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Neuroprotective effect of a ginseng (*Panax ginseng*) root extract on astrocytes primary culture

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Abstract

A standardized aqueous extract of *Panax ginseng radix* was tested for its antioxidant effect on primary astrocytes culture on an oxidant stress model generated by H_2O_2. The results indicated that this extract had a significant effect on the reduction of astrocytic death induced by H_2O_2. Dose–response experiments revealed that this ginseng extract increased cell viability at a wide range of concentrations. Therefore, we investigated the effects of this extract on antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidases (GPx) and glutathione reductase (GR), on glutathione content (reduced and oxidized forms and red/ox index) and on the intracellular reactive oxygen species (ROS) formation. Exposure of astrocytes to H_2O_2 decreased the activities of antioxidant enzymes, and increased ROS formation. Ginseng root extract reversed the effect of almost all of these parameters in H_2O_2-injured primary cultures of rat astrocytes.

Keywords: Ginseng; Ginsenosides; Astrocytes; Primary culture; Hydrogen peroxide; Antioxidants

1. Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer (*Araliaceae*), is a traditional medicine in Korea, China and Japan that has been shown to produce a variety of medicinal effects on both the nervous system and the non-nervous system. This crude drug has been empirically used as a psychic energizer and a general tonic in traditional medicine to increase vitality, health and longevity, especially in older persons, and for its cancer-preventing potential (Wang and Joseph, 1999; Shin et al., 2000). Much interest has been focused on the effects of ginseng as an adaptogen, a substance which helps the body to resist the adverse influences of harmful factors and improves the restoration of homeostasis irrespective of the direction of the altered physiological function. Some ginseng's active compounds exert beneficial effects on aging, central nervous system disorders (CNS) and neurodegeneration (Lian et al., 2005; Radad et al., 2006). The pharmacological effects of ginseng have been demonstrated in the CNS and in the cardiovascular, endocrine and immune systems (Tang and Eisenbrand, 1992; Attele et al., 1999; Shah et al., 2005; Wang et al., 2006). Ginseng appears to act mainly on the hypothalamus and has a sparing action on the adrenal cortex, mediated through anterior pituitary and ACTH release. In addition, ginseng and its constituents have been thought to possess antineoplastic, antistress and antioxidant effects (Seong et al., 1995; Kitts et al., 2000).

Evidence pointing out the medicinal efficacy of ginseng has been closely linked to its protective properties against free radicals (Facino et al., 1999; Bastianetto and Quirion, 2002; Kim et al., 2002). Recent results suggest that *Panax ginseng* extracts may protect neuronal cells from oxidative injury (Lee et al., 2004). However, the antioxidant capabilities of ginseng root on astrocytic cultures have not been established in literature.

The present research has tested whether ginseng root extract could protect astrocytes from oxidant stress generated by H_2O_2 because of the critical role of those cells in the neuron survival. The neuroprotective activities of astrocytes include expression of antioxidant enzymes, transport and metabolism of glucose that yields reducing equivalents for antioxidant regeneration and lactate for neuronal metabolism, synthesis of glutathione and recycling of vitamin C (Wilson, 1997). We have utilized primary astrocytes culture as a convenient way to identify the astroprotective compounds.

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2. Materials and methods

2.1. Animals

Newborn Wistar rats (0–2 days old) were obtained from Charles River Inc. (Wilmington, MA). The procedures followed the guidelines recommended by the European Community for the Care and Use of Laboratory Animals (Council Directive 86/609/EEC).

2.2. Plant material

Lyophilized aqueous ginseng root extract, normalized in total ginsenosides (9.5%, expressed in ginsenoside Rg_1 valued by Real Farmacopea Española (2005) HPLC method, was purchased from Burgundy Botanical Extracts (BUH-0630020), Reyssouze, France.

2.3. Reagents

Basal medium Eagle (BME) with Glutamax-I, antibiotics penicillin–streptomycin (10,000 IU/ml penicillin and 10,000 UG/ml streptomycin), foetal bovine serum (FBS) and Dulbecco's Phosphate buffered saline (PBS) were obtained from GibcoBRL Life Technologies (Barcelona, Spain). D-(+)-Glucose, dimethyl sulphoxide (DMSO), ethylenediaminetetraacetic acid disodium salt 2-hydrate and sodium piruvate were obtained from Panreac (Barcelona, Spain). 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT), hydrogen peroxide (H_2O_2), pirogallol, 2′,7′-dichlorofluorescein diacetate, β-nicotinamide adenine dinucleotide reduced form (NADH), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), glutathione reduced form (GSH), glutathione oxidized form (GSSG), *o*-phtaldialdehyde (OPT) and sodium azide were purchased from Sigma Chemicals Co. (Madrid, Spain).

Ginseng extract stock solution (10 mg/ml) was prepared in PBS and diluted in 1% heat-inactivated foetal bovine serum-BME medium (1% BME).

2.4. Preparation of glial cultures

Astrocytes were cultured from newborn Wistar rats. Rats were decapitated and the meninges removed. Cortex, striatum, and ventral mesencephalon were isolated and minced. A single-cell suspension was obtained by mechanical dissociation. The cells were further passed sequentially through 135 μm and then through 20 μm pore size sterile nylon meshes. The mixture was diluted to one hemisphere per 75 cm^2 culture flask (Sarstedt, USA). After seeding, the cells were incubated in a humidified incubator under 5% CO_2/95% air at 37 °C. The culture medium was composed of Basal medium Eagle containing Glutamax-I and supplemented with 10% heat-inactivated foetal bovine serum, glucose (33 mM), penicillin (20 UI/ml) and streptomycin (20 μg/ml). The medium was changed twice weekly. Astrocytes were harvested after 10–12 days of incubation, when the cells became confluent. Microglia, oligodendrocytes and other non-adherent cells were removed by shaking at 260 rpm overnight at 37 °C in an orbital shaker (Genesys Instrumentacion, Spain). Prior to the biochemical assays, astrocytes were subcultured onto 24-multiwell, 96-multiwell plates or Petri dishes for 24 h at different cell-plating densities per well. The cultures contained over 95% astrocytes.

2.5. Ginseng extracts activity

Cells were plated in at an appropriate density according to each experimental scale and treated during different periods (24 or 48 h) with a 1% BME medium containing ginseng extract. Ginseng doses were 0.0001, 0.001, 0.01, 0.05, 0.1, 0.5 and 1 mg of extract/ml of the culture medium. Cell injury and cell viability were determined using the LDH and MTT assays. Survival of vehicle-treated control groups (not exposed to ginseng extract) was defined as 100% surviving, and the number of dead cells in the treated groups was expressed as a percentage of control groups.

2.6. Assessment of cell injury and cell viability

The cytotoxicity of ginseng extract on astrocytes was determined by the release of lactate dehydrogenase (LDH) into the incubation medium. LDH activity was measured through the reduction of pyruvate to lactate with simultaneous oxidation of reduced nicotinamide adenine dinucleotide (NADH) at 340 nm wavelength. The rate of decrease in absorbance due to the formation of nicotinamide adenine dinucleotide NAD is directly proportional to the LDH activity in the sample. Here, astrocytes were seeded in 24-multiwell plates at a density of $1.6 \pm 0.2 \times 10^5$ cells/well. Ginseng extract was added (0.0001, 0.001, 0.01, 0.05, 0.1, 0.5 and 1 mg/ml) to the wells and incubated 6, 24 or 48 h. The supernatant were collected for LDH measurement in the cell-free medium. The cells remaining in each well were lysed in 0.5 ml lysis buffer (0.5% Triton X-100 in PBS). The release of intracellular LDH to the extracellular medium was measured at 340 nm wavelength using a Digiscan 340 microplate reader (Assys Hitech GmbH, Austria) and expressed as a percentage of total enzyme activity.

The MTT assay is used as an index of cell survival (Mosmann, 1983). 3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) is an indicator of the mitochondrial activity of living cells. MTT is reduced to a coloured compound (formazan) by mitochondria. Astrocytes were seeded in 24-multiwell plates at a density of $33 \pm 0.25 \times 10^4$ cells/well. Ginseng extract was added to the wells (0.0001, 0.001, 0.01, 0.05, 0.1, 0.5 and 1 mg/ml) and incubated 6, 24 or 48 h. At the indicated time after the treatment, the free-cell medium was removed and an MTT-1% BME solution (4 mg/ml final concentration) was added. After 1 h of 37 °C incubation, MTT solution was removed and formazan was dissolved in DMSO. MTT reduction in living cells was quantified at 550 nm wavelength using a Digiscan 340 microplate reader (Assys Hitech GmbH, Austria).

2.7. Hydrogen peroxide-induced toxicity

Cells were treated, after 80% confluence, for 30 min with PBS containing H_2O_2 (0.5 mM). Astrocytes were exposed to H_2O_2 0.5 mM for 6 or 24 h. The effect of varying the order in which the extract and the stressing agent was added was also investigated by carrying out the assays with the extract added first followed by the H_2O_2 0.5 mM and the assays in which this order was reversed.

2.8. Evaluation of antioxidant capacity

The evaluation of the *in vitro* antioxidant activity of ginseng extract was performed by measuring the intracellular reactive oxygen species (ROS) formation, the enzymatic activity of catalase, superoxide dismutase (SOD), glutathione peroxidases (GPx) and glutathione reductase (GR), and glutathione content (reduced and oxidized forms and red/ox index)

2.8.1. Measurement of intracellular reactive oxygen species (ROS) formation

Intracellular oxidant stress was monitored by 2′,7′-dichlorofluorescein diacetate (DCFH/DA) with a modification of the method previously described (Wang and Joseph, 1999). Here, cells seeded onto 96-multiwell plates (Sarstedt, USA) were incubated with DCFH/DA-PBS (final concentration 10 μM) for 30 min. At the end of the incubation, DCFH/DA-PBS solution was removed and different ginseng extract doses were added. H_2O_2 0.5 mM was used as a free radical generator in this cellular model. The fluorescence intensity (relative fluorescence units) was measured at 485 nm excitation and 530 nm emission wavelength in a FLX800TBI Microplate Fluororescence Reader (Bio-Tek Instruments Inc., Vermont, USA). Data points were taken every hour for 6 h.

2.8.2. Determination of antioxidant enzyme activity

To determine the antioxidant capacity of ginseng extract, dishes (100 × 20) were prepared containing 4×10^6 cells/ml of cells. Cells were treated, for 30 min with PBS containing H_2O_2 (0.5 mM). Following this treatment period, cells were incubated with a 1% heat-inactivated foetal bovine serum-BME medium for 24 h. The same protocol was used before the treatment with the test drug to assay its protective effect—cells were pre-treated with ginseng root extract (0.05, 0.01, 0.5 or 1 mg/ml) according to the viability results. Following these treatment periods, astrocytes were exposed to H_2O_2 0.5 mM for 24 h. The effect of varying the order in which the extract and the stressing agent was added was also investigated by carrying out the assays with the extract added first followed by the H_2O_2 0.5 mM and the assays in which this order was reversed.

For enzyme assays, the cells were suspended for 20 min in lysis buffer (25 mM of Tris, 150 mM of sodium chloride, 1 mM of EDTA and Triton X-100 0.1% at pH 7.4) and were ruptured.

The catalase activity was determined according to the method of Aebi (1984) based on H_2O_2 decomposition. A total of one unit of catalase decomposed 1 μmol of H_2O_2/mg of protein in 1 min at pH 7.4. The superoxide dismutase (SOD) activity was determined according to the method of Marklund and Marklund (1974) by measuring the enzymatic inhibition of pirogallol autoxidation. A total of one unit of SOD is defined as the amount of enzyme that inhibits the rate of pirogallol autoxidation in 50% per gram of protein. Glutathione peroxidases (GPx) activity was determined according to the method of Paglia and Valentine (1967) by qualifying the rate of oxidation of GSH to GSSG by cumene hydroperoxide – a reaction catalyzed by total Gpx, and the rate of oxidation of GSH to GSSG by hydrogen peroxide – a reaction catalyzed by Se-GPx. Glutathione reductase (GR) activity, based on the reduction of glutathione disulfide (GSSG) by GR and NADPH, was measured according to the method of Barja et al. (1990). Absorbance was measured in an Uvikon 930 spectrophotometer (Kontron).

2.8.3. Measurement of glutathione content

Intracellular glutathione content was monitored by *o*-phtaldialdehyde (OPT) with a modification of the method previously described (Hissin and Hilf, 1976) and adapted to 96-multiwell plates, with *o*-phtaldialdehyde (OPT) as fluorescent marker. Petri dishes (100 × 20) were prepared containing 4×10^6 cells/ml of cells. Cells were treated with ginseng root extract (0.05, 0.01, 0.5 or 1 mg/ml) according to the viability results. To assay the protective properties, following these treatment periods, astrocytes were exposed to H_2O_2 0.5 mM for 24 h. Cells were collected and lysed ($HClO_4$ 60% in phosphate buffer 0.1 M-EDTA 5 mM) and supernatants incubated 15 min with OPT (pH 8 for GSH and pH 12 for GSSG). The fluorescence was measured at 350 nm excitation and 420 nm emission wavelength in a FLX800TBI Microplate Fluororescence Reader (Bio-Tek Instruments Inc., Vermont, USA).

2.8.4. Protein assay

The protein content was measured using bicinchoninic acid (BCA) with bovine serum albumin as a standard.

2.9. Statistical analysis

Data were expressed as Means ± S.D. values. The one-way ANOVA followed by a Newman Keuls' multiple comparison test was used to compare control and treated groups with p values <0.05 being considered statistically significant.

3. Results

3.1. Effect of ginseng extract on primary culture of glial cells

The effect of ginseng aqueous extract on glial cells was examined after 6, 24 and 48 h of treatment with ginseng root aqueous extract. These effects were time and dose-dependent. No significant changes were observed on viability of cells treated with ginseng extract; this was determined by using the LDH release assay at 6, 24 and 48 h incubation time although 1 mg/ml ginseng extract weakly decreased the LDH release (Fig. 1).

Using the MTT reduction assay, cellular viability was increased in cells exposed to ginseng extract at a concentration

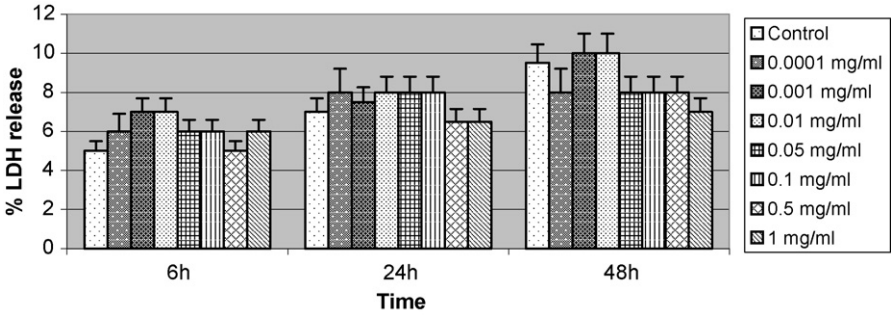

Fig. 1. Cytotoxicity of ginseng root aqueous extract (1–0.0001 mg/ml) on isolated rat astrocytes primary culture for 6, 24 and 48 h of incubation (%LDH released). Differences are significant at $*p < 0.05$ against vehicle-treated control (each value represents mean ± S.E.).

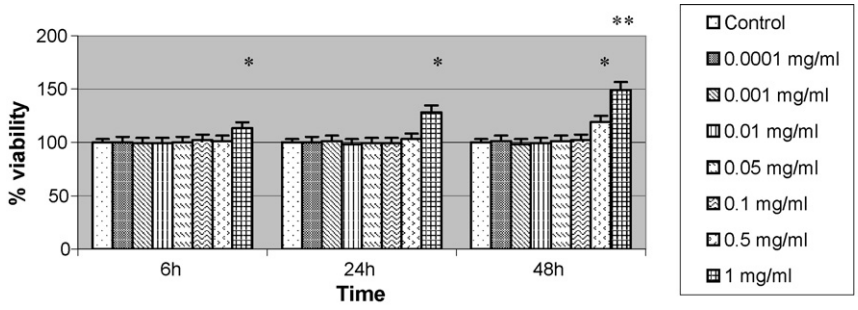

Fig. 2. Sensitivities of astrocytes to ginseng root aqueous extract (1–0.0001 mg/ml) for 6, 24 or 48 h. Results are expressed as %viability of vehicle-treated control (MTT assay). Data are mean ± S.E. ($*p < 0.05$; $**p < 0.01$).

of 1 mg/ml at 6 h and 24 h treatment (12 and 30%, respectively) and 1 and 0.5 mg/ml at 48 h incubation time (50 and 20%, respectively) (Fig. 2).

The concentration of H_2O_2 selected to test the astroprotective effect of ginseng extract and isolated ginsenosides was 0.5 mM.

3.2. Ginseng extract protective activity against H_2O_2-induced cytotoxicity

Astrocytes were pretreated with the tested ginseng root extract doses 24 or 48 h before their exposure to 0.5 mM H_2O_2 for 6 or 24 h. A ginseng protective effect was observed by LDH release assay on cells treated with ginseng extract at concentration of 1 mg/ml at 24 h pretreatment before 6 h H_2O_2 incubation, and at concentrations of 1 and 0.5 mg/ml before 24 h H_2O_2 incubation time; 48 h pretreatment showed a decrease of LDH release at 1 mg/ml concentration before 6 h H_2O_2 incubation and at 1 and 0.5 mg/ml concentration before 24 h H_2O_2 incubation (Table 1).

MTT assays showed a ginseng-protective effect on cells treated with ginseng extract at concentration of 1 mg/ml at 24 h pretreatment before 6 h H_2O_2 incubation time and at concentration of 1 mg/ml at before 24 h H_2O_2 incubation times; 48 h pretreatment showed protection at 1 mg/ml concentration before 6 h H_2O_2 incubation and at 1, 0.5 and 0.1 mg/ml concentration before 24 h H_2O_2 incubation (Table 2).

Table 1
Protective effect on astrocytes of ginseng extract pretreatment (24 and 48 h) on cytotoxicity of 6 or 24 h H_2O_2 (0.5 mM) exposure (%LDH released of cells exposed to Triton X-100)

	24 h treatment		48 h treatment	
	6 h H_2O_2	24 h H_2O_2	6 h H_2O_2	24 h H_2O_2
Control	38.8 ± 4.2	55.7 ± 6.1	44.3 ± 4.5	59.2 ± 6.4
Ginseng (mg/ml)				
0.0001	44.4 ± 4.7	54.0 ± 5.6	46.5 ± 4.7	52.1 ± 5.4
0.001	43.3 ± 4.5	56.9 ± 5.9	44.4 ± 4.6	55.0 ± 5.5
0.01	44.5 ± 3.9	56.0 ± 4.9	45.4 ± 4.5	54.0 ± 5.6
0.05	45.4 ± 4.1	54.0 ± 5.1	48.7 ± 4.9	52.1 ± 5.3
0.1	45.4 ± 4.6	45.3 ± 5.3	46.5 ± 4.6	51.1 ± 5.1
0.5	41.2 ± 4.2	38.6 ± 3.9*	39.0 ± 4.0	42.4 ± 4.6*
1	29.3 ± 3.2*	22.9 ± 2.7*	19.5 ± 2.2*	20.4 ± 2.8*

Differences are significant at $*p < 0.05$ values against control H_2O_2 0.5 mM (each value represents mean ± S.D.).

Table 2
Protective effect on astrocytes of ginseng extract (24 and 48 h) pretreatment at different concentrations on cytotoxicity of 6 or 24 h H_2O_2 (0.5 mM) exposure (MTT assay)

	24 h treatment		48 h treatment	
	6 h H_2O_2	24 h H_2O_2	6 h H_2O_2	24 h H_2O_2
Control (H_2O_2)	77.1 ± 7.8	59.0 ± 6.3	75.2 ± 7.9	56.3 ± 6.1
Ginseng (mg/ml)				
0.0001	71.6 ± 7.4	57.6 ± 5.9	67.6 ± 6.9	56.4 ± 5.7
0.001	71.8 ± 7.2	58.8 ± 6.0	67.1 ± 6.6	57.6 ± 5.8
0.01	72.0 ± 7.5	59.4 ± 6.1	68.2 ± 6.7	61.8 ± 5.9
0.05	74.0 ± 7.6	60.6 ± 6.3	70.6 ± 7.1	65.1 ± 6.4
0.1	71.6 ± 7.3	61.2 ± 6.2	71.2 ± 7.2	94.5 ± 8.1*
0.5	71.8 ± 7.4	72.6 ± 7.6	73.8 ± 7.5	98.2 ± 9.2*
1	73.2 ± 7.2	76.8 ± 7.7*	89.4 ± 9.4	100.6 ± 9.8*

Results are expressed as %viability of vehicle-treated control. Differences are significant at $*p < 0.05$ value against Control H_2O_2 0.5 mM (data are mean ± S.D.).

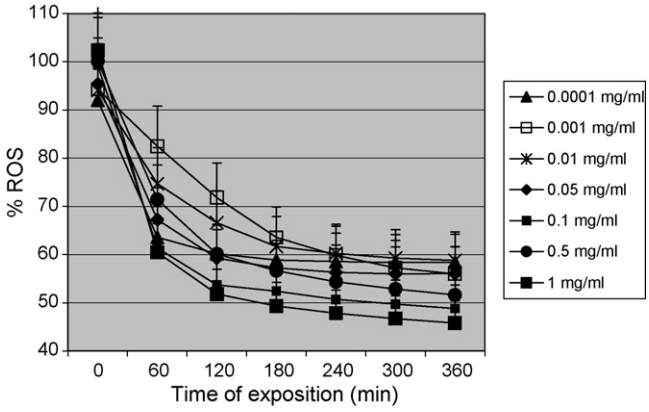

Fig. 3. Protective effects on astrocytes of ginseng extract co-treatment on ROS production of H_2O_2 (0.5 mM) exposure. Results are expressed as percentage of ROS production of cells exposed to H_2O_2 (mean ± S.D.). ($p < 0.001$).

3.3. Effect on intracellular reactive oxygen species (ROS) formation

For the study of intracellular ROS formation preventive effect, cells were co-incubated with ginseng extract doses and H_2O_2 0.5 mM. The assay was performed for 6 h and the DCF fluorescence formation was determinated by taking data every hour. The maximum ROS production of H_2O_2 was at 60 min. The addition of ginseng extract reduced the percentage of ROS formation starting at the first hour of incubation for all the doses in a dose-dependent manner, because, the main inhibition of ROS production was observed in highest concentration (1 mg/ml) with an important reduction (54.2%) in fluorescence at 6 h exposition time (Fig. 3).

3.4. Determination of antioxidant enzyme activity

Exposure of astrocytes to H_2O_2 decreased the activities of all the antioxidant enzymes measured. The enzymatic activities were determined in astrocytes before the treatment with the test drug to assay its protective effect: cells were treated for 24 h with ginseng root extract (0.05, 0.01, 0.1 or 1 mg/ml) after or before the exposure to H_2O_2 0.5 mM for 24 h. None of the treatments after H_2O_2 exposure showed differences with H_2O_2 treated cells. However, ginseng pretreatment showed protection on antioxidant enzymes activities (Table 3).

The ginseng pretreatment showed an enhancement on catalase activity at ginseng 0.5 mg/ml dose.

The superoxide dismutase (SOD) activity was improved after ginseng 0.5 mg/ml pretreatment.

Glutathione peroxidases (GPx) activity showed slight differences. Total GPx activity was improved with ginseng 0.5 and 1 mg/ml doses and Se-GPx was improved with 0.5 and 1 mg/ml pretreatment.

Glutathione reductase (GR) activity was improved after ginseng 1 mg/ml pretreatment.

3.5. Determination of intracellular glutathione content

The GSH and GSSG content and red/ox balance were determined in astrocytes before the treatment with the test drug to assay its protective effect: cells were treated for 24 h with ginseng root extract (0.05, 0.01, 0.1 or 1 mg/ml) before or after the exposure to H_2O_2 0.5 mM for 24 h.

Glutathione content in astrocytes with the ginseng treatment: GSH content did not show significant statistical differences but GSSG content was improved with 0.5 and 1 mg/ml pretreatment. That results yield differences in Red/ox balance in treatment with ginseng extract 0.5 and 1 mg/ml (Table 4).

Table 3
Effects on astrocytes enzymatic activity of 24 h H_2O_2 (0.5 mM) exposure after 24 h ginseng pretreatment at different concentrations

	Catalase (μmol H_2O_2/min mg prot)	SOD (U/min mg prot)	Total Gpx (nmol NADPH/min mg prot)	Gpx-Se (nmol NADPH/min mg prot)	GR (nmol NADPH/min mg prot)
Vehicle-treated control	40.5 ± 3.3	3.5 ± 0.28	15.7 ± 1.08	8.5 ± 0.75	21.9 ± 1.94
Control	31.7 ± 3.3	2.7 ± 0.27	9.0 ± 0.89	4.2 ± 0.39	16.6 ± 1.09
Ginseng (mg/ml)					
0.05	34.6 ± 2.1	2.9 ± 0.27	10.3 ± 0.97	4.5 ± 0.47	17.8 ± 1.15
0.1	34.2 ± 2.5	3.2 ± 0.3	9.2 ± 0.76	4.5 ± 0.46	16.5 ± 1.28
0.5	38.5 ± 3.3*	3.3 ± 0.3*	11.4 ± 1.17*	5.7 ± 0.71*	18.9 ± 1.37
1	37.7 ± 2.8	3.2 ± 0.3	12.8 ± 1.24*	5.8 ± 0.64*	19.7 ± 1.84*

Differences were considered significant at $*p < 0.05$ values against control H_2O_2 0.5 mM (each value represents mean ± S.D.).

Table 4
Effects on astrocytes glutathione content and red/ox index of 24 h ginseng treatment at different concentrations

	GSH (nmol/mg prot)	GSSG (nmol/mg prot)	Red/ox index (GSSG/GSH + GSSG)
Vehicle-treated control	55.5 ± 4.1	49.9 ± 1.8	0.47 ± 0.023
Ginseng (mg/ml)			
0.05	52.8 ± 3.7	49.1 ± 6.5	0.48 ± 0.063
0.1	47.3 ± 4.4	42.2 ± 5.5	0.47 ± 0.055
0.5	54.8 ± 7.4	33.6 ± 1.1*	0.38 ± 0.013*
1	57.5 ± 4.5	37.6 ± 5.3*	0.40 ± 0.054*

Differences were considered significant at $^{*}p < 0.05$ values against vehicle-treated control (each value represents mean ± S.D.).

Table 5
Effects on astrocytes glutathione content and red/ox index of 24 h H_2O_2 (0.5 mM) exposure after 24 h ginseng pre-treatment at different concentrations

	GSH (nmol/mg prot)	GSSG (nmol/mg prot)	Red/ox index (GSSG/GSH + GSSG)
Vehicle-treated control	69.5 ± 5.3	59.9 ± 2.6	0.46 ± 0.023
Ginseng 0.05 mg/ml + H_2O_2 0.5 mM	80.9 ± 5.1$^{\#}$	75.4 ± 4.4	0.48 ± 0.046$^{\#}$
Ginseng 0.1 mg/ml + H_2O_2 0.5 mM	78.1 ± 7.7$^{\#}$	67.6 ± 0.6$^{\#}$	0.46 ± 0.008$^{\#}$
Ginseng 0.5 mg/m + H_2O_2 0.5 mM	65.1 ± 4.4	78.3 ± 5.5	0.54 ± 0.055
Ginseng 1 mg/ml + H_2O_2 0.5 mM	69.7 ± 4.5$^{\#}$	82.1 ± 5.4	0.54 ± 0.054
Control H_2O_2 0.5 mM	56.7 ± 4.1*	80.1 ± 5.9*	0.59 ± 0.059*

Differences were considered significant at $^{*}p < 0.05$ values against vehicle-treated control and at $^{\#}p < 0.05$ values against control H_2O_2 0.5 mM (each value represents mean ± S.D.).

Glutathione content in cells exposed to H_2O_2 0.5 mM for 24 h after the treatment for 24 h with ginseng root extract: results showed slight differences. GSH content was improved with 0.05, 0.1 and 1 mg/ml pretreatment and GSSG content shown differences at the dose of 0.1 mg/ml with control H_2O_2 0.5 mM. Those results yielded significant statistical differences in Red/ox balance in treatment with ginseng extract 0.05 and 0.1 mg/ml (Table 5).

4. Discussion

Stress exposure of experimental animals has been reported to lead to oxidative injury in brain lipids (Li et al., 1999). Oxidative stress has been proposed as one major factor leading to cell death. It has been proposed that reactive oxygen species (ROS) production, including hydrogen peroxide (H_2O_2), may in turn initiate neurotoxic events. Thus, the excess of free radicals may lead to peroxidative impairment of membrane lipids and consequently disrupt neuronal functions and cause cellular death.

Exogenous H_2O_2 produces great cell injury, because of its reactivity with intracellular metal ions (iron or copper) creating highly toxic hydroxyl radicals which cause DNA damage and can justify in part H_2O_2-mediated cell death (Nassi-Calo et al., 1989; Ferger et al., 2001; Bastianetto and Quirion, 2002; Kim et al., 2002; Shao et al., 2004). H_2O_2 induces differential protein activation suggesting varied biological effects depending on the site of action of the exogenous H_2O_2 concentration (Pantopoulos et al., 1997).

Ginseng components have shown protective effects in experimental models of neurodegeneration (Lian et al., 2005). Ginseng root has been studied for its antioxidant potential, and is known to scavenge ROS, to chelate metal ions and to prevent LDL peroxidation via distinct concentration-dependent mechanism. Many reports refer to its capacity to scavenge free radicals (hydroxyl radicals or DPPH), to chelate metal ions and to protect against lipoprotein oxidation (Seong et al., 1995; Kitts et al., 2000). The antioxidant activity of ginseng extract and their components in other experimental models has also been studied (Chen et al., 1987; Facino et al., 1999; Voces et al., 1999). However, the antioxidant capabilities of ginseng root on astrocytic protection have not been established in literature.

The present work has tested whether ginseng root extract could protect astrocytes from oxidant stress generated by H_2O_2. In our research, the dose of exogenous H_2O_2 chosen to produce cell damage was 0.5 mM.

We have utilized dissociated astrocytes culture for astrocytic primary cultures. The selected model as a convenient way to identify the potential astroprotective compounds was primary astrocytes culture because of the critical role of those cells in neuroprotection. Oxidative stress causes cell death when intracellular levels of metabolic and antioxidant enzymes (especially glutathione related enzymes) and substrates (glutathione, glucose and ATP) are exhausted. Astrocytes are especially resistant to oxidative damage because of their relatively high antioxidant contents and their capacity to regenerate glutathione and ascorbate with reducing equivalents from glucose metabolism. Moreover, the neuroprotective role of astrocytes appears most evident following reactive gliosis (Wilson, 1997). Thus, in postischemic rat brain, reactive astrocytes upregulate expression of CuZn and Mn superoxide dismutase isoforms (Liu et al., 1993); in the brain of Alzheimer′s patients – where amyloid deposits generate free radicals – the reactive astrocytes surrounding senile plaques are enriched in Mn superoxide dismutase (Furuta et al., 1995). Astrocytes also respond to prolonged oxidative stress by upregulating plasma cystine transport activity, using this amino acid for glutathione synthesis and increasing their glutathione content (Sagara et al., 1996). As we

can see, astrocytes maintain high intracellular concentrations of certain antioxidants, making these cells resistant to oxidative stress relative to oligodendrocytes and neurons (Wilson, 1997).

As the first step to assay the ginseng extract and isolate ginsenosides protective activity, we have determined by LDH release and MTT reduction assays the optimal dose for this research of the selected toxic (H_2O_2), and the non-toxic doses of ginseng and ginsenosides.

Oxidant stress produced significant cell death. However, when astrocytes were pretreated – before the H_2O_2 exposition – with the ginseng root extract, this death was reduced by using the highest ginseng doses previously tested with the viability assays. This evidence indicates a significant reduction of oxidant stress.

When the cells were exposed only to exogenous H_2O_2, the DCF fluorescence increased significantly. However, co-treatment of H_2O_2 with ginseng root extract significantly attenuated the DCF fluorescence at all the tested doses, especially at the higher concentrations. H_2O_2 can traverse cell membranes to exert its biological effects intracellularly (Fridovich, 1986). Although some H_2O_2 may be scavenged by cellular catalase and glutation peroxidase, it can directly cause oxidation of various intracellular targets including the fluorescence probe, DCF. Formation of hydroxyl radicals mediated by intracellular heavy metal ions could also contribute to the increased DCF fluorescence in response to H_2O_2 (Nassi-Calo et al., 1989). Since DCF fluorescence is produced by the reaction between fluorescence probe and the H_2O_2/hydroxyl radical, it suggests that H_2O_2 that was added extracellularly traversed the cell membrane to cause intracellular oxidant stress. The results suggest that ginseng root extract exerts its antioxidant effects in the intracellular compartment. This claim is supported by the fact that DCF fluorescence represents intracellular activity, and therefore, the increased fluorescence is suggestive of the intracellular oxidant stress (Carter et al., 1994).

Exposure of astrocytes to H_2O_2 decreased the activities of all the antioxidant enzymes measured (Table 3). The enzymatic activities were determined in astrocytes before the treatment with the test drug to assay its protective effect: cells were treated 24 h with ginseng root extract after or before the exposure to H_2O_2. The sequential activities of SOD and glutathione peroxidase are the principal mechanisms for removal of ROS from cells. In addition to glutathione peroxidase, catalase activity is an important antioxidant pathway in the removal of hydro, but not organic, peroxides. Catalase is a more efficient scavenger of H_2O_2 at higher concentrations, whereas, glutathione peroxidase activity is favoured at lower H_2O_2 concentrations (Ehrhart and Zeevalk, 2001). Although no treatments after H_2O_2 exposure showed differences with H_2O_2 treated cells, ginseng pre-treatment showed protection on antioxidant enzymes activities.

GSH is involved in the removal of hydro- and organic-peroxides that are formed as products of normal cellular processes or toxic insults. During normal functioning of the respiratory chain <2% of mitochondrial O_2 is reduced and released as superoxide anion, which is converted to H_2O_2 by SOD and then further reduced by glutathione peroxidase. The reduction of peroxide by glutathione peroxidase results in the oxidation of GSH to GSSG. Oxidized GSSG is reduced back to GSH by the NADPH-dependent activity of glutathione reductase, thereby recycling GSH and limiting the accumulation of GSSG in cells (Ehrhart and Zeevalk, 2001). The treatment of astrocytes with ginseng extract improved the intracellular glutathione content and red/ox balance (Table 4); after H_2O_2 exposure the lowest doses yielded the best results (Table 5).

Our results suggest that ginseng root extract could protect astrocytes from oxidant stress generated by H_2O_2 which is consistent with the reports of antioxidant effects observed in ginseng root extracts in other cellular types (Himi et al., 1989; Huong et al., 1998; Liao et al., 2002; Shao et al., 2004). *Panax ginseng* saponins have shown a suppressive action on the lipid peroxidation caused by radical generating systems in tissue preparations, and attenuate lipid peroxidation in the rat liver homogenate.

Ginseng saponins may modulate the activity of the root in its proliferative and antioxidant effects. Ginsenosides alleviated oxidative stress by scavenging of free radicals, inhibiting of NO production which usually accompanies glutamate excitotoxicity, inducing superoxide dismutase (SOD1) and catalase genes and reducing lipid peroxidation (Braughler et al., 1988; Chang et al., 1999). Rudakewich et al. (2001) concluded that ginsenosides Rb_1 and Rg_1 potentiate NGF-induced neurite outgrowth in cell culture. Ginsenoside Rg_1 was shown to interrupt dopamine-induced elevation of reactive oxygen species (ROS) or NO generation in pheochromocytoma cells (PC12) (Chen et al., 2003). Moreover, ginseng radix attenuated MPP^+-induced apoptosis as it decreased the intensity of MPP^+-induced DNA laddering in PC12 cells and ginsenoside Rg_1 had protective effects against MPTP-induced apoptosis in the mouse substantia nigra (Chen et al., 2002; Kim et al., 2003). It has been reported that ginsenosides Rb_1, Rg_1, Rc, and Re inhibited tyrosine hydroxylase activity and exhibited anti-dopaminergic action since they reduced the availability of dopamine at presynaptic dopamine receptors (Kim et al., 1999).

Ginsenosides also exhibit protection against free radical-induced damage (Li et al., 1999). Some reports showed that neuroprotection by ginseng may be, in part, due to its effect on glial cell populations. In this respect, it has been reported that ginseng total saponins prevented astrocytic swelling induced by glutamate (Seong et al., 1995) and ginsenoside Rg_1 inhibited microglial respiratory burst activity and decreased the accumulation of NO produced by activated microglia (Gong and Zhang, 1999). Even though the exact mechanism of protection against oxidative stress remains unclear, we are performing experiments to elucidate it.

In summary, in the present study, we have demonstrated the protective effect of a normalized aqueous *Panax ginseng* root extract on hydrogen peroxide-induced oxidative damage in astrocytic primary cultures. Our results showed that the root of Korean ginseng is endowed with significant antioxidant properties and this is the base for its glioprotection against acute oxidant stress. Ginseng root extract is effective in reducing astrocytic death induced by H_2O_2. As a result of our investigation, ginseng extract may attenuate pathophysiological changes caused by oxidative stress exposure.

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