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Inhibitory Effects of Ginseng Total Saponins on Hypoxia-induced Dysfunction and Injuries of Cultured Astrocytes

Yeon Hee Seong[1]* and Hack Seang Kim[2]
[1]College of Veterinary Medicine, [2]College of Pharmacy, Chungbuk National University, Cheongju, 360-763, Korea

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The effects of ginseng total saponins (GTS) on hypoxic damage of primary cultures of astrocytes were studied. Hypoxia was created by placing cultures in an air tight chamber that was flushed with 95% N_2/5% CO_2 for 15 min before being sealed. Cultures showed evidence of significant cell injury after 24 h of hypoxia (increased lactate dehydrogenase (LDH) content in the culture medium, cell swelling and decreased glutamate uptake and protein content). Addition of GTS (0.1, 0.3 mg/ml) to the cultures during the exposure to hypoxic conditions produced dose-dependent inhibition of the LDH efflux. GTS (0.1, 0.3 mg/ml) also produced significant inhibition of the increased cell volume of astrocytes measured by [^{3}H]O-methyl-D-glucose uptake under the hypoxic conditions. Decreased glutamate uptake and protein content was inhibited by GTS. These data suggest that GTS prevents astrocytic cell injury induced by severe hypoxia *in vitro.*

Key words : Astrocytes, Hypoxia, Cell injury, Ginseng total saponins (GTS)

INTRODUCTION

The brain needs a continuous supply of oxygen and glucose for its functional integrity. Interruption of the supply of these substances during various pathological conditions, such as ischemia and stroke, initiates a sequence of biochemical events that can lead to leakage of intracellular material, and structural as well as functional damage of brain cells (Baethmann, 1978; Nemoto, 1985; Rothman and Olney, 1986). The injury caused by ischemia, which includes severe hypoxia, substrate deprivation and failure to remove toxic metabolic products, is multifactorial (Ljunggren *et al.*, 1974). There is ultrastructural evidence that astrocytic swelling is an early and primary event following cerebral ischemia (Chiang *et al.*, 1968; Kimelberg and Ransom, 1986). Astrocytes are important in the control of extracellular water content and ion concentrations in mammalian brain (Hertz and Schousboe, 1975; Hertz, 1982; Kimelberg and Ransom, 1986). Astrocytes also contain receptors and high affinity uptake systems for various neurotransmitters, which provide normal physiological microenvironments for neurons to function properly. Dysfunction of astrocytes would lead to a sequence of pathological events such as loss of cellular volume control, an increase of brain tissue volume, both cellular and vasogenic edema, a rise in intracranial pressure, cerebral herniation, and finally, arrest of cerebral circulation (Hossmann, 1985).

Primary culture system of rat cerebral cortical astrocytes has been well established and used by investigators for various biochemical, physiological, and pharmacological studies of brain cells (Hertz and Schousboe, 1975; Schousboe *et al.*, 1980; Hertz, 1981; Kimelberg, 1983; Kimelberg and Ransom, 1986). Thus, cultured astrocytes were used as a *in vitro* model to examine the dysfunction of astrocytes under severe hypoxia. Some investigators have elucidated multifactorial causes involved in hypoxic injury of astrocytes in studies using cultured brain astrocytes (Yu *et al.*, 1989; Callahan *et al*, 1990; Gregory *et al.*, 1990; Tholey *et al.*, 1991; Juurlink and Hertz, 1993). They have demonstrated that severe hypoxic conditions produce increase of lactate dehydrogenase (LDH) content in the culture medium, cell swelling, and decrease of glutamate uptake and protein content. The studies of the protective effects of the several agents on hypoxic astrocytes demonstrated that some of them were beneficial. Verapamil, furosemide and indomethacin were effective in reduction of the release of LDH from hypoxic astrocytes (Yu *et al.*, 1989). High glucose and fructose-1,6-bisphosphate protected astrocytes from hypoxic damage (Gregory *et al.*, 1989; Callahan *et al.*, 1990)

Much attention has been paid to ginseng saponins,

Correspondence to: Yeon Hee Seong, College of Veterinary Medicine, Chungbuk National University, Cheongiju 360-763, Korea

main effective component of ginseng, because of their multiple pharmacological actions. They have central actions such as suppression of exploratory and spontaneous movements (Saito *et al.*, 1977), prolongation of hexobarbital sleeping time (Takagi *et al.*, 1972) and inhibition of reverse tolerance development of dependence-liable drugs (Tokuyama *et al.*, 1992). There are several reports of *in vitro* actions of some active components extracted from *Panax Ginseng* on cultured cell line. Ginsenosides Rb1 and Rd, saponins isolated from *Panax Ginseng,* were reported to potentiate the nerve growth factor-mediated neurite extension in organ cultures of chicken embryonic dorsal root ganglia and lumbar sympathetic ganglia (Takemoto *et al.*, 1984). A promotive effect of the lipophilic components of *Panax Ginseng* on the differentiation of rat pheochromocytoma (PC 12) cells, was also reported (Mohri *et al.*, 1991). We reported that ginseng total saponins (GTS), as an active component fraction of ginseng extract, prevented the cell swelling of cultured astrocytes induced by L-glutamate, excitotoxic amino acid (Seong *et al.*, 1995). The present study was performed to investigate the effect of GTS on hypoxic damage of cultured astrocytes.

MATERIALS AND METHODS

Materials

[^{3}H]O-Methyl-D-glucose ([^{3}H]OMG) and [^{3}H]glutamate were purchased from Amersham (Arlington Heights, IL, U.S.A.). Eagle's minimum essential medium (MEM) and fetal bovine serum were from Gibco (U.S.A.). Dibutyryl cyclic AMP (dBcAMP), phroletin and NADH were purchased from Sigma Chemical Co. (ST. Louis, MO, U.S.A.). GTS from *Panax Ginseng,* extracted and purified by the method of Namba *et al.* (1974), was supplied from Korea Ginseng and Tobacco Research Institute. All other chemicals used were of the highest grade available.

Cell cultures

Cultured rat astrocytes were prepared from cerebral cortices of 1-to 2-d old Sprague-Dawley rats by the method of Frangakis and Kimelberg (1984). Briefly, the dissociated cells were suspended in the culture medium (MEM with 10% fetal bovine serum) and plated in 12-well tissue culture plates (Corning) at a density of 10^4 cells/cm^2. The cells were grown in a 5% CO_2/95% air humidified atmosphere at 37°C and medium was changed twice a week. Cells were grown to be confluent for 2 weeks and then further differentiated by the addition of 0.25 mM dBcAMP. The treatment with dBcAMP caused morphological changes in the astrocytes, as indicated by the process formation. The cultures were used for experiments after they were 3 weeks old. At this stage, more than 90% of cells were positively stained with antiserum against glial fibrillary acidic protein, a marker of astrocytes.

In vitro model of hypoxia

The *in vitro* model of hypoxia was similar to that described by Yu *et al.* (1989). Briefly, culture plates were placed in a humidified modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA, U.S. A.) that was purged with 95% N_2/5% CO_2 for 15 min and sealed. The chamber was placed inside an incubator and maintained at 37°C. In the present experiments, cultures were incubated under severe hypoxia for 24 h. To study the effects of GTS on the hypoxic damage of astrocytes, stock solution of GTS was added directly to the culture medium before placing the culture into the modular incubator chamber. Control cultures were incubated for the same period without exposure to hypoxia.

Measurement of OMG uptake

Astrocytic swelling was quantitatively studied by measuring the intracellular water space by [^{3}H]OMG equilibrium uptake in normoxic or hypoxic astrocytes by the method of Kletzien *et al.* (1975). The technique takes advantage of the fact that non-metabolizable hexose, OMG, is transported into cells and reaches an intracellular concentration equal to the extracellular concentration. Phloretin is used to inhibit back diffusion of OMG during washing (Kletzien *et al.*, 1975). The method requires the determination of the amount of hexose taken up at equilibrium vs. the protein content of the culture. After the incubation period of 24 h, the culture medium was removed and the cells were rinsed with HEPES-buffered Krebs-Ringer solution (HBKR (in mM); NaCl 156, KCl 5.6, $NaHCO_3$ 11, D-glucose 10, $MgSO_4$ 1, $CaCl_2$ 1, NaH_2PO_4 1, HEPES-Na 20, pH 7.4). Following the rinsing, HBKR containing 1 mM [^{3}H]OMG (0.5 μCi/well) was added to the culture and the incubation was carried out at 37°C for 20 min. The uptake was terminated by aspiration of the medium and subsequent rinsing 3 times with ice-cold HBKR containing 0.1 mM phloretin. The cells were then digested by 0.1 N NaOH and the subsequent aliquot was taken for protein determination (Lowry *et al.*, 1951) and scintillation counting. The radioactivity of [^{3}H]OMG was converted to intracellular water space according to the calculation of Kletzien *et al.* (1975). The results were expressed as μl H_2O per mg protein.

Lactate dehydrogenase (LDH) measurement

LDH activity was measured spectrophotometrically

with pyruvate and NADH in a phosphate buffer according to the method of Bergmeyer and Bernt (1974). One unit of LDH activity is defined as the amount of enzyme that catalyzes the formation of 1 mol of NADH/min. LDH activity of the incubation media was determined immediately after collection. Total LDH activity of the cell and media was measured after treatment with 0.1% triton X-100.

Percent LDH efflux into the media (% LDH efflux) was calculated for each of the hypoxic experimental and the normoxic control conditions by the following formula:

$$\frac{\text{LDH activity of media}}{\text{total LDH activity (cells and media)}} \times 100 = \%\ \text{LDH efflux}$$

Glutamate uptake studies

Uptake of glutamate was determined by the method of Yu *et al.* (1984, 1986). To determine the effects of hypoxia on glutamate uptake, cultures were incubated under severe hypoxia, as described above, for 24 h in the modular incubator chamber. At the end of the hypoxia incubation period, 50 μM [^{3}H]glutamate (0.1 μCi/ml) was added directly to the culture. Cells were incubated for five minutes, short enough to insure initial uptake (Hertz *et al.*, 1978) and to minimize the loss of accumulated amino acid as carbon dioxide, a metabolic process that may be quite pronounced with glutamate (Yu *et al.*, 1982). After the incubation, cells were rapidly washed twice with ice-cold phosphate-buffered saline. Cells were then digested with 0.1 N NaOH and the subsequent aliquot was taken for protein determination (Lowry *et al.*, 1951) and scintillation counting. The uptake in 5 min was calculated from the radioactivity per milligram of protein and the specific activity in the incubation media.

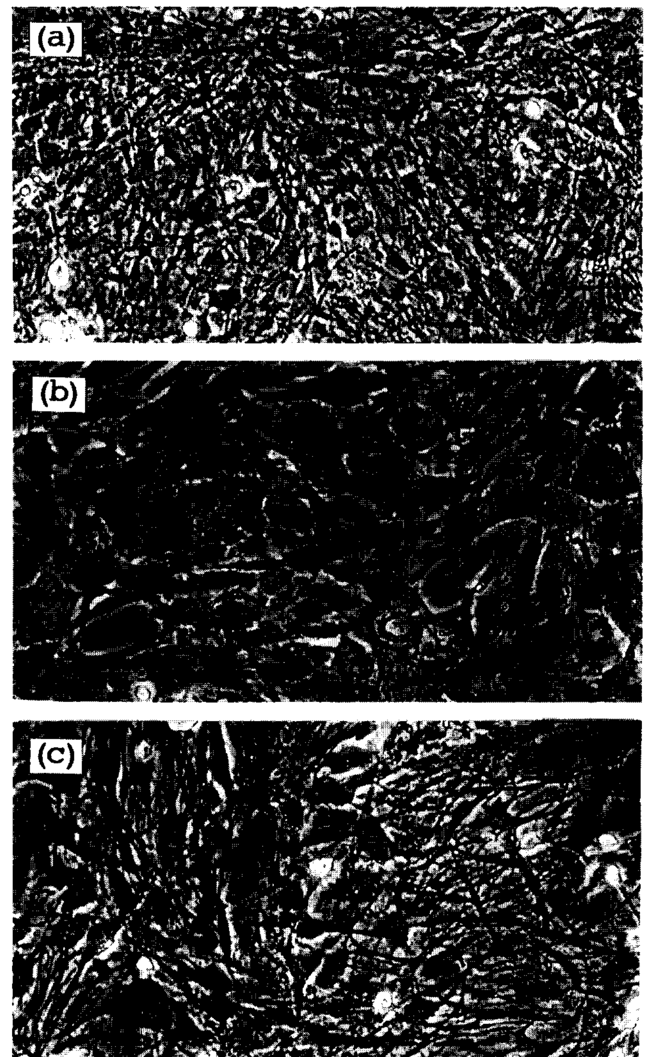

Fig. 1. Phase-contrast micrographs of cultures of cerebral cortical astrocytes. (A) Normal control culture without exposure to hypoxia; (B) culture after 24 h of hypoxia; (C) culture after 24 h of hypoxia in the presence of GTS (0.3 mg/ml). These cultures were over 25 days old and treated with dBcAMP after they were 2 weeks old.

RESULTS

Fig. 1 shows the morphological changes of the cells observed under a phase-contrast microscope. Fig. 1A shows a normal culture of astrocytes having many finely developed processes. Fig. 1B shows a similar culture after 24 h of severe hypoxia. Obvious structural dissociation was evident. Many cells were swollen, characterized by disappearance of astrocytic processes. Some cells were burst or detached from the culture plates. However, GTS (0.3 mg/ml), when added to the cultures exposed to 24 h of hypoxia, inhibited the cell damage (Fig. 1C). Many cells were still process-bearing.

The intracellular water space of astrocytes was measured under normoxia and hypoxia. Under normal conditions, the average intracellular water space was 9.03±0.46 μl/mg protein. As shown in Fig. 2, the intracellular cell volume almost doubled after the culture was exposed to hypoxia for 24 h. GTS (0.1, 0.3 mg/ml) reduced the increase of intracellular water space in hypoxic cultures. It was recovered to almost normal level (11.1±0.62 μl/mg protein) by 0.3 mg/ml of GTS. The protein contents of cultures under normoxia and hypoxia were also compared. The average protein content of cultures of astrocytes under normoxia was 0.128±0.003 mg/well. After 24 h of hypoxia, it decreased to 0.047±0.004 mg/well. GTS (0. 1, 0.3 mg/ml) significantly prevented the deprivation of protein in hypoxic cultures (Fig. 3).

Callahan *et al.* (1990) suggested that percent LDH efflux was shown to be an excellent correlate of cell

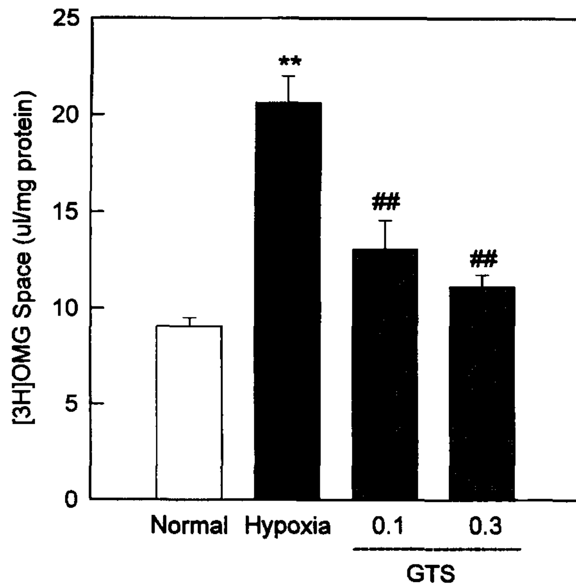

Fig. 2. Effect of GTS on hypoxia-induced increase in intracellular water space. Cultured astrocytes were subjected to 24 h of hypoxia with or without GTS (0.1, 0.3 mg/ml) in the culture medium. Values are means±S.E.M. of 3-4 different cultures. **p<0.01; compared to normal cells, ##p<0.01; compared to hypoxic control cells.

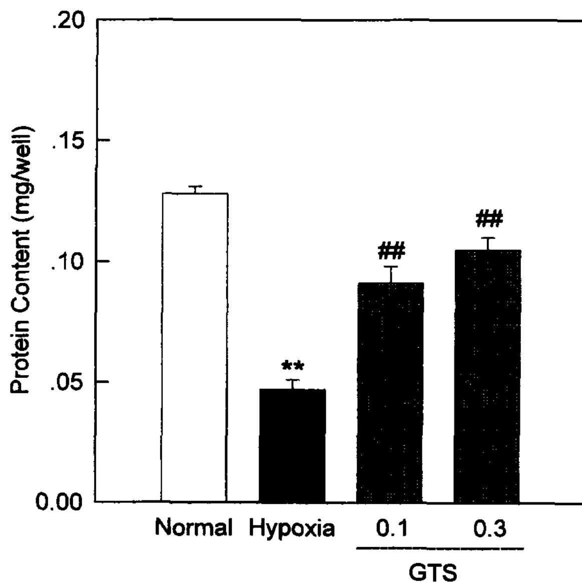

Fig. 3. Effect of GTS on hypoxia-induced decrease in protein content of each well. Cultured astrocytes were subjected to 24 h of hypoxia with or without GTS (0.1, 0.3 mg/ml) in the culture medium. Values are means±S.E.M. of different cultures. **p<0.01; compared to normal cells, ##p<0.01; compared to hypoxic control cells.

death, determined either morphologically or by measurement of total protein derived from cells remaining on the surface of the flasks. Koh and Choi (1987) similarly have demonstrated the value of LDH efflux in the quantitation of cortical neuronal injury in cell cul-

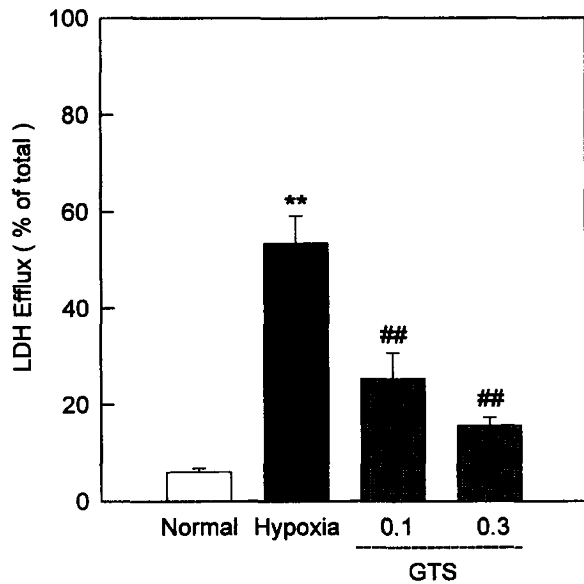

Fig. 4. Effect of GTS on hypoxia-induced LDH efflux. Cultured astrocytes were subjected to 24 h of hypoxia with or without GTS (0.1, 0.3 mg/ml) in the culture medium. Values are means±S.E.M. of 3-5 different cultures. **p<0.01; compared to normal cells, ##p<0.01; compared to hypoxic control cells.

Table 1. Uptake of [^{3}H]glutamate in normal cultures of astrocytes and cultures exposed to 24 h of severe hypoxia with or without GTS

	Glutamate uptake
	nmol/mg protein
normal control	44.23±2.7
hypoxic control	7.77±0.7**
+ GTS (0.3 mg/ml)	42.56±3.2##

Results are means±S.E.M. of 3-4 different cultures. ** p<0.01; compared to normal cells, ##p<0.01; compared to hypoxic control cells.

ture. Fig. 4 shows the increase of LDH content in the culture medium of hypoxic condition. The percent LDH efflux of the normoxic control cultures for 24 h was 6.08±0.67%. In the culture medium exposed to 24 h of severe hypoxia, LDH efflux was increased to 53.5±5.7%. We next evaluated the protective effect of GTS on the LDH efflux. The increased LDH efflux was decreased to 15.6±1.7% by GTS (0.3 mg/ml).

The average 5 min uptake of glutamate in this astrocytic preparation was 44.23±2.69 nmol/mg protein. Glutamate uptake was reduced by about 80% after 24 h of hypoxia. The decrease of glutamate uptake was significantly inhibited by GTS (0.3 mg/ml) (Table 1).

DISCUSSION

It was reported that many astrocytes were swollen,

and others had either burst or detached from the culture dish by over 18 h of hypoxia, and cells in the culture almost disintegrated into debris by 24 h of severe hypoxia (Yu *et al.*, 1989). In agreement with this report, the present experiments clearly demonstrated that cerebral cortical astrocytes in primary culture were significantly injured by 24 h of severe hypoxia. The uptake of glutamate decreased and LDH release from the cell increased after 24 h of severe hypoxia. The dysfunction was further evidenced by the cell swelling and the loss of protein in the hypoxic culture. This hypoxia-induced astrocytic injury was partly prevented by containing GTS in the medium. GTS inhibited the increase of intracellular cell volume and LDH release, and the decrease of protein content and glutamate uptake. The mechanism underlying the beneficial effect of GTS are not clear at present.

Inhibition of glutamate uptake indicates derangement of one of the important biochemical functions of astrocytes (Hertz, 1982). The inhibition may be caused by an energy failure of hypoxic astrocytes (Rothman and Olney, 1986), or by disruption of the integrity of the cell membrane, which is indicated by the leakage of the intracellular LDH into the extracellular medium. The defect of glutamate uptake in astrocytes would lead to an accumulation of this excitotoxic amino acid in the extracellular space and subsequent receptor-mediated neuronal cell death (Olney, 1983; Meldrum, 1985; Rothman and Olney, 1986). GTS inhibited the decrease of glutamate uptake by an unknown mechanism, showing a protective effect on the cell death. In addition, high concentrations of extracellular glutamate can induce depolarization of astrocytes (Bowman and Kimelberg, 1984) that would open ion channels (Kimelberg and Ransom, 1986) and allow the entry of sodium and water into the cells. This would also result in astrocytic swelling which may lead cell lysis.

The mechanisms for hypoxia-induced astrocytic injuries are not clear at present. There are a number of factors known to play a role. It has been shown that the increase in intracellular calcium and chloride following the increase of sodium influx with various insults is important for cell swelling and cell death (Kimelberg and Ransom, 1986; Rothman and Olney, 1986). In the present experiments, GTS significantly reduced the increase of intracellular water space and LDH efflux into the culture medium in hypoxic cultures. And, in previous report, we demonstrated that GTS reduced glutamate-induced astrocytic swelling (Seong *et al.*, 1995). Together with this report, it is likely that GTS reduced Na^+ influx triggered by extracellularly accumulated glutamate during the severe hypoxic conditions, resulting in the blockade of water influx. Since it is not known whether GTS contains active substances which specifically interact with Na^+ channels, the action of GTS on hypoxic astrocytic swelling and glutamate-induced astrocytic swelling may be due to a membrane stabilizing effect. Callahan *et al.* (1990) observed that high glucose prevented hypoxic injury of astrocytes. Thus, the protective effect of GTS might be due to the beneficial effect of glucose derived from each ginsenoside which contains more than one molecule of glucose in each structure.

In previous reports, we indicated the central actions of GTS administered systemically (100-200 mg/kg) in mice. GTS injected intraperitoneally showed an antagonism of opioid agonists-induced antinociception (Kim *et al.*, 1992a; Kim *et al.*, 1992b). Also, GTS blocked the development of cocaine- and methamphetamine-induced reverse tolerance and dopamine receptor supersensitivity in mice (Kim *et al.*, 1995a, Kim *et al.*, 1995b). These findings suggest that active components of GTS can pass through the blood-brain barrier and thus interact with brain cells. In support of the present results, Liu *et al.* (1992) have reported that ginsenosides could significantly increase the survival rate in mice exposed to acute hypoxia.

In conclusion, the present results indicate that GTS have a protective effect on hypoxic cell damage and dysfunction of astrocytes *in vitro*. To assess the pharmacological aspect of this action of GTS, it is necessary to determine the active components of GTS.

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