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Laboratory Investigation

The Combined Effects of *Ginkgo Biloba* Extracts and Aspirin on Viability of SK-N-MC, Neuroblastoma Cell Line in Hypoxia and Reperfusion Condition

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Objective : The purpose of this study is to investigate the combined effects of *ginkgo biloba* extract, ginkgolide A and B and aspirin on SK-N-MC, human neuroblastoma cell viability and mRNA expression of growth associated protein43 (GAP43), Microtubule-associated protein 2 (MAP2), B-cell lymphoma2 (Bcl2) and protein53 (p53) gene in hypoxia and reperfusion condition.

Methods : SK-N-MC cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) media in 37°C, 5% CO_2 incubator. The cells were cultured for 8 hours in non-glucose media and hypoxic condition and for 12 hours in normal media and O_2 concentration. Cell survival rate was measured with Cell Counting Kit-8 (CCK-8) reagent assay. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to estimate mRNA levels of GAP43, MAP2, Bcl2, and p53 genes.

Results : The ginkgolide A and B increased viable cell number decreased in hypoxic and reperfused condition. The co-treatment of ginkgolide B with aspirin also increased the number of viable cells, however, there was no additive effect. Although there was no increase of mRNA expression of GAP43, MAP2, and Bcl2 in SK-N-MC cells with individual treatment of ginkgolide A, B or aspirin in hypoxic and reperfused condition, the co-treatment of ginkgolide A or B with aspirin significantly increased GAP43 and Bcl2 mRNA levels. In MAP2, only the co-treatment of ginkgolide A and aspirin showed increasing effect. The mRNA expression of p53 had no change in all treating conditions.

Conclusion : This study suggests that the combined treatments of *Ginkgo biloba* extracts and aspirin increase the regeneration of neuroblastoma cells injured by hypoxia and reperfusion.

Key Words : *Ginkgo biloba* extract · Aspirin · GAP43 · MAP2 · Bcl2 · p53.

INTRODUCTION

Stroke is one of the leading causes of death and disability. It is estimated that approximately 600,000 people suffer a new or recurrent stroke each year in the United States. Strokes killed an estimated 160,000 people in the United States during 1996 and ranked behind heart disease and cancer, as the third leading cause of death. Also, ischemic strokes account for approximately 80% to 85% of all strokes[25]. Based on a report of the National Statistical Office in Korea, cerebral vascular disease follows cancer as the second leading cause of death among Koreans[15]. The prevalence of cerebral vascular disease is increasing because of 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 either artherothrombotic 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

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system[17]. Many studies have been carried out since 1960s and it was found that *ginkgo biloba* extract could increase rat cerebral blood flow[14], improve the mice memory impaired by ischemia[1,3,20,30], and protect the cerebral function[16,27]. Moreover, GbE showed an anti-oxidation effect[9,29]. GbE can eliminate free oxygen radicals[16], reduce lipid peroxidation and facilitate the synthesis and release of epoprostenol[5]. GbE helps to alleviate the subcellular damages of cerebral ischemia[22] 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[12]. It was reported that GbE could diminish the coagulation of platelets to inhibit thrombosis by antagonizing the platelet activating factor[17], and GbE is capable of inhibiting the adhesion of monocytes and neutrophils to cultured cerebral microvascular endothelial cells[26]. Therefore, GbE might be useful as a preventive therapy or as a post-ischemic treatment to reduce the damaging effects of stroke[19].

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[13]. 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[18]. Aspirin also inhibits the formation of prostaglandin G2, a cyclic endoperoxide intermediary of prostaglandin biosynthesis in human platelets[11], and prostaglandin G2 production is stimulated by thrombin, moreover, prostaglandin G2 will cause platelet aggregation and the release reaction[24].

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 without side-effects to prevent and treat the disease is considered necessary. Although there were several research experiments involving either *ginkgo biloba* extract or aspirin, the investigation on Ginkgo and aspirin collectively has not been reported yet and this was the primary reason why our study was initiated. Our aim was to elucidate the combined effects of *ginkgo biloba* extracts, ginkgolide A, B and aspirin in the cell model of cerebral infarction through the evaluation of transcription levels for GAP43 and MAP2 related to neuronal regeneration and p53 and Bcl2 participated in apoptosis.

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 Jeil Biotech services Inc. (Seoul, Korea) and GIBCO (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). Maxime 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% CO_2 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 O_2 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 10^4 cells per well, and then cells were treated with various concentrations of ginkgolide A or B extracts or aspirin for 48 hours in 37°C CO_2 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'-TAAAGCTCATAAGGCCGCAA-3', GAP43 reward primer; 5'-ATCACCCTCCCCCTTCTTCT-3', MAP2 forward primer; 5'-ACTGGTTCATCGAAATGCCA-3', MAP2 reward primer; 5'-AGTGAAGCTTCCCCCTTGAA-3', Bcl2 forward primer; 5'-TCTCCCCGCGACTCCTGATT-3', Bcl2 reward primer; 5'-CAGCGTGCGCCATCCTTC-3', p53 forward primer; 5'-GCGGATTACTTGCCCTTACT-3', p53 reward primer; 5'-TGTCACCGTCGTGGAAA-3', GAPDH forward primer; 5'-CGGAGTCAACGGATTTGGTCGTAT-3', GAPDH reward primer; A5'-GCCTTCTCCATGGTGGTGAAGAC-3'.

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.

RESULTS

The change of cell viability according to several conditions of hypoxic and reperfusion time

When the treating conditions of hypoxia/reperfusion were 5 hours/5 hours and 5 hours/10 hours in each, the viable cell number was significantly decreased by the level of 82.13-84.38% of normal control. In addition, when the hypoxia and reperfusion time was prolonged for 8 hours and 12 hours, the viable cell number was remarkably decreased by 26.87% of the control group (Fig. 1). So, we proceeded with the study in the condition of 8 hours for hypoxia and 12 hours for reperfusion.

The change of cell numbers and cell morphology in several culture conditions

In the culture conditions of free glucose media without hypoxia and reperfusion condition and normal media with hypoxia and reperfusion, there were almost no differences in cell morphologies, however, the numbers of viable cells were lowered to 50 and 53.2% in each compared to normal control. Additionally, when the cells were incubated in free glucose media with hypoxia and reperfusion, the number of viable cells was decreased by 14.6% of normal control (Fig. 2).

The change of cell viability in the treatment of ginkgolide A, B, and aspirin of various concentrations

When neuroblastoma cells were treated with ginkgolide A in the concentration range of 0.05-1,000 μmole/mL, the cell viability increased in the concentration range of 0.05-100 μmole/mL and set the proper concentration as 1 μmole/mL. On the other hand, the viability was increased in whole concentration range of 0.1-1,000 μmole/mL when we treated ginkoglide B to cells and set the proper concentration as 0.5 μmole/mL. When the cells were treated by aspirin in the concentration range of 10-1000 μmole/mL, the viability was increased in the concentration range of 20-100 μmole/mL and set the proper treating concentration as 50 μmole/mL (Fig. 3).

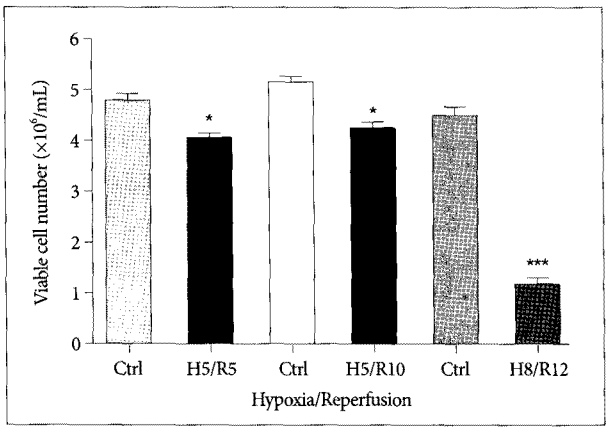

Fig.1. The change of viable cell number in various hypoxia/reperfusion conditions. The condition of hypoxia 8hours and reperfusion 12hours induced most remarkable the decrease of cell viability (*p<0.05, ***p<0.001).

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 differnce 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

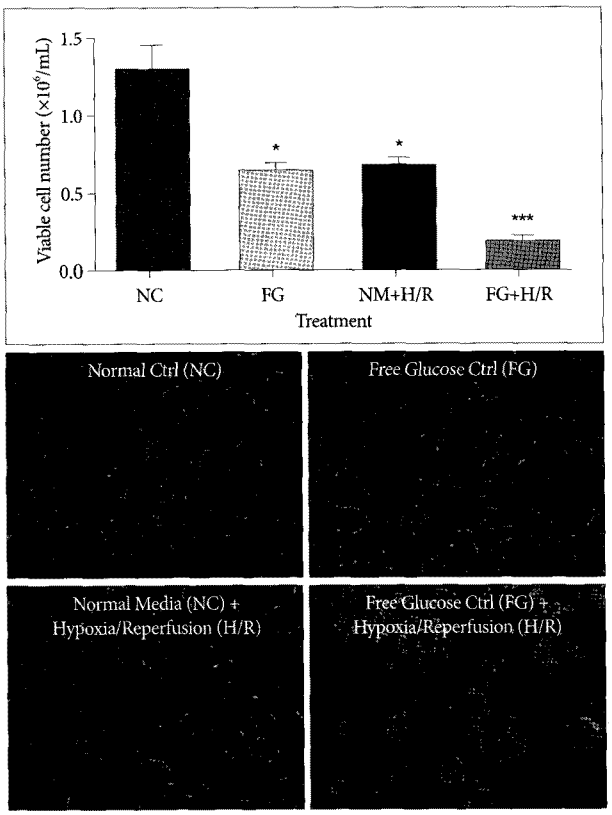

Fig. 2. The change of cell viability and shape in several culture conditions. Many dead cells are floated, and the number of viable cells are decreased by 14.6% of normal control level in the condition of free glucose and hypoxia 8 hours/reperfusion 12 hours. All images were viewed by light microscope (original magnification ×200) (*p<0.05; NC vs. FG, NC vs. NM+H/R, ***p<0.001; NC vs. FG+H/R, NM+H/R vs. FG+H/R).

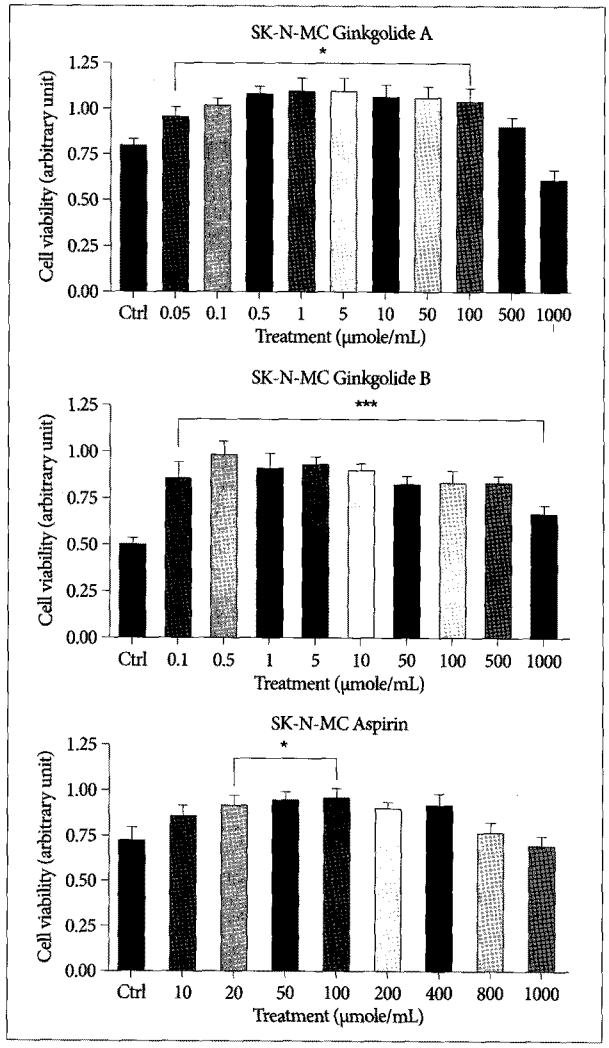

Fig. 3. The change of cell viability in the treatment of ginkgolide A, B, and aspirin of various concentrations. Ginkgolide A increased the cell viability in the range of 0.05-100 μmole/mL concentrations. Ginkgolide B elevated the cell viability in all treating concentrations. Aspirin increased the cell viability in the range of 20-100 μmole/mL concentrations (*p<0.05; Ctrl vs. ginkgolide A and aspirin treatments of various concentrations, ***p<0.001; Ctrl vs. ginkgolide B treatments in all concentrations).

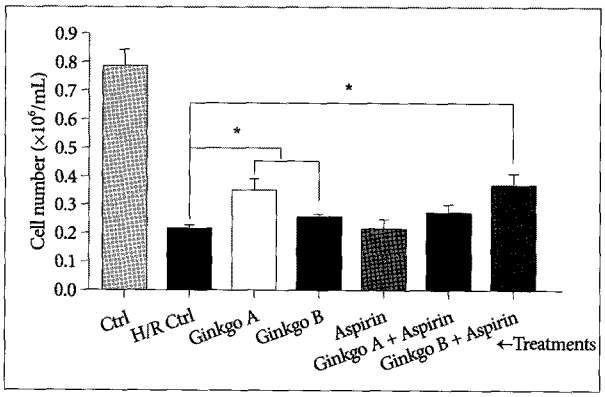

Fig. 4. The effects of ginkgolide A, B and aspirin on cell viability in the condition of hypoxia and reperfusion. Ginkgolide A and B increased the number of viable cells. The co-treatment of ginkgolide B and aspirin increased cell viability, however, there was no additive effect (*p<0.05).

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[6], and caspase activation and cytochrome c release may play roles in hypoxia-induced neuronal apoptosis[2].

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[29].

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[7], and its neuro-protective action is dependent on heme oxygenase 1 in ischemic reperfusion brain injury[19,31].

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[28].

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-

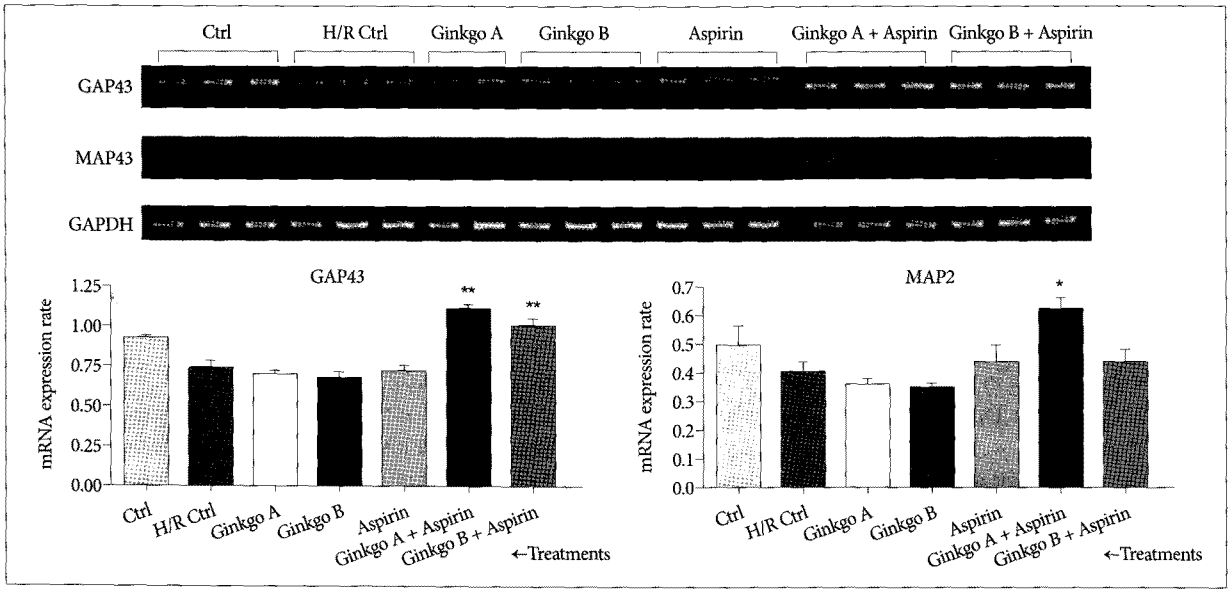

Fig. 5. The effects of ginkgolide A,B and aspirin on mRNA expression of GAP43 and MAP2 in the condition of hypoxia and reperfusion. The co-treatments of ginkgolide A or B and aspirin increased the mRNA expression of GAP43. The MAP2 mRNA level was elevated by the co-treatment of ginkgolide A and aspirin (*p<0.05; H/R Ctrl vs. ginkgolide A and aspirin, **p<0.01; H/R Ctrl vs. ginkgolide A or B).

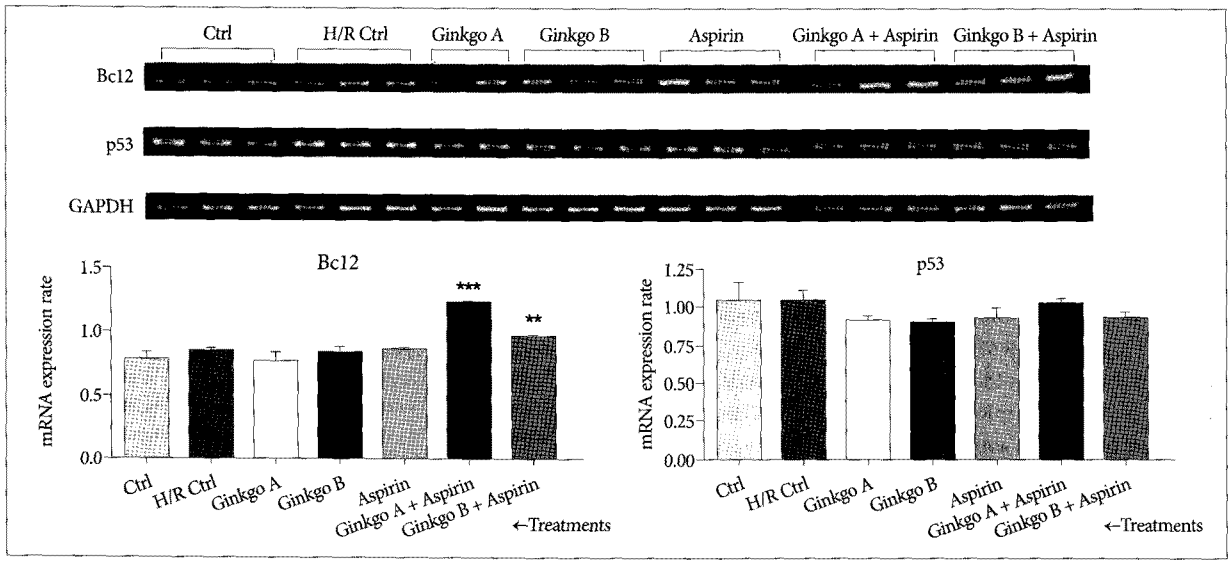

Fig. 6. The effects of ginkgolide A, B and aspirin on mRNA expression of Bcl2 and p53 in the condition of hypoxia and reperfusion. The co-treatments of ginkgolide A or B and aspirin increased the mRNA expression of Bcl2. The p53 mRNA level was not changed by individual treats of ginkgolide A, B and aspirin, and the co-treatment of ginkgolide A or B and aspirin (**p<0.01; H/R Ctrl vs. ginkgolide B and aspirin, ***p<0.001; H/R Ctrl vs. ginkgolide A and aspirin).

rons to ischemia and anoxic damage[17].

Some researches proved that after cerebral infarction, the cell death was involved in apoptosis which related with p53 and Bcl2 gene. Based on the research, expression of p53 gene was increased as result of cerebral infarction and Bcl2 gene played a role as antagonist on apoptosis[8]. But, there have not been any researches on the effect of GbE and aspirin on expression of p53 and Bcl2 gene.

In one study, aspirin (30 mg/kg) administration reduced the infarct volume significantly when administered immediately after middle cerebral artery occlusion (MCAO) compared with vehicle-treated rats[23].

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)[21].

Numerous studies have demonstrated the efficacy of aspirin in preventing the recurrence of ischemic stroke among the survivors of ischemic stroke[13]. 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 4weeks, compared with the effect of aspirin alone[10]. 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[4].

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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