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Evaluation of the Neurotoxicity of Strontium and *Glycyrrhiza Glabra*: First Report

Burcak SOYLEMEZ[1], Serap SAHIN-BOLUKBASI[2]

[1]Sivas Cumhuriyet University, Faculty of Health Sciences, Department of Neurosurgery, Sivas, Turkey
[2]Sivas Cumhuriyet University, Faculty of Pharmacy, Department of Biochemistry, Sivas, Turkey

Corresponding author: Burcak SOYLEMEZ ✉ burci1986@gmail.com

ABSTRACT

AIM: To investigate the neurotoxic effects of strontium (Sr) compounds and *Glycyrrhiza glabra* (licorice, *G. glabra*).

MATERIAL and METHODS: In this study, we conducted neurotoxicity assays on the human cortical neuronal cell line HCN-2 (CRL-10742) to determine the potential neurotoxic effects of Sr and *G. glabra*.

RESULTS: No significant decrease in HCN-2 cell viability was observed with longer Sr exposure or Sr concentrations up to 2000 µg/mL. The IC_{50} values of Sr for 24 and 48 hours of exposure were >2000 µg/mL, and 936.9 ± 0.09 µg/mL for 72 hours. However, we observed a significant reduction in HCN-2 cell viability with longer exposure and higher concentrations of *G. glabra*. The IC_{50} values of *G. glabra* for 24, 48, and 72 hours were 545.1 ± 0.03 µg/mL, 398.1 ± 0.03 µg/mL, and 393.3 ± 0.02 µg/mL, respectively.

CONCLUSION: Additional studies are needed to further investigate the neurotoxicity of Sr and *G. glabra* and elucidate the pathway by which these compounds exert their therapeutic effects in pathological conditions.

KEYWORDS: *Glycyrrhiza glabra*, Human cortical neuronal cell line HCN-2, Neurotoxicity, Strontium

ABBREVIATIONS: DMEM: Dulbecco's modified Eagle medium, **FBS:** Fetal bovine serum, ***G. glabra*:** *Glycyrrhiza glabra* (licorice), **HCN-2:** Human cortical neuronal cell line, **IC_{50}:** The half maximal inhibitory concentration, **MTT assay:** 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue, **Sr:** Strontium

INTRODUCTION

Strontium (Sr) is a naturally occurring element that was first discovered as a result of lead mining in Scotland in the 18th century and is physically and chemically similar to calcium (12). Sr is also a trace element and abundant in earth's crust, groundwater, and ocean water. Sr is a normal component of the human diet; the richest sources of Sr are leafy greens, grains, and seafood (28,34). In 1870, it was observed that Sr was naturally incorporated into the bones of animals fed small doses of the element (23). Some of these studies indicated that Sr has an anti-inflammatory effect, increases osteoblastic activity, reduces osteoclastic activity, and prevents skin irritation (2,11,25,27). Since these physiological functions were observed, screening Sr and related compounds for biological applications has been the subject of numerous studies.

In recent years, there has been a growing trend towards traditional herbal remedies for the alternative treatment of diseases due to the unwanted symptoms, side effects, and toxic effects of most drugs (7,8). Herbal drugs are used for the treatment of many human and animal diseases, especially in Africa and Asia (5,6). The use of licorice as traditional medicine and folk remedies are associated with certain geographical areas and periods (3). *G. glabra* has been used from ancient times to the present day. *G. glabra* is known to have antiviral, antibacterial, anti-inflammatory, antioxidant, neuroprotective, sedative, antidepressive, anticarcinogenic, and antidiabetic effects (20,24,30). *G. glabra* is one of the medical plants

Burcak SOYLEMEZ : 0000-0001-8626-6915 Serap SAHIN-BOLUKBASI : 0000-0003-1057-2558

belonging to the Fabaceae family, and *G. glabra* extracts are currently used in the pharmaceutical and food industries (30).

Although the therapeutic effects of Sr and *G. glabra* have been investigated in other tissues, it has not been possible to clarify whether they were neurotoxic, which has impeded studies related to the nervous system. There is very little information available on the neurotoxicity of Sr and *G. glabra*. To the best of our knowledge, this is the first study of the neurotoxic effects of Sr and *G. glabra*. In this study, we conducted neurotoxicity assays on the human cortical neuronal cell line HCN-2 (CRL-10742) to determine the potential neurotoxic effects of Sr and *G. glabra* on humans.

MATERIAL and METHODS

The toxic effects of Sr and *G. glabra* on HCN-2 (1A), a healthy (normal) human brain cortical neuronal cell line, were evaluated with MTT assay (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) (30). The MTT assay is a colorimetric method frequently used in studies determining antiproliferative activity. It is based on the conversion of yellow MTT to insoluble purple formazan by dehydrogenase, which is one of the mitochondrial enzymes of living cells. Therefore, the MTT assay is used as a measure of cell viability.

The HCN-2 (1A) cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin in an incubator at 5% CO_2, 37°C, and 95% humidity. HCN-2 (1A) healthy (normal) human brain cortical neuron cells were passaged and seeded in 96-well plates at $1x10^5$ cells/ml. After incubating at 5% CO_2, 37°C, and 95% humidity for 24 hours to allow adherence to the plates, Sr and *G. glabra* were applied at different concentrations (1–2000 µg/mL). Toxic effects were determined by MTT assay at 24, 48, and 72 hours after applying Sr and *G. glabra*. The half-maximal inhibitory concentration (IC_{50}) values of Sr and *G. glabra* were calculated using GraphPad Prism7.

Statistical Analysis

All assays conducted in the study were performed with nine replicates (n=9), and the results were expressed as mean ± standard error of the mean (SEM). The IC_{50} values of Sr and *G. glabra* were calculated using the GraphPad Prism7 graph and statistics software (GraphPad Software, San Diego, CA, USA). One-way analysis was performed and *p<0.05, **p<0.005, ***p<0.0005, and ****p<0.0001 were selected as levels of significance compared to the control.

RESULTS

Cell viability results for HCN-2 cells treated with Sr or *G. glabra* for 24, 48, and 72 hours are shown in Figures 1 and 2, respectively. The x-axis shows the applied concentrations of Sr or *G. glabra*, and the y-axis shows the percentage of viable cells compared to the control. The concentrations at which Sr and *G. glabra* killed 50% of the HCN-2 (1A) cells (IC_{50} values) were determined based on these data. For Sr, there was no significant dose- or time-dependent decrease in HCN-2 cell viability after exposure to Sr at concentrations up to 2000 µg/mL. The IC_{50} values of Sr were >2000 µg/mL for 24 and 48 hours and 936.9 ± 0.09 µg/mL for 72 hours (Table I). There was a significant reduction in HCN-2 cell viability with time and increase in *G. glabra* concentration. The IC_{50} values of licorice root for 24, 48, and 72 hours were calculated as 545.1 ± 0.03 µg/mL, 398.1 ± 0.03 µg/mL, and 393.3 ± 0.02 µg/mL (Table I).

Low IC_{50} values indicate high cytotoxic activity. In order to discuss high cytotoxic activity, IC_{50} values are expected to below. Sr in particular was found to have very high IC_{50} values. According to the results in Table I, neither strontium nor *glycyrrhiza glabra* exhibited cytotoxic effects on HCN-2 (1A) healthy (normal) human brain cortical neuron cells. However, *G. glabra* was found to have a relatively greater cytotoxic effect on HCN-2 (1A) cells compared to strontium.

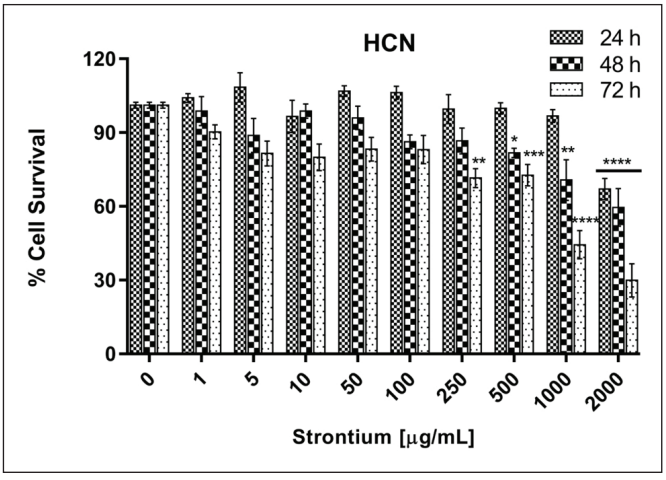

Figure 1: The cytotoxic effect of strontium on HCN-2 (1A) healthy (normal) human brain cortical neuron cells at 24, 48, and 72 hours (*p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001 compared to the control).

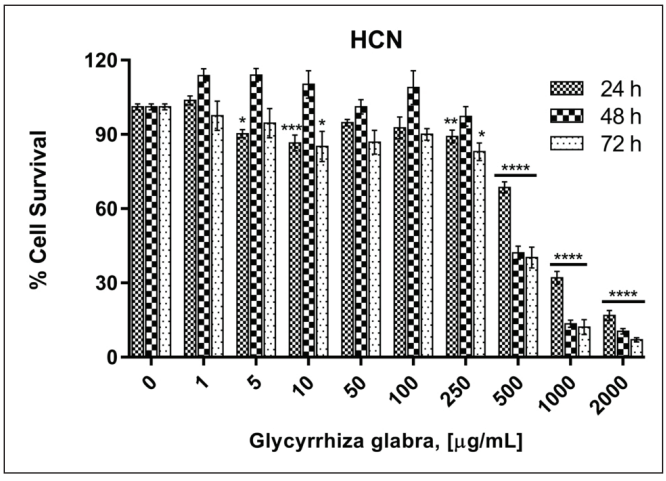

Figure 2: The cytotoxic effect of *Glycyrrhiza glabra* on HCN-2 (1A) healthy (normal) human brain cortical neuron cells at 24, 48, and 72 hours (*p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001 compared to the control).

Table I: The IC_{50} Values for Sr and *Glycyrrhiza glabra* on a Normal Human Brain Cortical Neuronal Cell Line (HCN-2 [1A]) at 24, 48, and 72 Hours After Application

Cells line	Time	Strontium (IC_{50}, µg/mL)	*Glycyrrhiza Glabra* (IC_{50}, µg/mL)
HCN-2	24 h	> 2000	545.1 ± 0.03
	48 h	> 2000	398.1 ± 0.03
	72 h	936.9 ± 0.09	393.3 ± 0.02

DISCUSSION

There has been little research on the biological effects of stable Sr. It was reported that patients who received 1 to 1.5 g/day of Sr gluconate showed higher serum Sr levels and a 100-fold increase in SR:calcium ratio. In rats, studies indicated that increased intracellular Sr level following supplementation resulted in mitochondrial protection. It was also determined that metastatic cancer patients who received stable Sr supplementation showed increased density of bone lesions. An inverse relationship was seen between the incidence of dental caries and high Sr content in drinking water (31). A substantial proportion of patients with osseous metastases experience suffer from bone pain. In a study by Ashayeri et al., 41 patients with multiple osseous metastases of prostate and breast cancer were treated with strontium chloride (Sr-89) and over two-thirds of the patients responded favorably (4). In another study, 93 patients with hormone-refractory metastatic prostate cancer were enrolled in a prospective study to measure pain reduction and changes in quality of life (QoL) after the administration of 150 MegaBequerel (MBq) Sr-89. Although there was limited QoL improvement in the 3 months following Sr-89 treatment, the 53 patients (63%) with reduced pain reported significant improvement in QoL within 6 weeks of receiving Sr-89 compared to patients with stable or worsening bone pain. These results indicate that pain reduction following Sr-89 treatment is accompanied by better QoL (33).

Sr can be released into the air by natural processes or as a result of human activities. Average Sr concentrations in air are generally below 0.1 µg/m^3. For adult humans, the total daily intake of Sr in many parts of the world is estimated to be up to about 4 mg/day (13). Sr is an alkaline metal that is found in nature in an oxidized state with a valence of +2. An adult human has 0.3–0.4 g of Sr, 99% of which is found in the bones (13). Sr metabolism is similar to that of calcium (Ca) in terms of intestinal absorption, metabolic pathways in bone deposition, and renal reabsorption. Sr salts suppress keratinocyte-induced tumor necrosis factor-alpha, interleukin-1 alpha, and interleukin-6 in in vivo cultures and directly affect C-type nerve fibers (1,9). Sr is known to primarily affect the bones (9), which it does through calcium-sensitive receptors (CaSR) (25). A study on Sr showed that high-dose oral Sr intake resulted in bone and cartilage anomalies, impaired calcification, reduced mineral content, increased acidic phospholipid complexes, non-mineralized regions, large epiphyseal plates, smaller bones, and rickets (13). However, we could not find any studies on the carcinogenicity, genotoxicity, immunotoxicity, neurotoxicity, or reproductive/developmental toxicity of Sr compounds (13). The only exception is a study conducted in 1977 that demonstrated an increase in thyroid weight in males and a decrease in pituitary weight in females associated with high-dose oral Sr intake (17).

We evaluated the toxic effect of Sr on the HCN-2 Human Cortical Neuron (CRL-10742) cell line after 24, 48, and 72 hours of exposure in order to assess its neurotoxicity. We found that Sr showed no cytotoxic effects and had particularly high IC_{50} values. Although Sr acts on bone tissue through CaSRs, it is still not clear exactly how it affects neuronal tissue.

G. glabra has been used medicinally since ancient times for its antiviral, antibacterial, anti-inflammatory, antioxidant, neuroprotective, sedative, antidepressive, anticarcinogenic, and antidiabetic effects (24). These effects have been attributed to enoxolone, a pentacyclic triterpenoid a glycan metabolite of glycyrrhizin, a constituent of the glycyrrhiza glabra plant. *G. glabra* is one of the medical plants belonging to the Fabaceae family (30). The anti-inflammatory mechanism of *glycyrrhiza glabra* is through an increase of glucocorticoids resulting from 11beta-hydroxysteroid dehydrogenase (11ßHSDH) inhibition (20,24,30).

Two mouse studies conducted between 2006 and 2013 demonstrated improvements in the learning and memory capacities of mice given *G. glabra*. Its effect on learning and memory was attributed to its antioxidant, anti-inflammatory, anticholinesterase, and neuroprotective effects (30).

It has been reported that aqueous, methanol, ethanol, flavonoid, ethyl acetate, chloroform, and acetone extracts of *G. glabra* have shown potent antibacterial effects toward different types of bacteria (14,16,21,26,29,32). Methanol extract of licorice also showed potent anti-fungal effectiveness against *Chaetomium funicola* M002 and *Arthrinium sacchari* M001, which was attributed to the active compound, glabridin (10). Researchers have studied the effects of licorice and its main components on breast, prostate, cervix, uterus, and melanoma cancer cells (20). Ethanol extract of licorice showed a cytotoxic effect on Vero cells (63% at 24 mg/mL) (19). In another study, the IC_{50} values of full licorice extract were found to be about 50 µg/mL for CT29 and HT29 cells and 200 µg/mL for HEK293 cells (15). Lee et al. reported that roasted licorice extract reduced the cell viability of MDA-MB-231 breast cancer cells *in vitro* and suppressed tumor growth and bone destruction *in vivo* (18). Another study showed that *G. glabra* decreased cell proliferation of the HT-29 cell line in a dose-dependent and time-dependent manner (52, 62, and 68% after 24, 48, and 72 hours, respectively) (22).

Different dose ranges of *G. glabra* have been reported in previous studies. For example, in studies of its neuroprotective effect under *in vivo* conditions, *G. glabra* extract was applied at concentrations of 75-300 mg/kg for 7 days, while *in vivo* studies of its anti-inflammatory effect used *G. glabra* at concentrations of 75 mg/kg, 1-100 µmol, or 50-200 µg/ml (24). Toxic effects associated with the oral use of *G. glabra* occur secondary to hypertension, hypokalemia, and metabolic

alkalosis. Furthermore, neurologic syndromes develop as a result of the induction of alkalosis (24,30).

In our experiment, we observed a decrease in HCN-2 cell viability as *G. glabra* exposure time and concentration increased. However, calculation of IC_{50} values for *G. glabra* at 24, 48, and 72 hours showed that *G. glabra* is not neurotoxic.

CONCLUSION

The results of this study suggest that Sr and *G. glabra* are not neurotoxic. Additional studies are needed to further investigate the neurotoxicity of Sr and *G. glabra* and elucidate the pathway by which these compounds exert their therapeutic effects in pathological conditions.

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