL-1β + A; 5, JST (1 mg/ml) + IL-1β + A. *P < 0.05 is different from the unstimulated cells. ***P < 0.01 is different from the IL-1β + Astimulated cells.

4. Effect of JST on Concentrations of IL-8 Production on U373MG Cells

To determine whether JST can modulate IL-1β plus A-induced IL-8 production, the cell were pretreated with various concentrations of JST for 30 min prior to IL-1β plus A. IL-8 production decreased with JST pretreatment (about 11.58% at 0.01 mg/ml JST 20.42% at 0.1 mg/ml JST 34.45% at 1 mg/ml JST, $P < 0.05$) in a dose-dependent manner, but at 0.01-0.1mg/ml JST, there was no significant difference (Fig. 4).

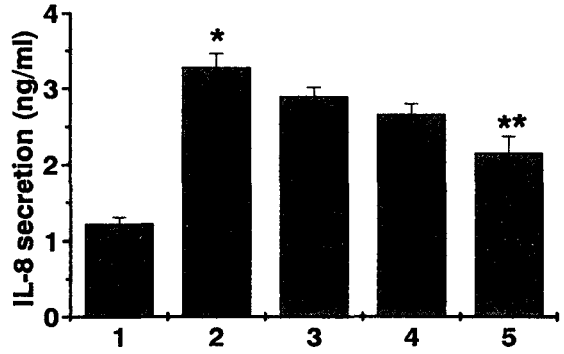

Fig. 4. Effect of JST on IL-8 production in U373MG cells at various concentrations. JST (0.01 - 1mg/ml) was tested after 24 h of activation with IL-1β (10 ng/ml) plus A (30 μ M). Data represent mean SEM of six independent experiments. 1, unstimulated cells; 2, IL-1β + A; 3, JST (0.01 mg/ml) + IL-1β + A; 4, JST (0.1 mg/ml) + IL-1β + A; 5, JST (1 mg/ml) + IL-1β + A. *P < 0.05 is different from the unstimulated cells. **P < 0.05 is different from the IL-1β + A stimulated cells.

5. Effect of JST on expression of COX-2 in U373MG cells

Finally, we examined the effect of JST on the IL-1β plus A-induced expression of COX-2 protein in U373MG cells using Western blotting. As a result, pretreatment of 1 mg/ml JST inhibited the expression of COX-2 in co-stimulated cells with IL-1β plus A (Fig. 5).

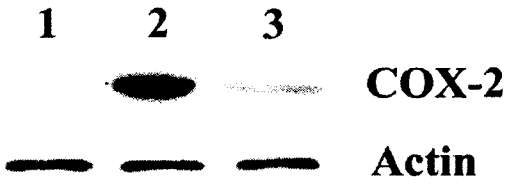

Fig. 5. Effect of JST on Expression of COX-2 in U373MG Astrocytoma Cells. JST (1 mg/ml) was pretreated before IL-1β (10 ng/ml) plus A (30 μ M). One hundred microgram of cell lysates was separated by SDS-polyacrylamide gelelectrophoresis and immunoblotted with antibody to COX-2. 1, unstimulated cells; 2, IL-1β + A; 3, JST (1 mg/ml) + IL-1β + A.

Discussion

In this work, we demonstrated that JST inhibits the production of cytokine (IL-6 and IL-8) on IL-1β plus A-stimulated U373MG cells. We also showed that JST decreased the COX-2 expression in IL-1β plus A-stimulated U373MG cells. These results indicate that JST has a regulatory effect of cytokine production and COX-2 expression in in vitro AD model, which might explain it's beneficial effect in the treatment of AD.

Human brain cells are capable of initiating and amplifying a brain specific inflammatory response involving the synthesis of cytokines, acute-phase proteins, complement proteins, prostaglandins and oxygen radicals[21]. In AD, all signs of an inflammatory microglial and astroglial activation are present inside and outside amyloid depositions and along axons of neurones with neurofibrillary tangles[22,23]. Cell culture and animal models suggest a bidirectional relationship between inflammatory activation of glial cells and the deposition of A. Although it remains unclear which of the different pathophysiological processes in AD may be the driving force in an individual case, the inflammatory activation may increase the speed of cognitive decline. Activated glia-derived pro-inflammatory cytokines are important pathologic factors of progression of AD, neuropathologic changes, and inflammation in the CNS.[5] Thus, the anti-inflammatory drug can reduce AD risk and delay some forms of AD pathology. A peptide potentiates pro-inflammatory cytokine and chemokine production in rat astrocytes and human astrocytoma cells[6,7]. IL-1β is a critical inflammatory cytokine in AD, and IL-1β -expressing cells are responsible for microglia stimulated with A[14].

In the present study, the production of IL-6 and IL-8 in response to A was synergistically potentiated by co-stimulation

of IL-1β. Data from other investigators basically agree with these findings. The expression of IL-6 mRNA is indeed increased in brain areas where A deposition and astroglia activation are prominent in AD patients and increased IL-6 levels in the brain have been implicated in early stages of plaque formation[24,25]. IL-8 is low-molecular-weight proinflammatory proteins produced by a wide range of cells, including microglia, and astrocytes. IL-8 was expressed locally in response to inflammatory stimuli and may amplify subsequent tissue reactions through their activating and chemotactic activities.

The pretreatment of JST not only inhibited the production of IL-6 and IL-8 but also had no toxic effects in human astrocytoma cells. Therefore, our data indicate that JST has an ability of regulation in inflammatory response and neurotoxicity in astrocytoma cells.

It was also reported that A peptide could induce the production of COX-2 in neuroglial cells[26]. Increased levels of COX-2 expressionin astrocytes surrounding A contribute to neurotoxicity and damage in AD brain[27]. COX-2 is continuously expressed within a distinct population of neurons in the brain, which is an attribute common in enzymes involved in physiological functions of the CNS such as memory, sensory integration, and autonomic regulation and may suggest this role for COX-2[28]. COX-2 upregulation is thought to mediate neuronal damage presumably by producing excessive amounts of harmful prostanoids and free radicals[29].

In our study, JST inhibited the expression of COX-2 in cells co-stimulated with IL-1β and A. Therefore, these results suggest that JST might have a beneficial effect in treatment of COX-2-mediated AD.

JST consists of 18 different herbs. Other studies reported that each medicine herb has a different effect. Scrophularia buergeriana has significant protective effects against glutamate-induced neurodegeneration in primary cultures of rat cortical neurons[30]. Asparagus cochinchinensis has Inhibitory effect of tumor necrosis factor-alpha-induced apoptosis in Hep G2 cells[31], Poria cocos has antitumor activities against Sarcoma in vivo[32], Acorus gramineus has inhibitory effect of fragrance inhalation on central nervous system[33], Biota orientalis has protective activity against glutamate-induced neurotoxicity in primary cultures of rat cortical cells[34]. Even though those herbs are frequently used to treat the various brain diseases, these effects are never same with JST. This prescription was also composed on the basis of the theory of traditional Korean medicine to maximize its efficacy.

Overall our results suggest that JST is thought to be applicable to the treatment of the AD. Nevertheless, the exact mechanisms of JST should be established in the future.

Acknowledgements

This work results from the performance of the 2003 industrial-educational consortium project supporting business of the academic research fund of dongeui university

References

1. Ferencik M., Novak M., Rovensky J., Rybar I., Bratisl. Lek. Listy., 102, 123-132, 2001.
2. Iversen L. L., Mortishire-Smith R. J., Pollack S. J., Shearman M. S., Biochem. J., 311, 1-16, 1995.
3. Mark R, E., Sheng J. G., Griffin S. T., Hum. Pathol., 26, 816-823, 1995.
4. Aschner M., Toxicol. Lett., 102-103, 283-287, 1998.
5. Raivich G., Bohatschek M., Kloss C. U. A., Werner A., Jones L. L., Kreutzberg G. W., Brain Res. Rev., 30, 77-105, 1999.
6. Forloni G., Mangiarotti F., Angeretti N., Lucca E., De Simoni M. G., Cytokine, 9, 759-762, 1997.
7. Gitter B. D., Cox L. M., Rydel R. E., May P. C., P. Natl. Acad. Sci. U.S.A., 92, 10738-10741, 1995.
8. Eikelenboom P., Bate C., Van Gool W. A., Hoozemans J. J., Rozemuller J. M., Veerhuis R., Williams A., Glia, 40, 232-239, 2002.
9. Sutton E. T., Thomas T., Bryant M. W., Landon C. S., Newton C. A., Rhodin J. A., J. Submicrosc. Cytol. Pathol., 31, 313-323, 1999.
10. Kaltschmidt B., UherekM., Volk B., Baeuerle P. A., Kaltschmidt C., P. Natl. Acad. Sci. U.S.A., 94, 2642-2647, 1997.
11. Bauer J., Strauss S., Schreiter-Gasser U., Ganter U., Schlegel P., Witt I., FEBS Lett., 285, 111-114, 1991.
12. Gruol D. L., NelsonT. E., Mol. Neurobiol., 15, 307-339, 1997.
13. Yamada K., Kono K., Umegaki H., Yamada K., Iguchi A., Fukatsu T., Neurosci. Lett., 186, 219-221, 1995.
14. Akama K. T., Van Eldik L. J., J. Biol. Chem., 275, 7918-7924, 2000.
15. Hayashi M., Luo Y., Laning J., Strieter R. M.,Dorf M. E., J. Neuroimmunol., 60, 143-150, 1995.
16. Ransohoff R. M., Hamilton T. A., Tani M., Stoler M. H., Shick H. E., Major J. A., Estes M. L., Thomas D. M., Tuohy V. K., FASEB J., 7, 592-600, 1993.
17. Pasinetti G. M., Aisen P. S., Neuroscience, 87, 319-324, 1998.

18. Yasojima K., Schwab C., McGeer E. G., McGeer P. L., Brain Res., 830, 226-236, 1999.
19. Rohrenbeck A. M., Bette M., Hooper D. C., Nyberg F., Eiden L. E., Dietzschold B., Weihe E., Neurobiol. Dis., 6, 15-34, 1999.
20. Jeong H. J., Na H. J., Hong S. H., Kim H. M., Endocrinology, 144, 4080-4086, 2003.
21. Hull M., Lieb K., Fiebich B. L., Expert Opin. Inv. Drug., 9,671-683, 2000.
22. Meda L., Bonaiuto C., Szendrei G. I., Ceska M., Rossi F., Cassatella M. A., J. Neuroimmunol., 59, 29-34, 1995.
23. Pike C. J., CummingsB. J., Cotman C. W., Exp. Neurol., 132, 172-179, 1995.
24. Bauer J., Strauss S., Schreiter-Gasser U., Ganter U., Schlegel P., Witt I., FEBS Lett., 285, 111-114, 1991.
25. Huell M., Strauss S., Volk B., Berger M., Bauer J., Acta Neuropathol, 89, 544-551, 1995.
26. Bazan N. G., Lukiw W. J., J. Biol. Chem., 277, 30359-30367, 2002.
27. Nogawa S., Takao M., Suzuki S., Tanaka K., Koto A., Fukuuchi Y., Hayakawa I., International Congress Series, 1252, 363-372, 2003.
28. Kaufmann W. E., Andreasson K. I., Isakson P. C., WorleyP. F., Prostaglandins, 54, 601-624, 1997.
29. Butterfield D. A., Curr. Med. Chem., 10, 2651-9, 2003.
30. Kim S. R., Lee K. Y., Koo K. A., Sung S. H., Lee N. G., Kim J., Kim Y.C., J. Nat. Prod., 65, 1696-1699, 2002.
31. Koo H.N., Jeong H. J., Choi J. Y., Choi S. D., Choi T. J., Cheon Y. S., Kim K. S., Kang B. K., Park S. T., Chang C. H., Kim C. H., Lee Y. M., Kim H. M., An N. H., Kim J. J., J. Ethnopharmacol., 73, 137-143, 2000.
32. Jin Y, Zhang L, Zhang M, Chen L, Cheung P. C., Oi V. E., Lin Y., Carbohydr. Res., 338, 1517-1521, 2003.
33. Koo B. S., Park K. S., Ha J. H., Park J. H., Lim J. C., Lee D. U., Biol. Pharm. Bull., 26, 978-982, 2003.
34. Koo K. A., Sung S. H., Kim Y. C., Chem. Pharm. Bull., 50, 834-836, 2002.