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J Ginseng Res xxx (2016) 1–11

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research article

The beneficial effect of ginsenosides extracted by pulsed electric field against hydrogen peroxide-induced oxidative stress in HEK-293 cells

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ARTICLE INFO

Article history:
Received 16 September 2015
Received in Revised form
30 January 2016
Accepted 26 February 2016
Available online xxx

Keywords:
antioxidant activities
ginsenosides
oxidative stress
Panax ginseng
pulsed electric field

ABSTRACT

Background: Ginsenosides are the main pharmacological components of *Panax ginseng* root, which are thought to be primarily responsible for the suppressing effect on oxidative stress.
Methods: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity and oxygen radical absorption capacity were applied to evaluate the antioxidant activities of the ginsenosides. Human embryonic kidney 293 (HEK-293) cells were incubated with ginsenosides extracted by pulsed electric field (PEF) and solvent cold soak extraction (SCSE) for 24 h and then the injury was induced by 40μM H_2O_2. The cell viability and surface morphology of HEK-293 cells were studied using MTS assay and scanning electron microscopy, respectively. Dichloro-dihydro-fluorescein diacetate fluorescent probe assay was used to measure the level of intracellular reactive oxygen species. The intracellular antioxidant activities of ginsenosides were evaluated by cellular antioxidant activity assay in HepG2 cells.
Results: The PEF extracts displayed the higher 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity and stronger oxygen radical absorption capacity (with an oxygen radical absorption capacity value of 14.48 ± 4.04μM TE per μg/mL). The HEK-293 cell model also suggested that the protective effect of PEF extracts was dose-dependently greater than SCSE extracts. Dichloro-dihydro-fluorescein diacetate assay further proved that PEF extracts are more active (8% higher than SCSE extracts) in reducing intracellular reactive oxygen species accumulation. In addition, scanning electron microscopy images showed that the HEK-293 cells, which were treated with PEF extracts, maintained more intact surface morphology. Cellular antioxidant activity values indicated that ginsenosides extracted by PEF had stronger cellular antioxidant activity than SCSE ginsenosides extracts.
Conclusion: The present study demonstrated the antioxidative effect of ginsenosides extracted by PEF *in vitro*. Furthermore, rather than SCSE, PEF may be more useful as an alternative extraction technique for the extraction of ginsenosides with enhanced antioxidant activity.
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1. Introduction

Reactive oxygen species (ROS) have been implicated with cell oxidative stress injury, which result in disorders of physiological functions of DNA, proteins, lipids, and other macromolecules, and subsequently many diseases. When the maintenance of redox homeostasis is overwhelmed, exogenous antioxidants play a significant role in the body's redox homeostasis system. ROS were released and cyclin D1 was degraded by ubiquitin when human embryonic kidney 293 (HEK-293) cells were exposed to excessive H_2O_2, which was found to contribute to the induction of the cell cycle arrest in the G2 phase [1]. Exogenous extracts of antioxidants from dietary sources could be extremely useful in suppressing the accumulation of oxidative stress injury [2].

Panax ginseng as "the king of herbs" has been used as a Chinese traditional medicine for thousands of years in East Asia, known for its various beneficial effects on cardiovascular systems, central nervous, endocrine systems, and on sexual function [3]. Ginsenosides have been regarded as the main active ingredients of *P. ginseng*, and are used as a marker for assessing the quality of

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http://dx.doi.org/10.1016/j.jgr.2016.02.007

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ginseng. There are many reports dealing with ginsenosides' pharmacological effects on oxidative damage. Lu et al [4] have found that ginsenoside Rb1 can significantly and selectively reduce the hydroxyl radical, which is one of the strongest ROS, with unique molecular mechanisms in a cell-free system. Ni et al [5] have reported that ginsenoside Rb1 exhibits potent neuroprotective effects against oxidative injury induced by tert-butyl hydroperoxide. Jiang et al [6] investigated the effects of ginsenoside Ro in PC12 cells under an anoxic or oxidative environment. Cells treated with ginsenoside Ro had a lower content of ROS, and their survival ratio was higher with a lower apoptosis rate. Ma et al [7] investigated the potential beneficial effect of ginsenoside Rg1 on Schwann cells exposed to oxidative injury, which inhibited the detrimental effect of hydrogen peroxide on cell number and cell viability.

A variety of studies concentrate on yields of extractions and antioxidant mechanisms of individual ginsenoside; however, little is known about the underlying effect of different extraction methods on the biological activity of ginsenosides under the premise of higher yields. Pulsed electric field (PEF) has been used for the extraction of ginsenosides, which showed a higher yield than other common methods [8,9]. Several studies have demonstrated that specific treatments, such as heat processing, can improve the medicinal efficacies of ginsenosides such as antioxidant and anticancer activities, and indicated that the improvement of biological activities was related to the structural change of ginsenosides by heat processing [10–12].

Therefore, our study aimed to compare the antioxidant properties of ginsenosides extracted by PEF and solvent cold soak extraction (SCSE) against H_2O_2-induced oxidative stress. In this study, HEK-293 cells were selected for *in vitro* research. HEK-293 cells are immortalized human embryonic kidney cells, and their metabolic conditions are closer to normal human cells compared with tumor cells, thus showing a more realistic oxidative stress status. The HEK-293 cell line has been widely used for studying *in vitro* oxidative damage [13,14]. Previous researches have reported that H_2O_2 was used as a stable source of free radicals to induce oxidative stress in HEK-293 cells [13,15]. The following experiments were explored to investigate the effects of ginsenosides on oxidative damage, which was measured by determining the cell viability and production of ROS, detected by the MTS assay and laser scanning confocal microscopy, respectively. The studies reported here were performed to expand on previous studies to determine the influence of PEF extraction on the biological fate of ginsenosides following incubation with cells, and their effect on cell viability, intracellular ROS, surface morphology, and cellular antioxidant activity against oxidative damage.

2. Materials and methods

2.1. Materials and chemicals

The dried *P. ginseng* roots were obtained from Ji'an, Jilin province in China. The reference standard ginsenosides (Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd) were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), purity $\geq$ 98%. Chromatographic grade methanol, acetonitrile, and acetic acid (Thermo Fisher Scientific, Waltham, MA, USA) were used as received, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (trolox), fluorescein disodium, 2, 2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2, 2-diphenyl-picrylhydrazyl (DPPH), fluorescence probes 2′, 7′-dichloro dihydro fluorescein diacetate (DCFH-DA), and dimethyl sulfoxide were obtained from Sigma–Aldrich (St. Louis, MO, USA). HEK-293 cell lines were purchased from the American Type Culture Collection. Dulbeco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin solution (PSS), and MEM nonessential amino acids was obtained from Gibco (Life Technologies Inc., Grand Island, NY, USA). The Cell Titer 96 AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega Biotechnology Co. Ltd. (Madison, WI, USA). D101 macroporous resin was purchased from Tianjin Pesticide Co. Ltd (Tianjin, China). All other reagents with analytical grade were obtained from Beijing Reagent Company (Beijing, China).

2.2. Preparation of ginsenosides extracted by PEF and SCSE

The dried *P. ginseng* roots were powdered in a pulverizer, and passed through a 120-mesh sieve. The powder were weighed and mixed with 70% (v/v) ethanol–water solution. Subsequently, the mixture were pumped into the PEF system with the conditions of 60 KV/cm electric field intensity, pulse duration of 8 μs, and solid-to-liquid ratio was 1:100 at a flow velocity of 12 mL/min. However, in the SCSE method, the mixture of ginseng powders and ethanol–water solution were added into an erlenmeyer flask and were stirred for 12 h using a magnetic stirrer. When the extractions of the two methods were completed, the ethanol extracts were filtered and evaporated to dryness. The crude saponin fractions were suspended in water and mixed with ether to remove the lipids. Lastly, the ginsenosides were obtained after absorption and disadsorption of D101 macroporous resin and vacuum-rotary evaporation.

2.3. Determination of total ginsenosides contents

The contents of total ginsenosides extracted by PEF and SCSE were determination using the colorimetric method. The standard ginsenoside Re was used to construct a standard curve. The samples were diluted in methanol, and then were mixed with ethanol solution containing 16% vanillin and 77% sulfuric acid solution at 60°C for 15 min. The absorbance of total ginsenosides was detected with spectrophotometry at 544 nm, and the concentration was determined by a standard curve $y = 1.512x + 0.215$ (x, mg/mL, is the content of ginsenoside Re of solution for colorimetric analysis, and y is the absorbance at 544 nm). The experiment was carried out in triplicate and the results were averaged.

2.4. HPLC analysis of ginsenosides extracted by PEF and SCSE

The analysis of ginsenosides extracted by PEF and SCSE were measured according to a protocol described previously with modification [16]. The separation of the ginsenosides was performed on a C-18 analytical column (VP-ODS, 250 mm × 4.6 mm, internal diameter, 5 μm). The detection wavelength was set at 203 nm and the temperature of the column was controlled at 35°C. The gradient elution solvent consisted of acetonitrile (A) and water (B). The process of elution was carried out as follows: 0–24 min, 18–22% A, 82–78% B; 24–26 min, 22–26% A, 78–74% B; 26–30 min, 26–32% A, 74–68% B; 30–50 min, 32–35% A, 68–65% B; 50–55 min, 35–38% A, 65–62% B; and 65 min, 38% A, 62% B. The flow rate was kept at 1.0 mL/min, and the injection volume was 20 μL. Standard ginsenosides (Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd) were mixed and diluted with chromatographic methanol as well as the samples. All solutions were filtered with a nylon filter membrane (0.45 μm) prior to the HPLC analysis.

2.5. DPPH radical scavenging assay

DPPH radical scavenging activity of ginsenosides was measured with a modified version of colorimetric method [17]. One hundred and fifty microliters of 2mM DPPH solution in ethanol was mixed

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with different concentrations of the sample solution (2 mg/mL, 1 mg/mL, and 0.5 mg/mL) in 96-microwell plates. After the mixture was incubated for 30 min in darkness at room temperature, the absorbance of solution was determined with a multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA) at 517 nm. For the blank, 150 μL of ethanol was used instead of the sample. trolox was used as a positive control compound. The DPPH radical scavenging activity was calculated as the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{blank} - A_{sample})/A_{blank}] \times 100\% \quad (1)$$

2.6. Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay was conducted using fluorescein (FL) according to a protocol described previously [18] with some modifications. With the exception of ginsenosides which were prepared with 70% ethanol, all other reagents were prepared in 75mM phosphate buffered saline (PBS; pH 7.4). The final mixture contained 20 μL of 210nM FL, 60 μL of 36mM AAPH, 20 μL of ginsenosides (0.01 μg/mL, 0.1 μg/mL, 1 μg/mL, and 10 μg/mL), or PBS for a reagent blank, which were placed in black 96-microwell plates. Standards and samples were run in quadruplicate simultaneously using a multi-mode microplate reader (BioTek Instruments) immediately. The fluorescence value of the assay mixture was recorded every minute for 3 h at 37°C, with an excitation wavelength of 485 nm and emission wavelength of 528 nm. Final results were calculated using the relative area under the FL decay curves between the blank and sample. The results were then expressed as micromoles trolox equivalent (TE).

2.7. Cell culture

HEK-293 cells were cultivated in DMEM supplemented with 10% FBS, 1% PSS, and 1% MEM nonessential amino acids at 37°C in 5% CO_2 and 95% saturated atmospheric humidity. Growth medium was replaced once every 2–3 d until the cells attained confluence. HEK-293 cells were seeded in flat bottom 96-well plates (100 μL/well) at a density of 5–6 $\times$ 10^3 cells/well and then incubated in a CO_2 incubator overnight, until all cells adhered to the wall. HepG2 cells were cultivated in DMEM supplemented with 10% FBS and 1% PSS at 37°C in a humidified atmosphere of 5% CO_2. Growth medium was replaced once every 2–3 d until the cells attained confluence.

2.8. Cell viability assay

Cell viability was determined with the MTS assay as previously mentioned [19], which used the Cell Titer 96 AQueous One Solution Cell Proliferation Assay Kit (Promega Biotechnology Co. Ltd.) according to the manufacturer's instructions. MTS (20 μL) was added to the wells after treatment of four groups, followed by incubation at 37°C, 5% CO_2 for 1–6 h. The plates were then read in a multi-mode microplate reader (Bio Tek Instruments) at 490 nm wavelength. The results were expressed as the mean optical density of each group and dose. All the experiments were repeated at least three times.

2.9. Establishment of hydrogen peroxide-induced oxidative injury model

HEK-293 cells were cultivated in DMEM complete media, which was placed with 90 μL/well at 37°C, 5% CO_2 for 24 h. HEK-293 cells were treated with 100μM, 200μM, 300μM, 400μM, 500μM, 600μM, 700μM, and 800μM hydrogen peroxide (H_2O_2, 10 μL/well) for 6 h. Normal HEK-293 cells without H_2O_2 treatment were used as a control group.

2.10. Cytotoxic assessment of the ginsenosides

HEK-263 cells were seeded in a 96-well plate of 5 $\times$ 10^3 cells/well and incubated for 24 h. Then, the cells were treated with the ginsenosides dissolved in dimethyl sulfoxide and DMEM medium at different concentrations (10 μg/mL, 20 μg/mL, 40 μg/mL, and 100 μg/mL). They were then incubated for an additional 24 h at 37°C, 5% CO_2. Cell viability was determined using the MTS method as described above.

2.11. Protective effects of ginsenosides against oxidative stress in HEK-293 cells

HEK-263 cells were seeded in a 96-well plate of 5 $\times$ 10^3 cells/well and incubated for 24 h. The 10-μL culture medium which contained 10 μg/mL, 20 μg/mL, and 40 μg/mL ginsenosides (extracted from PEF and SCSE) were incubated with cell culture medium for 24 h. Subsequently, 400μM of H_2O_2 was added after preincubation with ginsenosides, after which culturing was performed for another 6 h. Normal HEK-293 cells without ginsenosides and H_2O_2 treatment were used as a control. DMEM and H_2O_2 were then added to the treatment without the addition of ginsenosides as a damage group. Cell viability was determined using the MTS method as described above.

2.12. Measurement of intracellular ROS

Production of intracellular ROS was assessed using DCFH-DA as described previously [20]. HEK-293 cells were seeded in a 24-well plated of 5 $\times$ 10^4 cells/well and incubated for 24 h. The 100-μL culture medium which contained 10 μg/mL, 20 μg/mL, and 40 μg/mL ginsenosides (extracted from PEF and SCSE) were incubated with cell culture medium for 24 h. Subsequently, 400μM of H_2O_2 was added after preincubation with ginsenosides, after which culturing was performed for another 6 h. The medium was removed after treatment of HEK-293 cells with ginsenosides and H_2O_2. After washing once with PBS, the cells were incubated with DCFH-DA (10μM) for 20 min at 37°C in the dark. The cells were washed with PBS three times. The DCFH-DA was oxidized to a highly fluorescent compound dichlorofluorescein (DCF) by intracellular H_2O_2 or low molecular-weight peroxides. The green fluorescence intensity of DCF was measured with laser scanning confocal microscopy (Olympus, Tokyo, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm, which was maintained at 37°C in the dark.

2.13. Microscopic analysis of HEK-293 cells surface morphology

The surface morphology of HEK-293 cells was characterized with scanning electron microscopy (SEM) using the methods described previously [21,22]. HEK-293 cells grown on round-glass coverslips in 24-well plates, which were rinsed three times with PBS after treatment with the ginsenosides and H_2O_2, were fixed with 2.5% glutaraldehyde at a pH 7.4 phosphate buffer for 1 h at 4°C, followed by postfixation in 1% osmium tetroxide for 30 min at 4 C and dehydrated through a series of alcohol from 30% to 100%. The attached cells were lastly dried by lyophilization and sputter coated with gold under vacuum before examination with Hitachi S-3400N (Hitachi, Tokyo, Japan) scanning electron microscope at 5.0 kV at different magnifications.

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2.14. Cellular antioxidant activity of ginsenosides

Cellular antioxidant activity (CAA) assay was performed as described in previous report [23] with a few modifications. Briefly, HepG2 Cells were seeded in a 96-well plate of 6×10^4 cells/well in 100 μL of growth medium. After incubating for 24 h at 37°C and 5% CO_2, the medium was removed and the cells were washed with PBS. The wells were treated for 1 h with 100 μL of treatment medium containing different concentrations of ginsenosides and 25μM DCFH-DA. The treatment media were removed and the wells were then washed with 100 μL of PBS three times, to remove the antioxidants in the medium not associated with the cells. Then, 100 μL of 600μM AAPH solution (dissolved in Hanks' balanced salt solution) was applied to the cells, and the 96-well plate was transferred to a multi-mode microplate reader at 37°C. The emission wavelength at 538 nm and excitation wavelength at 485 nm was read every 5 min for 1 h. This was repeated at least three times for all the groups. Each plate included a triplicate control and blank wells. The control group contained cells treated with DCFH-DA and oxidant, blank wells contained cells treated with dye and Hanks' balanced salt solution without oxidant.

After blank subtraction from the fluorescence readings, the area under the curve of fluorescence versus time was integrated to calculate the CAA value of each group as follows:

$$\text{CAA unit} = 100 - \left(\int_{SA} \Big/ \int_{CA}\right) \times 100 \quad (2)$$

where $\int_{CA}$ is the integrated area in the control curve and $\int_{SA}$ is the integrated area under the curve of different concentration of ginsenosides. The median effective concentration (EC_{50}) of ginsenosides was calculated from the plot of log (fa/fu) versus log (concentration), where fa is the fraction affected by the treatment (CAA unit) and fu is the fraction unaffected (100-CAA unit) by the treatment. In each experiment, trolox was used as a standard, and the results were expressed as μmol of TE per 100 μg of ginsenosides. EC_{50} values were used to convert to CAA values and were expressed as μM of TE per 100 μg of ginsenosides.

2.15. Statistical analysis

All of the assays were carried out in triplicate. Data were analyzed using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). The results were expressed as the mean ± standard deviation. The statistical significance of differences between two groups was determined with the one-way analysis of variance program. Probability values of < 0.05 were considered significant ($p < 0.05$, $p < 0.01$, $p < 0.001$).Significant differences between means were identified using least significant difference procedures.

3. Results and discussion

3.1. Analysis of ginsenosides extracted by PEF and SCSE

The colorimetric method was used to determine the total content of ginsenosides. The contents of total ginsenosides extracted by PEF and SCSE were 75.7275% and 73.0820%, respectively. HPLC was used to analyze the seven individual ginsenosides Rg1, Re, Rb1, Rf, Rc, Rb2, and Rd. The peaks of the extracts were identified by comparing with standard ginsenoside according to retention time, which were carried out under the same conditions. The observed changes in individual ginsenosides of PEF and SCSE extracts are shown in Table 1. The contents of the ginsenosides varied with PEF and SCSE extracts. The ginsenosides Rg1, Rf, and Rc contents of PEF extract was similar to the SCSE extract. The contents of ginsenosides Rb1, Rb2, and Re decreased with PEF treatment, whereas the ginsenoside Rd content of the PEF extract was higher than that of the SCSE extract. It seems that ginsenoside Rd increased due to the conversion of some other ginsenosides.

Table 1
Changes of ginsenoside content by pulsed electric field (PEF) and solvent cold soak extraction (SCSE) extraction

Ginsenoside	Retention time (min)	PEF extraction yield (%)	SCSE extraction yield (%)
Rg1	28.872	9.3155 ± 0.6348	9.3813 ± 0.3654
Re	29.387	9.8013 ± 0.3641	10.5913 ± 0.4369
Rf	37.058	2.5524 ± 0.2367	2.5096 ± 0.1364
Rb1	38.936	12.8518 ± 0.4637	14.2968 ± 0.5621
Rc	41.155	10.4107 ± 0.5651	10.5953 ± 0.3624
Rb2	44.016	5.5073 ± 0.1639	6.2748 ± 0.2031
Rd	51.548	10.5329 ± 0.5963	5.8520 ± 0.1934

Data represent the average value of three extraction samples ± standard deviations

3.2. DPPH radical scavenging activity of ginsenosides extracted by PEF and SCSE

DPPH is long-lived nitrogen radical, which is used as one of the few stable and accurate methods for measuring the antioxidant capacity of the nutrient contents from flora and fauna [24]. The DPPH radical scavenging activity of ginsenosides was investigated at concentrations of 0.5 mg/mL, 1 mg/mL, and 2 mg/mL. According to the results shown in Fig. 1, the scavenging capacity in the middle-dose group and high-dose group treated with PEF extracts were significantly higher than that with SCSE extracts ($p < 0.05$), which indicated that the free radical scavenging activity of extracts was affected by PEF treatment. Similar antioxidant scavenging trials also demonstrated that the ultrahigh pressure extraction method can produce ginsenosides with stronger DPPH radical scavenging activity compared with microwave extraction, ultrasound extraction, Soxhlet extraction, and heat reflux extraction methods [25]. Generally, one single chemical assay could not accurately reflect the antioxidant capacity of the substances, so different assay methods should be applied for comprehensive evaluation of antioxidant activity.

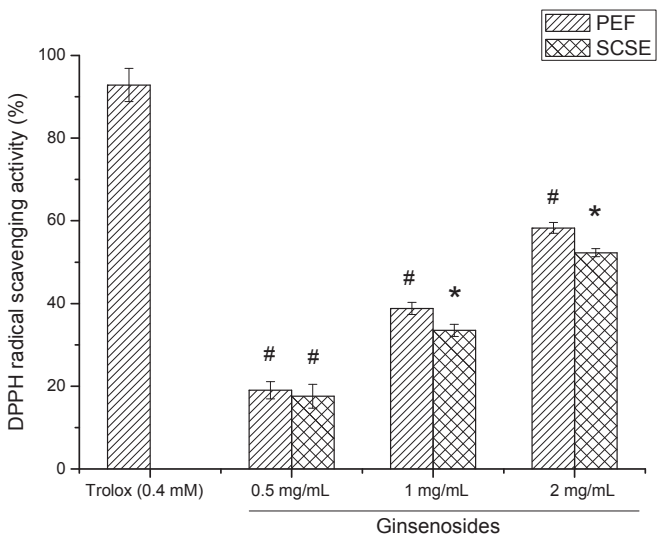

Fig. 1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of ginsenosides extracted by pulsed electric field (PEF) and solvent cold soak extraction (SCSE). Trolox (0.4mM) was used as a positive control. All tests were conducted in triplicate, and mean values are used. The vertical bars represent the standard deviation of each data point. Values marked by different symbols means significant ($p < 0.05$).

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3.3. ORAC of ginsenosides extracted by PEF and SCSE

The ORAC assay has been found to be widely used in measurements and the quantification of antioxidant capacity of botanical ingredients, which reflects relatively dynamic information on radical chain-breaking capacity with peroxy radicals [26].

In this study, the ORAC was evaluated to measure the capacity of ginsenosides extracts to scavenge peroxy radicals, which is based on hydrogen atom transfer reaction mechanism. ORAC values were expressed as TE. The effects of ginsenoside extracts on the time-dependent decay of fluorescein induced by AAPH are shown in Fig. 2. The results showed that both PEF extracts and SCSE extracts exhibited significant ORAC values, and had a concentration-dependent growth. The ginsenosides extracted by PEF were found to have higher inhibition of fluorescein decay than that extracted by SCSE, and it showed the highest ORAC value of 18.3μM trolox per 10 μg/mL PEF extracts (Table 2). It was noted that the ORAC value of PEF extracts was 8% (1.4μM trolox) greater than that of SCSE extracts (Table 2), which demonstrated that the PEF extract had better electron-donating capacity and stronger ORAC. This may be partially explained by the procedure used to prepare the ethanol extracts for the ORAC test. Furthermore, these data also indicated the potential effects of PEF treatment used in extracting, which may improve the extracting efficiency and change the composition of ginsenosides resulting in enhancing the antioxidant activity.

Table 2
Oxygen radical absorption capacity (ORAC) value of ginsenosides extracted by pulsed electric field (PEF) and solvent cold soak extraction (SCSE)

Method	ORAC (TE μM)			
	0.01 μg/mL	0.1 μg/mL	1 μg/mL	10 μg/mL
PEF	7.03 ± 1.87	11.80 ± 3.17	14.48 ± 4.04	18.30 ± 4.73
SCSE	5.57 ± 1.15	9.35 ± 1.47	12.45 ± 3.64	16.95 ± 2.29

Data represent the average value of three extraction samples ± standard deviations
TE, trolox equivalent

3.4. Cytotoxicity or proliferation promotion effects of ginsenosides extracted by PEF and SCSE on HEK-293 cells

An effective *in vitro* assay is an important and precious tool for clinical studies if it is combined with efficient oxidative stress biomarker assays [26]. Some *in vitro* assays have demonstrated that the antioxidant effects of ginsenosides may not only be dependent on the direct scavenging of free radicals, but also be mediated by activation and completion of intracellular antioxidant systems [27]. In this study, several methods were employed to determine whether treatment with the ginsenosides extracted by the two methods could alter the response of HEK-293 cells to H_2O_2.

To evaluate the activity of ginsenosides extracted by PEF on protecting HEK-293 from oxidative injury induced by H_2O_2, stimulation or cytotoxicity effects were tested first. The effects of ginsenosides extracted by PEF and SCSE on the viability of HEK-293 cells were assessed by the MTS assay. Fig. 3 showed that the ginsenosides of the two methods had no negative effect on the cell viability at concentrations from 10 μg/mL to 40 μg/mL, but the cell viability had a decrease at 100 μg/mL, which proved that cell viability was not affected by ginsenosides at a concentration less than 40 μg/mL, while the ginsenosides have cytotoxicity on HEK-293 cells at high concentrations. In this study, the results showed that the effects of PEF and SCSE extracts on the growth and inhibition of cultures were not significant under the concentration of 40 μg/mL (Fig. 3), and the concentrations below 40 μg/mL were chosen to use in subsequent experiments.

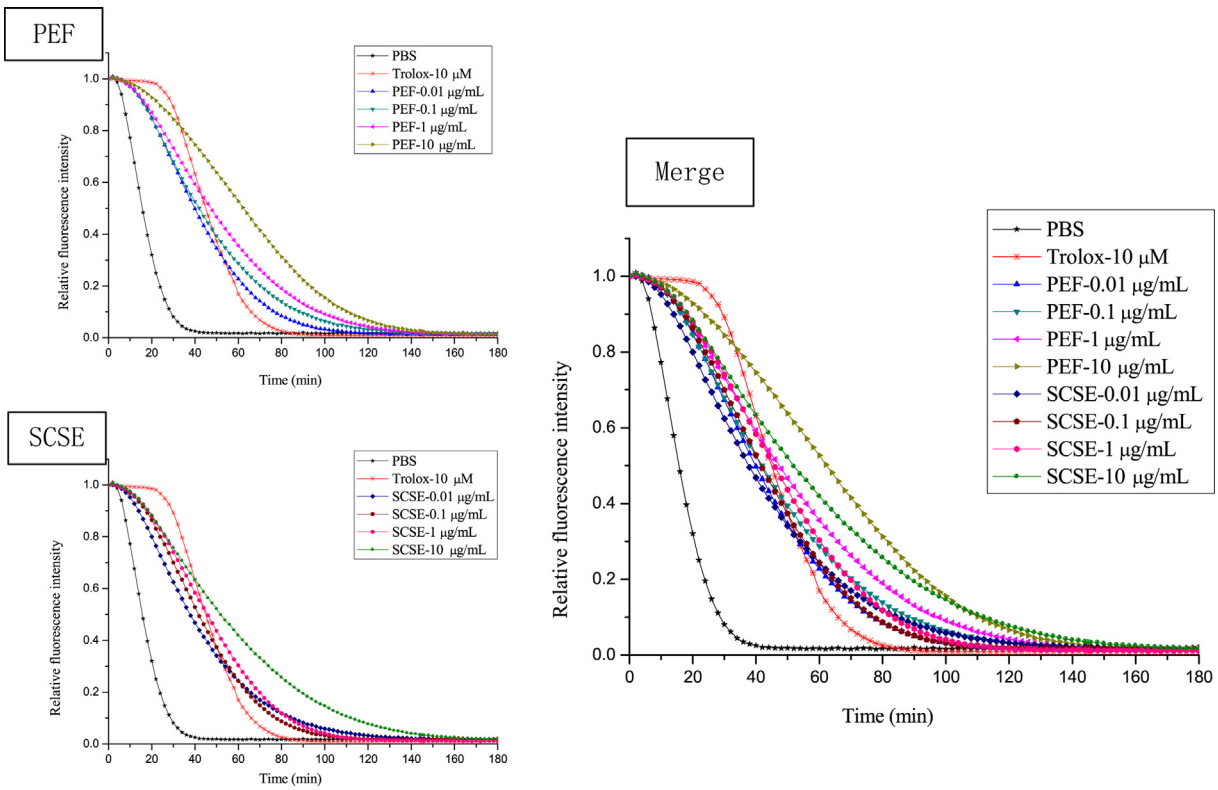

Fig. 2. Oxygen radical absorption capacity of ginsenosides extracted by pulsed electric field (PEF) and solvent cold soak extraction (SCSE; oxygen radical absorption capacity assay). Phosphate-buffered saline was used as a control group. Trolox (10μM) was used as a positive control.

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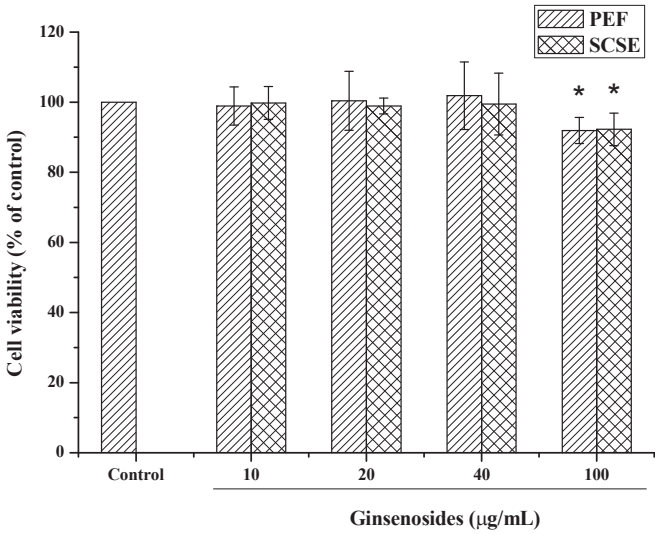

Fig. 3. Cytotoxicity effects of ginsenosides extracted by pulsed electric field (PEF) and solvent cold soak extraction (SCSE) on human embryonic kidney-293 cells. Cells were treated with the ginsenosides at the indicated concentrations (10 μg/mL, 20 μg/mL, 40 μg/mL, and 100 μg/mL) for 24 h. Cell viability was assessed by MTS assay. Vertical bars indicate mean values ± standard deviation. $^{*}p < 0.05$ compared with the control group.

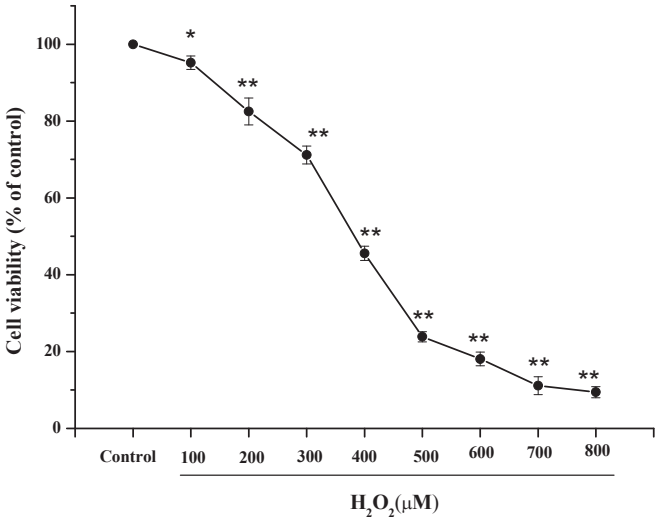

Fig. 4. Cytotoxicity effects of H_2O_2 on viability of human embryonic kidney-293 cells. Cells were treated with H_2O_2 at the indicated concentrations (from 100μM to 800μM) and after 6 h cell viability was assessed by MTS assay. $^{*}p < 0.01$ compared with the control group. $^{**}p < 0.001$ compared with the control group.

3.5. *Protective effects of ginsenosides extracted by PEF and SCSE against H_2O_2-induced cell damage*

Oxidative stress is caused by increased free radical production and poorer antioxidant defenses, which is thought to be one of the main contributing factors to aging, inflammation, and some chronic diseases. In this study, the capability of the extracts to scavenge free radicals in a cell culture model induced by H_2O_2 was also evaluated. H_2O_2 has been extensively used as an exogenous trigger of oxidative stress *in vitro* models, which is a potential source for hydroxyl radicals. Exogenous H_2O_2 can traverse cell membranes to generate the accumulation of ROS, react with intracellular metal ions, and cause DNA damage, and dysfunctional mitochondria through the cumulative damage, even in part H_2O_2-mediated cell death [28]. Therefore, H_2O_2 was used as a typical chemical for the investigation of oxidative stress [29].

In our study, H_2O_2-induced HEK-293 cell model was constructed to continue the biological research and exploration about oxidative stress inhibitory properties of ginsenoside extracts. The HEK-293 cells were treated with different concentrations of H_2O_2 (100–800μM) for 6 h and the cell viabilities were evaluated by MTS assay. In Fig. 4, the cell viability of HEK-293 cells exhibited a dose-dependent decrease. The minimum dose for H_2O_2 to significantly decrease was 100μM, and the cell viability was approximately decreased by 90.58% with treatment of the maximum dose. The cell viability decreased to 45% due to the oxidation of 400μM H_2O_2. In subsequent experiments, 400μM of H_2O_2 was chosen as the optimal injury concentration to evaluate the protective effect of ginsenosides against oxidative stress. Liu et al [13] observed a similar result when establishing the oxidative damaged HEK-293 cell model in a study of the antioxidative and antiapoptosis effects of egg white peptide.

Ginsenosides have already been shown to have the protective effects on intracellular defense against oxidative stress [30]. Xie et al [27] showed that ginsenoside Re can protect cardiomyocytes from oxidant injury, which may attribute to scavenging H_2O_2 and hydroxyl radicals. Previous studies have showed that other ginsenosides such as ginsenoside Rb1, Ro, Rd, and Rg1 could protect cells or help them recover from the H_2O_2-induced cell damage [6,7,31,32].

The ability of scavenging free radicals has been shown in the DPPH radical scavenging assay and ORAC assay in previous studies; therefore, we speculated that the PEF and SCSE extracts might have a potential intervention effect against with oxidative stress damage of H_2O_2. In this study, the abilities of ginsenosides extracted by PEF and SCSE to modulate H_2O_2-induced cell damage were examined. In Fig. 5, when HEK-293 cells were exposed to 400μM hydrogen peroxide without pretreatment of ginsenosides, the cell viability was decreased compared with the control group. By contrast, the cell viabilities of the pretreatment groups were higher than that of the damage group. However, the PEF extracts could alleviate the H_2O_2-induced damage in a dose-dependent manner, which showed significant protective effects not only at low concentrations but also at high ones ($p < 0.05$). While for the SCSE, it showed no significantly effective effects at any concentration (10 μg/mL, 20 μg/mL, and 40 μg/mL). The protective effects of ginsenosides extracted by PEF were significant stronger than that by SCSE ($p < 0.05$; Fig. 5). Similar research revealed that ginseng was more useful as a functional biomaterial with strong antioxidant activity and protective effects after treatment with steaming and drying [33]. It seems that the preventive effect of ginsenosides on oxidative damage to HEK-293 cells was greater after being treated with PEF. Therefore, we assumed that PEF treatment might enhance the ability of ginsenosides to protect cells from oxidative damage, just like super-high pressure and heating. To verify this hypothesis, further experiments were developed.

3.6. *Ginsenosides extracted by PEF and SCSE ameliorated H_2O_2-induced intracellular ROS accumulation*

The state of oxidative stress can be obviously changed by some oxidant compounds such as ROS and reactive nitrogen species, the radical chain reactions can be stopped by the antioxidant through scavenging the ROS or reactive nitrogen species, or prevented from being formed in the first place [26]. The ability of cells to maintain the level of ROS can indicate the status of cellular redox. The generation and elimination of ROS in biological systems was continuous, which is closely in touch with biochemical functions and

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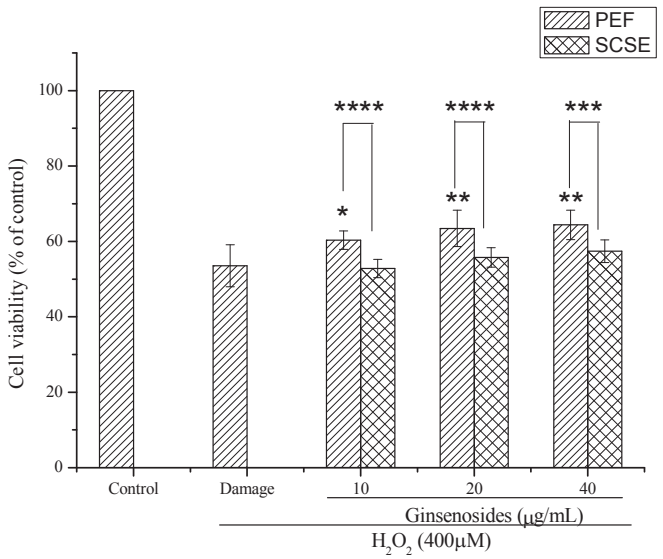

Fig. 5. Protective effects of ginsenosidesextracted by pulsed electric field (PEF) and solvent cold soak extraction (SCSE) against H_2O_2-induced oxidative damage in human embryonic kidney-293 cells. Cells were treated with the ginsenosides at the indicated concentrations (10 μg/mL, 20 μg/mL, and 40 μg/mL) for 24 h. The viability of cells after H_2O_2 treatment for 6 h was assessed by MTS assay. Vertical bars indicate mean values ± standard deviation. $^{*}p < 0.05$ compared with the H_2O_2 treated group. $^{**}p < 0.01$ compared with the H_2O_2 treated group. $^{***}p < 0.05$. $^{****}p < 0.01$ compared between PEF and SCSE group.

pathological processes of organisms [34]. H_2O_2 can traverse cell membranes and partly cause oxidation of various intracellular targets. DCFH-DA can cross cell membrane and be catalyzed by intracellular esterases, then oxidized by intracellular ROS and turn to DCF fluorescent. Therefore, the intracellular ROS levels can be represented by the intensity of DCF fluorescent. Previous studies over 10 yr have indicated that ginsenosides derived from *Panax* species target ROS; therefore, they may prevent several diseases induced by oxidative stress [35]. Exposure of astrocytes to H_2O_2 increased ROS formation, and all the tested ginsenosides (Rb1, Rb2, Rc, Rd, Re, and Rg1) reduced the ROS formation, with ginsenoside Re being the most active component [36]. Ginseng root extracts have similar effects with purified ginsenoside monomers. H_2O_2-induced ROS generation was significantly reduced by treatment with saponin fractions of white or red ginseng roots [33]. Red ginseng extract ameliorated ROS production in rats with gentamicin induced acute kidney injury [37].

To determine whether the ginsenosides extracted by PEF and SCSE decrease ROS production during oxidative stress induced by H_2O_2 in HEK-293 cells, cells were exposed to H_2O_2 (400μM) and DCFH-DA. As shown in Fig. 6A, compared with the control group (a), green fluorescence intensity displayed considerable increase after cells were incubated with H_2O_2 (b). However, the intensity of green fluorescence decreased markedly in the group that pretreated with ginsenosides compared with damage group, and exhibited a dose-dependent decrease (c, d, e, f, g, and h). Compared with the SCSE group, it was seen that the fluorescence intensity of the PEF group decreased even more.

As shown in Fig. 6B, the DCF fluorescence of the control group changed very little, indicating that the intracellular ROS formation was stable under normal physiological conditions. When HEK-293 cells were treated only with 400μM H_2O_2, the DCF fluorescence significantly increased. Pretreatment with PEF or SCSE extracts suppressed the increase of DCF fluorescence in a dose-dependent fashion (Fig. 6B). Moreover, PEF extracts were more potent than that of SCSE at the concentration of 10 μg/mL and 40 μg/mL, with approximately 46% and 38% fluorescence intensity reduction at the highest concentration (40 μg/mL) compared with the damage group, respectively (Fig. 6B). These data demonstrated that ginsenosides extracted by PEF and SCSE protect HEK-293 cells from H_2O_2-induced oxidative stress by eliminate intracellular ROS generation, and PEF extracts seem more active. The results of similar studies about other treatments implied that the antioxidant activities of hydroponic-cultured ginseng roots and leaves can be enhanced by heat treatment [38]. ROS formation was decreased by the pretreatment with ginsensosides of white ginseng and ginsensosides of red ginseng, and heat treated red ginseng was generally more effective than white ginseng in reducing hepatic damage by oxidative stress [33]. Our results are parallel to their study result that some treatment methods of ginseng are helpful for reducing the ROS level of cells.

3.7. The surface morphology of HEK-293 cells

The surface morphology of human aortic endothelial cells was observed with a scanning electron microscope, and the results of surface changes indicated that the total saponins of *P. ginseng* could exert an inhibitory effect on angiotensin II-induced damage of human aortic endothelial cells [22].

The SEM images of HEK-293 cells exposed to H_2O_2 and ginsenosides extracted by PEF and SCSE are shown in Fig. 7. Fig. 7A shows the physical characteristics of normal cells, the morphological characteristics of the control group maintained integral cell shape, and cell tight junctions were closely packed with no apparent intercellular spaces. It is clear that there are significant morphological changes in the damage group (Fig. 7B) compared with the control group. HEK-293 cells demonstrated obvious cell shrinkage and membrane fracture when exposed to 400μM H_2O_2, which suggests the occurrence of cell apoptosis. With the protection of the ginsenosides extracted by PEF (Fig. 7C) and SCSE (Fig. 7D), the cell maintained a relatively intact cell morphology and no obvious membrane damage was found. But compared with the control group, the cells showed slight shrinkage and the morphology changed from a polygonal shape to a round shape, and some cell tight junctions were partly destroyed and cells arranged loosely. Furthermore, the cells pretreated with PEF extracts showed better protection with normal morphological characteristics, which exhibited plumpness and were round in size. In this study, compared with the damage group, the ultrastructure of the cells in treatment with ginsenosides extracted by PEF and SCSE were more complete compared with the damage group (Fig. 7B), and displayed no obvious apoptosis morphology (Fig. 7). In addition, PEF extracts maintained a better intact cell shape compared with the SCSE group. These data further provided the evidence that ginsenosides extracted by PEF exhibited more efficient protection from the oxidative damage of cells induced by H_2O_2.

There was a study that demonstrated that different interactions between sugar moieties and the central structure generate various ginsenosides, which play different roles in antioxidative and prooxidative activity [39]. Many reports suggested that ginseng saponins are capable of accessing intracellular locations thanks to their steroid-like structures, justifying their ability to suppress oxidative stress caused by a variety of stimuli [39,40]. In addition, it has been known that heat processing can also facilitate production of ginsenoside 20 (S)-Rg3 and the generation of 20 (S)-Rg3 increases·hydroxyl radical scavenging activity [41]. Ginsenoside F2, F4, Rk3, Rh4, Rg3 (S form), Rg3 (R form), Rk1, and Rg5 were formed after heat treatment, which were absent in the raw ginseng [38]. The high molecular weight ginsenosides, such as Re, can be specifically hydrolyzed to the low molecular weight ones through an ultra high-pressure process [42]. The study about PEF on the

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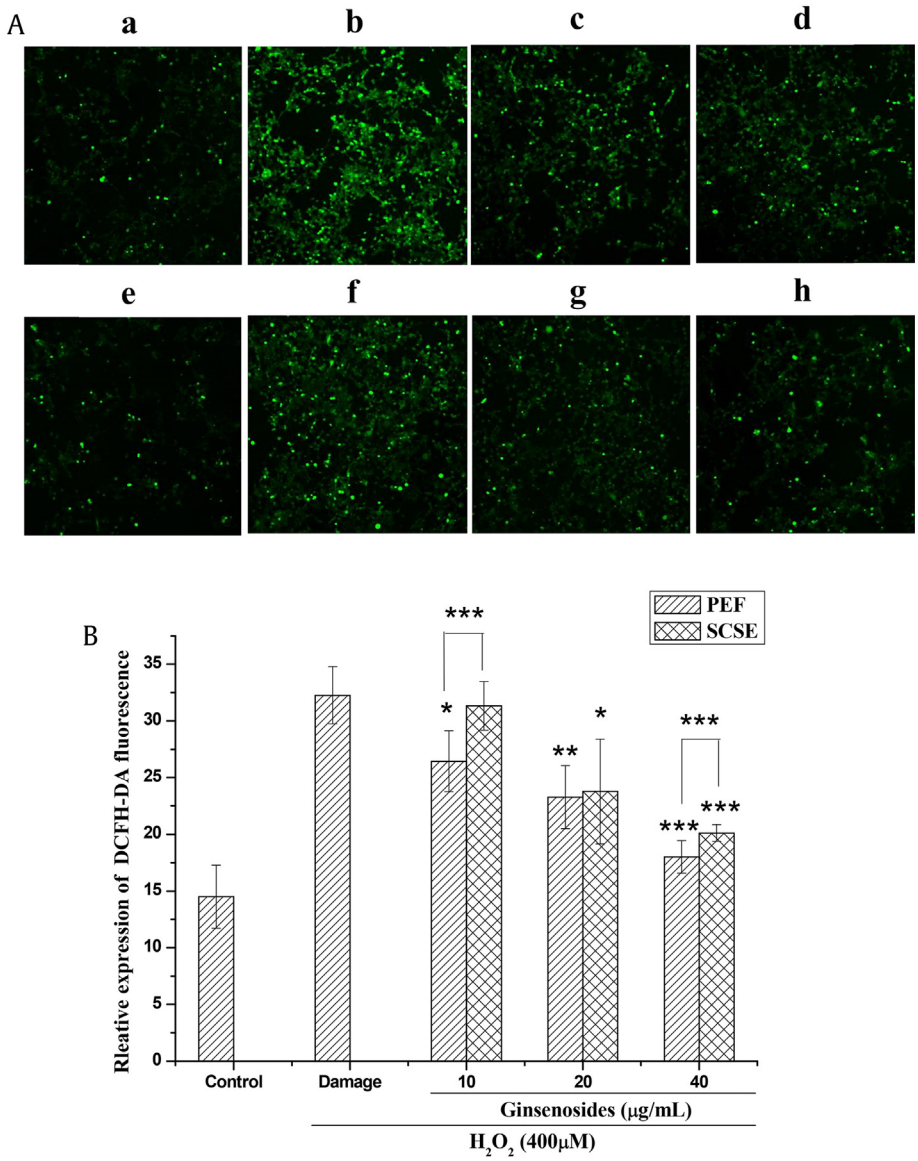

Fig. 6. Effects of Ginsenosides extracted by pulsed electric field (PEF) and solvent cold soak extraction (SCSE) on H_2O_2-induced reactive oxygen species overproduction. (A) Fluorescence images of 2′,7′-dichlorofluorescein in human embryonic kidney-293 cells were collected by laser scanning confocal microscopy. [(a), control group; (b), damage group (400μM H_2O_2 treated); (c), 10 μg/mL PEF extracts + 400μM H_2O_2; (d), 20 μg/mL PEF extracts + 400μM H_2O_2; (e), 40 μg/mL PEF extracts + 400μM H_2O_2; (f), 10 μg/mL SCSE extracts + 400μM H_2O_2; (g), 20 μg/mL SCSE extracts + 400μM H_2O_2; (h), 40 μg/mL SCSE extracts + 400μM H_2O_2.] (B) Production of intracellular reactive oxygen species was measured using the fluorescence probe dichloro-dihydro-fluorescein diacetate (DCFH-DA). The fluorescence intensity was determined by a multi-mode microplate reader (BioTek Instruments) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Vertical bars indicate mean values ± standard deviation. $^{*}p < 0.05$ compared with H_2O_2 treated group. $^{**}p < 0.01$ compared with H_2O_2 treated group. $^{***}p < 0.05$ compared between the PEF and SCSE groups.

extraction of ginseng soluble components indicated that the soluble solid content can be increased by application of PEF, and the sugar and free sugar content of the extract is significantly reduced compared with non-PEF-treated samples [9]. Therefore, relatively prominent antioxidant activity of PEF extracts in our study was thought to be due to a different composition of ginsenosides from ginseng. As some researchers have mentioned, the increased antioxidant activity of ginseng may result from the change of total phenolic compound contents and structure of ginseng after heating processing [43,44].

3.8. Cellular antioxidant activity of ginsenosides extracted by PEF and SCSE

The cellular antioxidant activities of ginsenosides extracted by PEF and SCSE were conducted by another biologically relevant assay, CAA assay, which was regarded physical characteristics more likely than common chemical antioxidant activity assays. For the CAA assay, the exogenous probe DCFH-DA was catalyzed to DCFH by cellular esterases in HepG2 cells, and then oxidized by AAPH-induced peroxyl radicals and which turns to DCF with fluorescence. The fluorescence intensity of intracellular DCF was proportional to the degree of oxidation. The attenuation of cellular fluorescence compared with the control group represents the antioxidant activity of the tested compounds [23,45].

As shown in Fig. 8, the dynamics of DCFH oxidation in HepG2 cells induced by 2,2′-azo-bis-amidinopropane radicals is well observed for ginsenoside extracts. The fluorescence intensity of intracellular DCF was attenuated by PEF and SCSE ginsenoside extracts in a dose-dependent manner for the concentration range of 1–200 μg/mL (Figs. 8A and 8B). The median effective concentration (EC_{50}) for the inhibitory action of ginsenosides on DCFH oxidation

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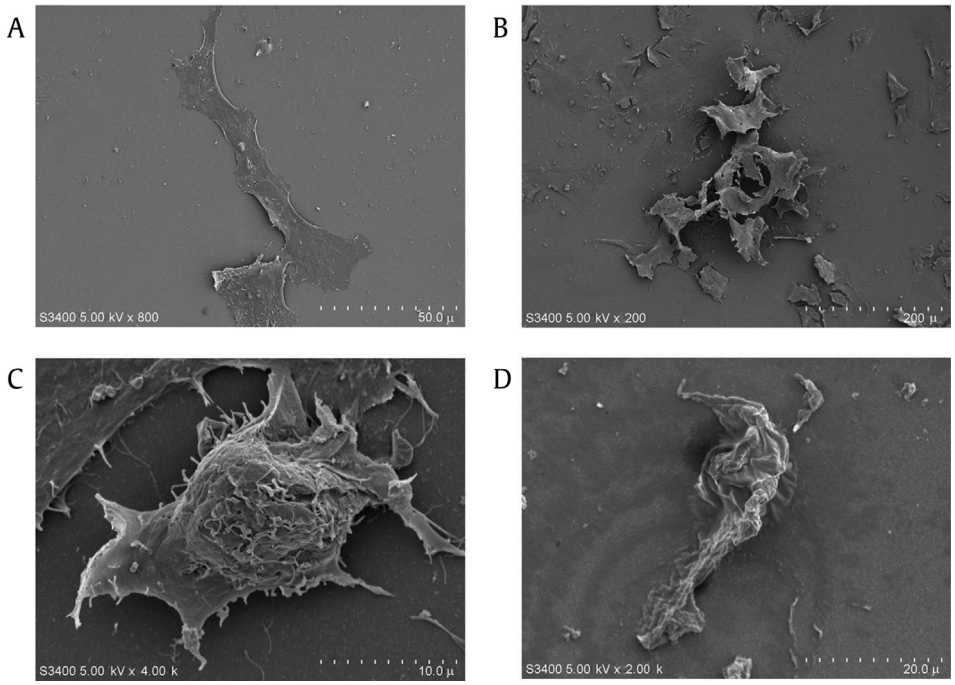

Fig. 7. The scanning electron microscopy images of human embryonic kidney-293 cells after H_2O_2 treatment in the absence or presence of ginsenosides. (A) The control group. (B) The damage group (400μM H_2O_2 treated). (C) The 40 μg/mL pulsed electric field extracts + H_2O_2. (D) The 40 μg/mL solvent cold soak extraction extracts + H_2O_2.

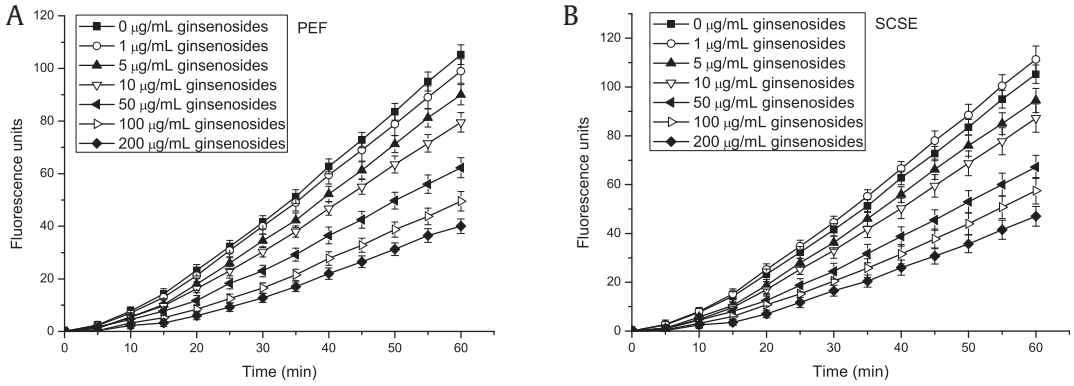

Fig. 8. The inhibition of peroxyl radical-induced DCFH oxidation by ginsenosides extracted through PEF (A) and SCSE (B) over time. The curves shown in each graph are from a single experiment (mean ± standard deviation, $n = 3$).

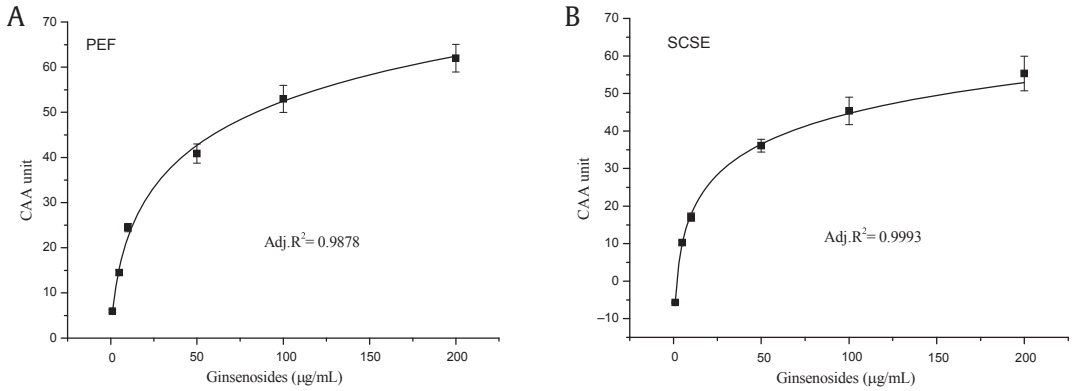

Fig. 9. Dose-response curves for inhibition of peroxyl radical-induced DCFH oxidation by ginsenosides extracted through PEF (A) and SCSE (B). The curves shown are each from a single experiment (mean ± standard deviation, $n = 3$). Adj., adjusted; CAA, cellular antioxidant activity.

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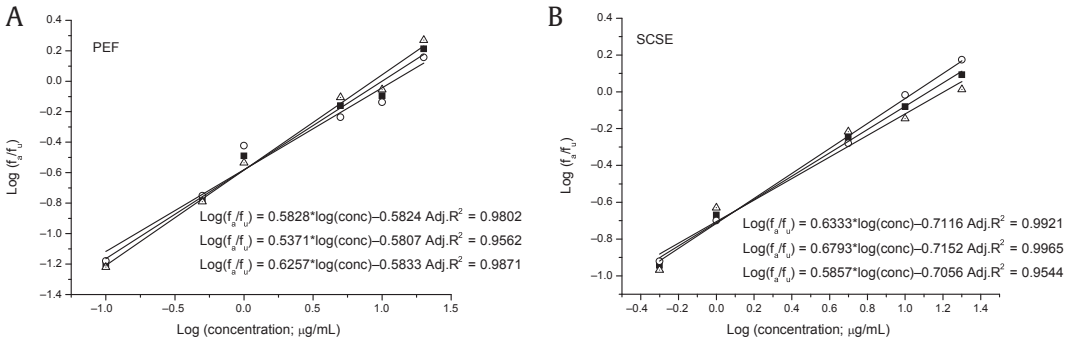

Fig. 10. Median effect plots for inhibition of peroxyl radical-induced DCFH oxidation by ginsenosides extracted through PEF (A) and SCSE (B). The curves shown are from a single experiment ($n = 3$). Adj., adjusted.

Table 3
Half maximal effective concentration (EC_{50}) values and cellular antioxidant activity (CAA) values for the inhibition of peroxyl radical-induced dichlorofluorescin oxidation by ginsenosides extracted through pulsed electric field (PEF) and solvent cold soak extraction (SCSE)[1)]

Method	EC_{50} (μg/mL)	CAA (μmol TE per 100 μg/mL ginsenosides)
PEF	60.22 ± 2.69[a]	47.53 ± 4.17[a]
SCSE	150.03 ± 4.03[b]	21.46 ± 2.55[b]

Data represent the average value of three extraction samples ± standard deviations
[1)] Values with no letters in common in a column are significantly different ($p < 0.05$)

was calculated from the dose-response curves and median-effect curve plotted for each concentration and are shown in Figs. 9, 10, respectively. On the basis of the fitting curves, the EC_{50} values and CAA values of ginsenosides extracted by PEF and SCSE are listed in Table 3. The EC_{50} value of ginsenosides extracted by PEF was 60.22 ± 2.69 μg/mL, which was more than twice lower than that of SCSE (150.03 ± 4.03 μg/mL, $p < 0.05$). This result indicating that PEF ginsenosides extracts had a stronger inhibition effect on DCFH oxidation. The CAA values were converted from EC_{50} values, expressed as μmol of quercetin equivalency per 100μmol of compound for pure antioxidant compounds or per 100 g for fresh fruits [23]. The CAA value of ginsenosides extracted by PEF was 47.53 ± 4.17μmol TE per 100μg/mL ginsenosides, which was higher than that of SCSE (21.46 ± 2.55μmol TE per 100μg/mL ginsenosides, $p < 0.05$), indicating that ginsenosides extracted by PEF had stronger cellular antioxidant activity than SCSE ginsenosides extracts. This higher activity probably results from the chemical structure changes of the different components [46]. A negative correlation was reported between the EC_{50} and CAA values, indicating a lower EC_{50} value is along with a higher CAA value [47], which was in accordance with these results.

4. Conclusion

In summary, the efficiencies of ginsenosides extracted from the ginseng roots by two alternative extraction techniques were compared. The present study demonstrated the protective effect of ginsenosides extracted by PEF on H_2O_2-induced oxidative stress of HEK-293 cells culture for the first time, and PEF extracts exhibited more effective antioxidant activities *in vitro*. The protection was, at least in part, mediated by the radical scavenging properties of ginsenosides, especially for DPPH, ORAC, and H_2O_2. ROS scavenging assay further proved that ginsenosides extracted by PEF exhibited more efficiency in inhibiting cell oxidative stress by reducing intracellular ROS accumulation. In addition, the morphology observed by SEM of HEK-293 cells under different treatments showed that H_2O_2 treatment displayed cell apoptosis morphology while PEF or SCSE extract pretreatment prevented this morphology alternation. The CAA value indicated that ginsenosides extracted by PEF had stronger cellular antioxidant activity than SCSE ginsenosides extracts. In conclusion, PEF extraction technique can enhance the antioxidant activity of ginsenosides *in vitro*. However, the exact mechanisms of PEF on protection against oxidative stress remain unclear. The expressions of genes and proteins of antioxidant enzymes should be measured to compare the antioxidant effects of these two extraction techniques, therefore further experiments are needed to elucidate it.

Conflicts of interests

All authors have no conflicts of interest to declare.

Acknowledgments

This work was partially supported by the National Natural Science Foundation of China (No. 31271907 and No. 31471597), and the Project of National Key Technology Research and Development Program (No. 2012BAD33B03).

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Please cite this article in press as: Liu D, et al., The beneficial effect of ginsenosides extracted by pulsed electric field against hydrogen peroxide-induced oxidative stress in HEK-293 cells, Journal of Ginseng Research (2016), http://dx.doi.org/10.1016/j.jgr.2016.02.007