changes to the dietary lifestyle of Koreans which include consuming excessive fat and insufficient exercise. A cerebral infarction is a type of ischemic stroke caused by a disturbance in the blood vessels supplying blood to the brain. The causes could be atherothrombotic or embolic. This loss of blood supply results in the death of tissue to that area of the brain. During the ischemic cascade, oxygen deprivation leads to the absence of ATP in the tissue of the brain. ATP proton pumps fail, allowing a massive influx of calcium ions into the cells. This results in the generation of reactive oxygen species, free radicals, and other harmful chemicals. Eventually, some of the cells begin to die via necrosis, triggering an inflammatory response which can itself cause further damage to brain tissue. The ischemic cascade can occur in any type of tissue, but the brain is considered most vulnerable due to its complete dependence on aerobic metabolism.

Ginkgo biloba extract (GbE) is found to have extensive protective effects on both the central nervous system and circulatory system.
system. Many studies have been carried out since 1960s and it was found that *ginkgo biloba* extract could increase rat cerebral blood flow, improve the mice memory impaired by ischemia, and protect the cerebral function. Moreover, GbE showed an anti-oxidation effect. GbE can eliminate free oxygen radicals, reduce lipid peroxidation and facilitate the synthesis and release of epoprostenol. GbE helps to alleviate the subcellular damages of cerebral ischemia and allows mitochondria to maintain their respiratory activity under ischemic conditions as long as some oxygen is present, thus delaying the onset of ischemia-induced damage. It was reported that GbE could diminish the coagulation of platelets to inhibit thrombosis by antagonizing the platelet activating factor, and GbE is capable of inhibiting the adhesion of monocytes and neutrophils to cultured cerebral microvascular endothelial cells. Therefore, GbE might be useful as a preventive therapy or as a post-ischemic treatment to reduce the damaging effects of stroke.

Aspirin (acetylsalicylic acid) is the most common and widely used anticoagulant and is considered an effective drug in the secondary prevention of ischemic stroke worldwide. The household analgesic, aspirin, has an alternate role as an inhibitor of platelet function and it has been shown to inhibit the platelet release reaction. Aspirin also inhibits the formation of prostaglandin G2, a cyclic endoperoxide intermediary of prostaglandin biosynthesis in human platelets, and prostaglandin G2 production is stimulated by thrombin, moreover, prostaglandin G2 will cause platelet aggregation and the release reaction.

Recently, ischemic stroke is shown to have a close relationship with diabetes mellitus, obesity and hypertension and its prevalence rate has been increasing. After a stroke occurs, that person may also suffer from severe sequelae. Due to the slow curative effect, person must endure the damage to their brain and the great financial burden. Therefore, the development of medicine could diminish the coagulation of platelets to inhibit thrombosis by antagonizing the platelet activating factor, and GbE is capable of inhibiting the adhesion of monocytes and neutrophils to cultured cerebral microvascular endothelial cells. Therefore, GbE might be useful as a preventive therapy or as a post-ischemic treatment to reduce the damaging effects of stroke.

**Materials and Methods**

**Materials**

The SK-N-MC neuroblastoma cell line was purchased from a Korean cell line bank (Seoul, Korea). Cell culture reagents were purchased from Invitrogen (Carlsbad, California, USA). Aspirin and ginkgolides were obtained from Sigma-ALDRICH (St. Louis, MO, USA). TRIzol® reagent and SuperScript III reverse transcriptase were purchased from Invitrogen (Carlsbad, California, USA). Maximum PCR Premix kit was obtained from iNtRON Biotechnology (Gyeonggi-do, Korea).

**SK-N-MC cell culture and the making hypoxia and reperfusion condition**

The cell line was incubated in DMEM media containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution in 37°C with 5% CO₂ incubator. The cell line was replaced with new DMEM media every 48-72 hrs. The cells cultured in general DMEM media containing 4500 mg/L D-glucose concentration were cultured in free glucose DMEM media under hypoxic condition for 8 hours. Then, the cells were again cultured with general DMEM media under normal O₂ concentration condition for 12 hours. With this process, the cells were set in condition of hypoxia/reperfusion state.

**Measurement of cell survival rate (CCK-8 assay)**

SK-N-MC cells were seeded on a 96-well plate at a concentration of 10⁴ cells per well, and then were treated with various concentrations of ginkgolide A or B extracts or aspirin for 48 hours in 37°C CO₂ incubator. After treatment duration, the CCK-8 assay reagent was added to culture media and incubated for 3 hours. Absorbance was read at 450 nm on a micro-plate reader.

**Reverse transcription polymerase chain reaction (RT-PCR) for the estimation of GAP43, MAP2, Bcl2, p53, and GAPDH mRNA levels**

Total RNA was extracted by the Trizol reagent according to manual. Complementary DNA was synthesized by SuperScript III reverse transcriptase from total RNA, and polymerase chain reactions for GAP43, MAP2, Bcl2, p53, and GAPDH were administered with PCR Premix kit. The primer sequences used for RT-PCR are as follows: GAP43 forward primer; 5′-TAAAGCTCATAAAGGCGCAA-3′; GAP43 reward primer; 5′-ATCACCCTCCCGCTTTCTCT-3′; MAP2 forward primer; 5′-ACTGGTTCATCGAATGCCA-3′; MAP2 reward primer; 5′-AGTGAAGCTTCCTCGTTTAATG-3′; Bcl2 forward primer; 5′-TCTCCCGGACTCTCTGATT-3′; Bcl2 reward primer; 5′-CGGCTGCGCCACATCTCTC-3′; p53 forward primer; 5′-CCGGATTTACTTGCCCTATC-3′; p53 reward primer; 5′-TGTCACCGTCGTGGAAA-3′; GAPDH forward primer; 5′-CATAAGGCCGCAA-3′; GAPDH reward primer; 5′-GCGGATTACTTGCCCTTACT-3′; GAPDH reverse primer; 5′-CAGCGTGCGCCATCCTTC-3′. The house

**Statistical analysis**

Data are presented as mean ± SEM (Standard Error of Measures). Statistically significant differences between two groups were calculated by the Student’s t-test and one-way ANOVA was used to certify the statistical differences among over three groups. A value of p<0.05 was considered significant.
The efficacy of ginkgolide A, B and aspirin to cell viability after hypoxia and reperfusion condition

After the cells were treated with ginkgolide A, ginkgolide B and aspirin individually, or together for 48 hrs, the cells were set under hypoxic condition for 8 hrs and then they were reperfused for 12 hrs. The numbers of viable cells in hypoxia and reperfusion control were decreased by 26% of the normal control. In the treat of ginkgolide A or B, the numbers of viable cells were 1.7 and 1.23 fold higher in each than hypoxia and reperfusion control. The group treated with ginkgolide B and aspirin collectively also had significantly increased the number of viable cells than hypoxia and reperfusion control, however, there was no the additive effect in the co-treatment of ginkgolide B and aspirin (Fig. 4).

The efficacy of ginkgolide A, B and aspirin on the mRNA expression of GAP43 and MAP2 in hypoxia and reperfusion condition

The mRNA expression levels of GAP43 and MAP2, which were involved in regeneration of the neural tissues, had no significant difference between the hypoxia and reperfusion control and the condition treated with ginkgolide A, B and aspirin individually. On the other hand, the level of mRNA expression of...
GAP43 was increased in the treatment of ginkgolide A or B with aspirin, and the level of mRNA expression of MAP2 was increased only in treatment of ginkgolide A with aspirin (Fig. 5).

The efficacy of ginkgolide A, B and aspirin on the mRNA expression of Bcl2 and p53 gene

The level of mRNA expression of Bcl2 had no significant change between hypoxia and reperfusion control and the condition treated with ginkgolide A, B and aspirin individually. On the other hand, the level of mRNA expression of Bcl2 was significantly increased in treatment of ginkgolide A or B with aspirin. However, the level of mRNA expression of p53 had no significant change in all groups (Fig. 6).

DISCUSSION

Postulative mechanisms for neuronal damage in brain ischemia were reported that interleukin-6·8 (IL-6·8), monocyte chemotactic protein-1 (MCP-1) and vascular endothelial growth factor (VEGF) secreted from hypoxic endotherial cells may play a significant role in breakdown of endothelial barrier and eventual neuronal damage observed in ischemic brain injury, and caspase activation and cytochrome c release may play roles in hypoxia-induced neuronal apoptosis.

It was reported that Ginkgolide A and B block the inhibition of cell growth and apoptosis induced by sodium nitroprusside, a nitric oxide (NO) donor, in human neuroblastoma cell line SK-N-SH.

Some reports suggested that ginkgo biloba extract, EGb 761 can prevent cell death due to brain injury and that EGb 761 protection is affected by preventing the injury-induced decrease of Akt phosphorylation, and its neuro-protective action is dependent on heme oxygenase 1 in ischemic reperfusion brain injury.

A few researches were focused on the effect of ginkgo biloba extract (GbE) on cerebral function following ischemic insult using electroencephalography (EEG), which reflected cerebral excitation. The studies tested the effect of GbE on EEG during cerebral ischemia and reperfusion instead of measuring cell survival rate and mRNA expression of GAP43, MAP2, Bcl2 and p53 gene. The studies also concluded that GbE protects the cerebral cell function against loss during ischemia.

Another study observed the dynamic changes of biochemical metabolism after acute cerebral ischemia and validate the effects of GbE on acute cerebral ischemia during early stage by magnetic resonance spectroscopy (MRS). On MRS, lactate level was related to the infarction volume. This study found that lactate level in the treatment group was significantly lower than that in the ischemic group, but the lactate level in the prophylactic group was the lowest among the groups demonstrating that GbE could protect the cerebral ischemic neurons. It was also found that the infarct volume in the prophylactic group was reduced more significantly as compared with that in the treatment group, implying that prophylactic GbE can enhance the tolerance of the neu-
A contributing mechanism of aspirin on neuroprotection was suggested that aspirin protects neuron injured by hypoxia and reoxygenation through inhibition of the sustained activation of extracellular-signal-regulated kinase 1/2 (ERK1/2).

Numerous studies have demonstrated the efficacy of aspirin in preventing the recurrence of ischemic stroke among the survivors of ischemic stroke. Even though there are many studies of ginkgo biloba extract and aspirin individually on cerebral ischemia, ginkgo biloba extract and aspirin combined study on cerebral ischemia and regeneration of neurons has not been investigated yet.

Someone might raise a question about coagulation and bleed...
ing tendency when combination of ginkgo biloba and aspirin used for prevention and treatment of cerebral infarction. There are several investigations upon effect of ginkgo biloba and aspirin on coagulation and bleeding showed that in older adults with peripheral artery disease (PAD) or cardiovascular disease risk, a relatively high dose of ginkgo biloba combined with 325 mg/day daily aspirin did not have a clinically or statistically detectable impact on indices of coagulation examined over 4 weeks, compared with the effect of aspirin alone. Thus, the bleeding tendency would not be increased in combined treatment of ginkgo biloba and aspirin.

In Taiwan, the trend in co-prescription of aspirin and ginkgo biloba is increasing for Taiwan’s elderly population during 1997-2003. However, there are no proper clinical investigations on combined treatment of Ginkgo biloba and aspirin for cerebral infarction yet. So, it needs more clinical experiences and researches of combined treatment of ginkgo biloba and aspirin on cerebral infarction.

In this study, the individual treatment of ginkgolide A and B to the normal neuroblastoma cells increased the viability of the cells in wide concentration range of 0.05-100 μmole/mL and 0.1-1000 μmole/mL in each. In contrast, aspirin had a relatively narrow concentration range of 20-100 μmole/mL to increase the viability of the cells compared to the ginkgo biloba extracts. Consequently, these three substances are helpful to elevation of SK-N-MC, neuroblastoma cellular viability in normal growth condition.

The viable cell numbers reduced by hypoxia and reperfusion condition were significantly increased through individual treatment of ginkgolide A and B, however, there was no additive effect in the co-treatment of ginkgolides with aspirin.

The investigation on the effectiveness of ginkgolide A, B and aspirin on the level of mRNA expression of GAP43 and MAP2, which are associated with regeneration of the neural cells in condition of hypoxia and reperfusion, showed that the individual treatment of ginkgolide A, B and aspirin had no significant increases to effectiveness on mRNA expression of GAP43 and MAP2. Alternatively, the mRNA expression of GAP43 in the co-treatment of ginkgolide A or B with aspirin was elevated significantly and the mRNA expression of MAP2 in the co-treatment of only ginkgolide A with aspirin increased. From this result, even though there is no the increasing effect of the individual treatment of ginkgolide A, B and aspirin on hypoxic and reperfused injury of the neural cells, the co-treatment of ginkgolide A or B with aspirin would increase the mRNA levels of GAP43 and MAP2 that regenerate the injured neural cells. This study assumes that using ginkgo biloba extracts and aspirin together would be helpful to the ischemic stroke patients.

The investigation into the effectiveness of ginkgolide A, B and aspirin on the transcription of Bcl2 and p53 that suppresses and induces apoptosis in each, showed that even though the individual treatment of ginkgolide A, B and aspirin did not have effectiveness on mRNA expression of Bcl2 and p53, the co-treatment of ginkgolide A or B with aspirin significantly increased Bcl2 transcription compared to the hypoxia and reperfusion control. However, there was no change in mRNA expression of p53 even in co-treatment of ginkgolide A or B with aspirin. From this study, the co-treatment of ginkgolide A or B with aspirin would suppress the apoptosis and increase the viability of neural cells through Bcl2 regulation.

**CONCLUSION**

Although the ginkgolide A and B increase the viability of injured neural cells in hypoxica and reperfusion condition, our study results suggests that the aspirin should be treated together with ginkgolide A or B to increase regeneration rate of the injured neural cell in hypoxia and reperfusion condition. In the management of stroke patients, reference to the results of this study would provide the theoretical basis for clinical prevention and treatment of stroke.

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