# Effect of Ginseng Saponins on the Survival of Cerebral Cortex Neurons in Cell Cultures

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**The effects of nerve growth factor (NGF) and saponins isolated from *Panax ginseng* C. A. MAYER on the survival of chick and rat embryonic cerebral cortex neurons were examined. Ginsenoside Rg1 (GRg1) exerted a survival-promoting effect on both chick and rat cerebral cortex neurons in cell cultures. Ginsenoside Rb1 (GRb1) also had an effect in the rat and displayed some influence in the chick. NGF alone exerted no effect on both neurons, although it did potentiate the GRb1 effect on chick embryonic cerebral cortex neurons, but did not alter the GRb1 effect on rat embryonic cerebral cortex neurons. NGF did not alter the survival-promoting effect of GRg1 on either chick or rat embryonic cerebral cortex neurons. The other saponins alone or with NGF exerted no effect on the survival of cerebral cortex neurons in either the chick or rat.**

**Keywords** nerve growth factor (NGF); ginseng saponin; chick; rat; cell culture; cerebral cortex; embryo; neuronal survival

## Introduction

*Panax ginseng* C. A. MAYER has been used as a constituent of many prescriptions in Chinese traditional medicine and is said to be effective for amnesia among its chief indications.[1] Ginseng saponins, such as ginsenosides Rb1 (GRb1), GRb2, GRc, GRd, GRe and GRg1, have been identified as components of ginseng and their pharmacological activities have been investigated. Nerve growth factor (NGF) exerts a marked effect on the neurite outgrowth of sympathetic and sensory neurons in organ cultures. Takemoto *et al.* reported that GRb1 and its analogues potentiated NGF-mediated nerve fiber production in organ cultures of chick embryonic dorsal root ganglia (DRG) and lumber sympathetic ganglia (Sym G).[2] Recently, NGF was found to be localized specifically in the basal forebrain regions of the central nervous system (CNS) and its relationship to memory is currently being evaluated.[3] We were therefore interested to determine whether ginseng saponins have a life-prolonging effect on CNS neurons in cell cultures. In the present study, we examined the effect of ginseng saponins on the survival of chick and rat embryonic cerebral cortex neurons.

### Materials and Methods

Sixteen-day-old rat embryos (Wistar strain, Shizuoka Laboratory Animal Center) and 8-day-old chick embryos (Ohmiya Co., Ltd.) were used. The cerebral cortices were removed from the embryos, minced into pieces smaller than 1 mm square, and digested with 0.25% trypsin (Difco 1:250) and 0.01% deoxyribonuclease I (DNase I) (Sigma D-0751) in phosphate-buffered saline (PBS) containing 0.5% glucose at 37 °C for 20 min with stirring every 5 min. Digestion was repeated once more with fresh enzyme solution. After removal of the supernatant, medium was added. The medium was composed of Dulbecco's modified Eagle's medium (DME: Gibco 430-2100) containing 8.2 mg/ml glucose (Wako), 0.3 mg/ml glutamine (Wako), 1.7 mg/ml sodium bicarbonate, 51 mg/l (Wako) penicillin-G sodium (Sigma), 85 mg/l streptomycin sulfate (Sigma) and 15% fetal bovine serum (FBS, Filtron). The cells were then dissociated by pipetting, collected by centrifugation, and washed again with fresh medium. The cell suspension was filtrated with a niron mesh to remove cell aggregates and diluted with medium to give about $6 \times 10^5$ cells/ml. One milliliter of cell suspension was placed in a 24-well plate (Falcon) with a coverglass ($3 \times 10^5$ cells/cm$^2$) or 0.5 ml of cell suspension was placed in a 48-well plate (Costar, $3 \times 10^5$ cells/cm$^2$). Each well had first been coated with poly-L-lysine (0.1 g/l of 0.1 M boric buffer pH 8.4; Sigma P-2636) and washed with water three times. Cells were incubated at 37 °C in 95% air and 5% $CO_2$. After 3 d of culture, the medium was changed every third day and the drugs were given at the same time. The culture durations were 11 d (chick) and 15 d (rat). NGF was isolated from adult male mouse submandibular glands as the 2.5 s subunit according to the procedure of Bocchini and Angeletti.[4] Purified ginseng saponins were provided by Takeda Chemical Co., Ltd. The NGF and ginseng saponins were dissolved in the medium just before administration. Surviving neuronal cell staining was performed by the following steps: fixation with 4% paraformaldehyde at 4 °C for 30 min, decomposition of the cell membrane with 0.1% Triton-X 100 (Wako) in PBS for exactly 15 min, inhibition of the internal peroxidase with 0.3% hydrogen peroxide in methanol for 20 min, inhibition of non-specific protein binding with 2% FBS in PBS for 20 min, binding of mouse monoclonal anti-neurofilament antibody (diluted ×200 with PBS; Labo-System) for 2 h, binding of rabbit anti-mouse IgG-antibody (diluted ×200 with Tris buffer saline, TBS; MBL) for 1 h, binding of peroxidase-anti-peroxidase (diluted ×20000 with TBS; Wako) for 1 h, and reaction with 0.5 mg/ml 3,3′-diaminobenzidine and 0.0013% hydrogen peroxide in 5 mM Tris–HCl buffer (pH 7.6) for about 1 h. All reactions except the fixation were performed at room temperature. In order to avoid background staining, thorough washing with the buffer to be used at the next step was necessary in the interval between each step. In the case of the 24-well plates, cover glasses were picked up dehydrated and mounted on the slide glass. The numbers of stained cells were counted as the surviving neurons in 10 visual fields (1.9 mm$^2$/10 visual fields) under a microscope in every well, and were expressed in cells/mm$^2$. Statistical analyses were performed with ANOVA followed by Duncan's multiple range tests.

## Results

**Period of Culture** The relationships between the period of cell culture and the numbers of surviving neurons of the chick and rat are shown in Figs. 1 and 2, respectively. In cell

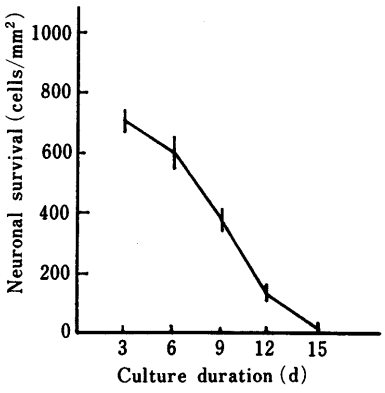



Fig. 1. Culture Duration and Number of Surviving Neurons of the Chick Embryonic Cerebral Cortex Neurons in Cell Cultures

Dissociated chick embryonic cerebral cortex cells were cultured. The medium was changed every third day. Cells were fixed and neurons were stained specifically by the PAP method using anti-neurofilament antibody after certain periods of culture. Six wells were used for each culture duration. The numbers of stained neurons were counted in 10 visual fields per well under a microscope and expressed in cells/mm$^2$.

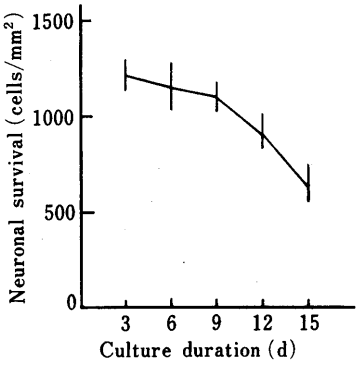



Fig. 2. Culture Duration and Number of Surviving Neurons of the Rat Embryonic Cerebral Cortex in Cell Cultures

Dissociated rat embryonic cerebral cortex cells were cultured. For other details, see Fig. 1.

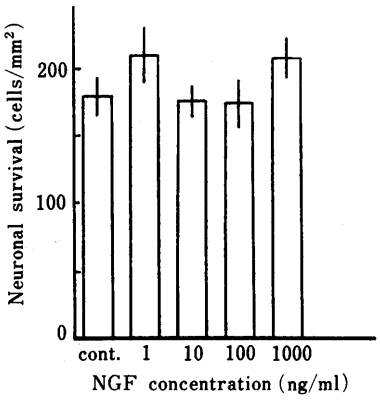



Fig. 3. Effect of NGF on the Survival of Chick Embryonic Cerebral Cortex Neurons in Cell Cultures

After 3 d of preincubation, the medium was changed and the cells were treated with NGF at various concentrations between 1 to 1000 ng/ml during the culture duration (for 12 d; total culture duration, 15 d). The medium was changed every third day and NGF was given at the same time. "cont." indicates the control treatment, in which the medium was given instead of NGF or saponin solution. The cells were stained and the surviving neurons were counted according to the method described in Fig. 1.

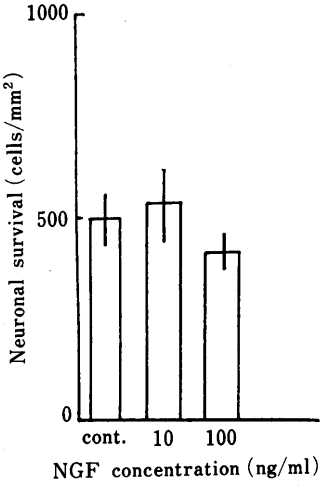



Fig. 4. Effect of NGF on the Survival of Rat Embryonic Cerebral Cortex Neurons in Cell Cultures

After 3 d of preincubation, the medium was changed and the cells were treated with NGF at various concentrations between 1 to 100 ng/ml during the culture duration (for 8 d; total culture duration, 11 d). The medium was changed every third day and NGF was given at the same time. "cont." indicates the control treatment, in which the medium was given instead of NGF or saponin solution. The cells were stained and the surviving neurons were counted according to the method described in Fig. 1.

cultures of chick embryonic cerebral cortex neurons, the stability of the system was lost after 12 d of culture (Fig. 1). In cell cultures of rat embryonic cerebral cortex cell neurons, the number of surviving neurons did not decrease greatly, but glia cells increased so much the number of neurons could not be counted after more than 18 d of culture (Fig. 2). We therefore set the period of cell cultures in subsequent experiments at 11 d for chick neurons and 15 d for rat neurons.

**Effect of NGF** NGF at concentrations between 1 and 1000 ng/ml had no effect in the cell cultures of both chick and rat embryonic cortex neurons, as shown in Figs. 3 and 4, respectively.

**Effect of Ginseng Saponins** The effect of ginseng saponins at a concentration of 30 $\mu$M on the survival in cell cultures of both chick and rat embryonic showed cerebral cortex neurons is illustrated in Figs. 5 and 6, respectively. Ginsenoside Rg1 exerted a marked life-prolonging effect of chick embryonic cerebral cortex neurons and tended to promote neuronal survival in the rat. Ginsenoside Rb1 revealed only a slight tendency to promote neuronal survival in chick embryonic cell cultures (Fig. 5) but had a marked effect in the rat (Fig. 6). The other saponins exerted

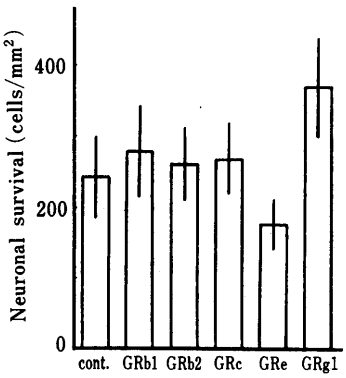



Fig. 5. Effect of Ginseng Saponins on the Survival of Chick Embryonic Cerebral Cortex Neurons in Cell Cultures

After 3 d of preincubation, the medium was changed and the cells were treated with various kinds of saponins at a concentration of 30 $\mu$M during the culture duration (for 8 d; total culture duration, 11 d). The medium was changed every third day and saponins were given at the same time. The cells were stained and the surviving neurons were counted according to the method described in Fig. 1.

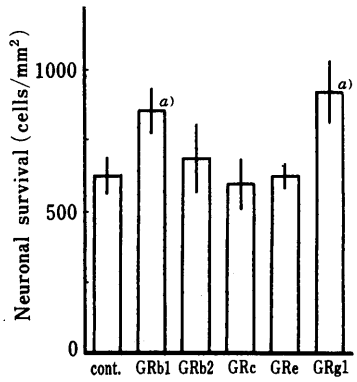



Fig. 6. Effect of Ginseng Saponins on the Survival of Rat Embryonic Cerebral Cortex Neurons in Cell Cultures

After 3 d of preincubation, the medium was changed and the cells were treated with various kinds of saponins at a concentration of 30 $\mu$M during the culture duration (for 12 d; total culture duration, 15 d). The medium was changed every third day and saponins were given at the same time. The cells were stained and the surviving neurons were counted according to the method described in Fig. 1. *a*) $p<0.05$.

no effect on neuronal survival in the chick and rat embryonic cultures.

**Interactions between NGF and Ginseng Saponins** Figures 7 and 8 show the effect of ginseng saponins (30 $\mu$M) plus NGF (100 ng/ml) on the survival of cerebral cortex neurons in chick and rat embryo cell cultures, respectively. Ginsenoside Rg1 plus NGF tended to promote neuronal survival in both the chick and the rat, but the effect of GRg1 plus NGF was similar to that of GRg1 alone when compared to the control (Figs. 5—8). Ginsenoside Rb1 with NGF revealed a marked life-prolonging effect on the chick embryonic cerebral cortex neurons (Fig. 7), but showed only a slight tendency in rat neurons (Fig. 8). The level of the survival-promoting effect of GRb1 plus NGF (Fig. 7) as compared to that of GRb1 alone (Fig. 5) was clearly improved in chick embryonic cell cultures, but the effect of GRb1 plus NGF (Fig. 8) was almost the same as that of GRb1 alone (Fig. 6) in rat embryonic cell cultures.

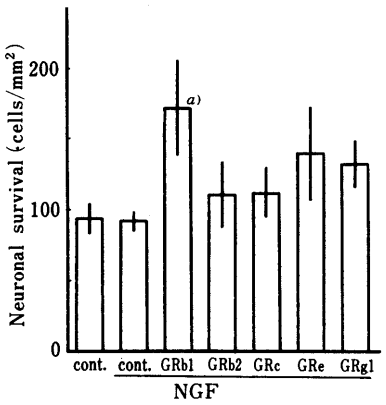



Fig. 7. Effect of Ginseng Saponins plus NGF on the Survival of Chick Embryonic Cerebral Cortex Neurons in Cell Cultures

After 3 d of preincubation, the medium was changed and the cells were treated with various kinds of saponins (30 $\mu$M) plus NGF (100 ng/ml) during the culture (for 8 d; total culture duration, 11 d). The medium was changed every third day and the drugs were given at the same time. "cont." (far left) indicates the control treatment, in which the medium was given instead of NGF or saponin solution. "cont." (underlined) was treated with NGF only. The cells were stained and the surviving neurons were counted according to the method described in Fig. 1. *a*) $p<0.05$.

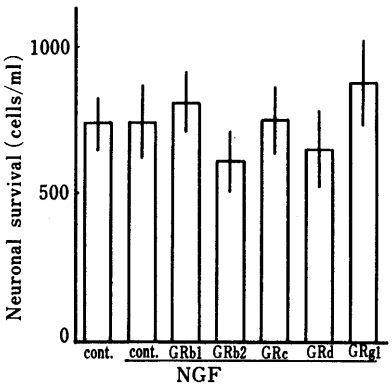



Fig. 8. Effect of Ginseng Saponins plus NGF on the Survival of Rat Embryonic Cerebral Cortex Neurons in Cell Cultures

After 3 d of preincubation, the medium was changed and the cells were treated with various kinds of saponins (30 $\mu$M) plus NGF (100 ng/ml) during the culture duration (for 12 d; total culture duration, 15 d). The medium was changed every third day and the drugs were given at the same time. "cont." (far left) indicates the control treatment, in which the medium was given instead of NGF or saponin solution. "cont." (underlined) was treated with NGF only. The cells were stained and the surviving neurons were counted according to the method described in Fig. 1.

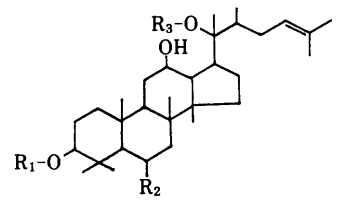
The other ginseng saponins plus NGF exerted no effect on neuronal survival.

## Discussion

The structures of each saponin are shown in Fig. 9. The rate of nerve fiber production in organ cultures of sensory and sympathetic ganglia has generally been used as an indicator of the NGF effect in bioassays. It has been reported that both GRb1 and GRd, which are 20 (*S*)-protopanaxadiol glycosides having glucose units in their two sugar side chains attached to $C_{(3)}$ and $C_{(20)}$, markedly potentiated the NGF effect in organ cultures of chick DRG and Sym G.[2] The rat was the only animal used in the investigations of the NGF effect on the CNS. It is known that NGF exerts effects on the cholinergic neurons in the basal forebrain region, including an increasing effect on the choline acetyltransferase activity and promoting effects on the neuronal survival and nerve fiber production.[5] Cerebral cortex neurons of both the chick and rat were therefore used to investigate the effect of ginseng saponins alone or with NGF. Sixteen-day-old rat embryos and 8-d-old chick embryos were selected as most suitable for the experiments, since chick embryos of more than 8 d old had braincases which were too hard for easy removal of the brain and the neurons of rat embryos of more than 16 d old did not survive long in the cell cultures. Glia cells of the 8- and 12-d-old chick embryonic cortex did not proliferate rapidly under our conditions. However, the glia cells of the 16-d-old rat embryonic cerebral cortex proliferated too rapidly to allow the number of neurons to be counted after more than 18 d of culture, although the neurons were specifically stained.

Nerve growth factor alone at concentrations between 1 ng/ml and 1 $\mu$g/ml did not have any effect on the survival of chick and rat embryonic cerebral cortex neurons in the cell cultures (Figs. 3 and 4). Our data from this experiment indicated that NGF exerted no effect on the survival of the cerebral cortex neurons as a whole, involving many kinds of neurons in terms of their possession of neurotransmitters.

Ginsenoside Rg1 itself had a life-prolonging effect on both chick and rat cerebral cortex neurons in the cell cultures. The effect of GRg1 alone was almost the same as that of GRg1 plus NGF. Ginsenoside Rg1 has been




| saponins | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 20(*S*)-protopanaxadiol glycosides | | | |
| ginsenoside Rb1 | glc–glc | H | glc–glc |
| ginsenoside Rb2 | glc–glc | H | ara(p)–glc |
| ginsenoside Rc | glc–glc | H | ara(f)–glc |
| ginsenoside Rd | glc–glc | H | glc |
| 20(*S*)-protopanaxatriol glycosides | | | |
| ginsenoside Re | H | rha–glc–O | glc |
| ginsenoside Rg1 | H | glc–O | glc |

Fig. 9. Structures of the Saponins

reported to have no effect itself or no promoting effect on NGF-mediated nerve fiber production in organ cultures of chick embryonic DRG or Sym G. This indicates that there was no interaction between GRg1 and NGF, but it is interesting that GRg1, which has been reported to have a promoting effect on learning,[6] exerted a promoting effect on the survival of neurons. Takemoto *et al.*[2] reported synergistic effects between NGF and GRb1 in organ cultures of DRG and in Sym G. In cell cultures of chick cerebral cortex neurons, GRb1 itself has a slight effect on cell survival (Fig. 5) and the effect was markedly potentiated by NGF (Fig. 7). On the other hand, in cell cultures of rat cerebral cortex neurons, GRb1 itself exerted a marked effect and GRb1 plus NGF showed an activity which was almost the same as that of GRb1 alone (Figs. 6 and 8). These findings might reflect a species difference, and NGF may have some activities only on chick cerebral cortex neurons. Ginsenoside Rd, which is of similar structure to GRb1, showed no effect in the present assay system. Structure–activity relationships, as in the case of the promoting effect of NGF-mediated nerve fiber production in organ cultures of chick embryonic DRG and Sym G,[2] were not found for the survival of rat and chick embryonic cerebral cortex neurons in the cell cultures. We observed direct promoting effects of GRg1 and GRb1 on the survival of neurons in the CNS. These might be related to the promoting effect of GRg1 on the process of learning[6] and memory and the tranquilizing effect of GRb1.[6]

**Acknowledgment** We are grateful to Takeda Chemical Co., Ltd. for providing the ginseng saponins.

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