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Selenium nanoparticles functionalized by mushroom polysaccharides potentiated neuroprotection of selenium against Parkinson's disease with lower toxicity

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ABSTRACT: There is an urgent need to develop novel, oral agents that can prevent the occurrence of Parkinson's disease (PD). Selenium (Se) plays a preventative role against PD, but there is great concern about its toxicity in clinical practice. We developed a novel extraction technology specifically tailored for the Cordyceps mycelium Cs4 polysaccharides-protein complex (PSP) and prepared the Se nanoparticles (Cs4-SeNPs) by using Cs4 PSP as capping agent, which has a well-characterized structure and high stability. The acute and sub-chronic toxicity of Cs4-SeNPs was demonstrated to be much lower than inorganic and organic Se. The preventative oral administration of Cs4-SeNPs could significantly improve motor functions and prevent dopaminergic neuronal loss in two PD animal models, but organic Se could not. Mechanism studies revealed that Cs4-SeNPs showed much higher antioxidant properties compared to inorganic or organic Se in PD animal and cell models. Cs4-SeNPs could protect against ROS-induced dopaminergic neuron apoptosis due to their strong antioxidant activities and by way of inhibition on the activation of ERs (endoplasmic reticulum stress) and PI3K/Akt/mTOR signaling pathway mediated autophagy in PD cell model. Our findings showed SeNPs functionalized by tailor-made Cs4 PSP enhanced the preventative neuroprotection of Se against PD with higher antioxidant activities and lower toxicity. This study provides solid scientific evidence for the development of an economical and safe oral agent in the prevention of PD.

Keywords: Parkinson's disease; *Cordyceps mycelium* Cs4; Cs4 polysaccharides; Selenium; Cs4-SeNPs; Selenoproteins

1. Introduction

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Parkinson's disease (PD) is the second most prevalent age-related neurodegenerative disorder after Alzheimer's disease (AD). It is characterized by motor symptoms like bradykinesia, rigidity, resting tremor, postural instability [1] and non-motor manifestations such as sleep behavior disorder, olfactory loss, constipation, and depression [2]. Today, PD affects over ten million people all over the world [3], and its etiology is not fully understood. Available drugs in clinical practice are mainly used in the symptomatic treatment of PD and cannot prevent the progression of the disease. As prevention of PD is more important than its therapy, there is an urgent need to develop novel agents that can impede the progression of the disease, especially during the early stages.

The main pathological feature of Parkinson's disease (PD) is the progressive death of dopaminergic (DA) neurons in the substantia nigra and this depletion of DA is associated with the formation of Lewy bodies. Oxidative stress (OS) is a major contributor to the loss of dopaminergic neurons in PD, and DA neurons are particularly sensitive to OS, making them more susceptible to cell death [4]. Therefore, reducing the level of OS in the brain is a crucial aspect of PD prevention.

Selenium (Se), an essential antioxidant trace element, is crucial for maintaining brain function [5]. When Se intake is insufficient, serum Se levels decrease, but the brain retains a priority for Se [6]. The cognitive-enhancing effects of Se have been demonstrated [7], and Se can also alleviate brain damage caused by stroke by inhibiting ferroptosis [8]. Se exercises its biological functions in humans mainly via 25 selenoproteins that have Sec at their active center [9]. Several selenoproteins are expressed in the brain, wherein the selenoproteins highly related to PD pathology are glutathione peroxidises (GPxs), foremost among them GPx4 and selenoprotein P (Selp), which are important antioxidant enzymes in the brain [6].

However, the role of Se in PD remains controversial. Many clinical observations have found that Se levels in the blood or cerebrospinal fluid (CSF) of PD patients are elevated [10-14], suggesting that Se may increase the risk of PD. Conversely, a cross-sectional study in USA including 15,660 adults aged over 40 years old indicated a link between elevated blood Se levels and a reduced occurrence of PD [15]. Plasma Se was found to be positively related to the coordination and motor speed performance of older subjects [16], suggesting the preventative potential of Se against PD. Moreover, some animal experiments have demonstrated that Se administration (inorganic Se, not organic Se) could prevent dopaminergic cell death and the decrease in DA and its metabolites in PD animal models [17-19]. Hence, elevated levels of Se in PD patients may have multiple causes, which do not necessarily contradict the preventive role of Se against PD. PD pathogenesis can lead to alterations in the body's metabolic processes, such as glucose or lipid metabolism [20], that might alter Se distribution and metabolism, leading to elevated levels of Se in the blood or CSF of PD patients. The body may attempt to counteract the OS and neuronal damage caused by PD by increasing Se concentrations. The true relationship between Se and PD requires further investigation, but the preventative role of Se against PD cannot be dismissed.

The efficacy and toxicity of Se depend on its chemical form [21]. Se occurs in nature as a combination of organic and inorganic forms. Inorganic Se compounds, including selenate (SeO_4^{2-}), selenite (SeO_3^{2-}) and

selenide (Se^{2-}) are present in soil and water; whereas organic Se compounds, such as selenomehionine (SeMet) and selenocysteine (SeCys), are synthesized in plants and animals [9]. The narrow window between the effective and toxic doses of both inorganic and organic Se greatly limits their wide application in human health. Organic Se is the commonly used Se form on the market currently. However, according to our research findings [19], it is inorganic Se, rather than organic Se, that is the form of Se effective in preventing PD. Nevertheless, inorganic Se is highly toxic and almost impossible to be applied. Se nanoparticles (SeNPs) have become a new form of Se supplement by virtue of its extremely low toxicity [22] compared to common food sources of organic and inorganic Se. Several studies have reported that SeNPs by using different preparation methods could protect from neurotoxin induced damage in PD cell or animal models [23-26], in which, polysaccharides from Sargassum fusiforme [23] or chitosan [24] or glycine [25] or human serum albumin (HSA) [26] were used to stabilize Se. However, these studies lacked the systematic comparisons of the efficacy and toxicity with ordinary Se and no stability data of SeNPs were provided.

By using the water-soluble polysaccharides-protein complex (PSP) from different mushrooms as the capping agent, we successfully prepared highly stable and size-controllable Se nanoparticles (US patent no.: 9,072,669) under a simple redox system, and proved their higher cellular uptake and anti-proliferation activity in cancer cells, notably, with very low toxicity [27]. Furthermore, our studies found that the decoration of SeNPs with various water-soluble polysaccharides extracted from different mushrooms can tune the cancer selectivity of SeNPs in a tissue-specific manner [28].

Cordyceps sinensis (Berk.) Sacc. (also called “Dong Chong Xia Cao” in China) is a precious medicinal fungus in traditional Chinese medicine. Researchers at the Chinese Academy of Sciences successfully cultivated 4th isolated mycelium (*Cordyceps* mycelium, Cs4), which led to a significant drop in its price and the broadening of its application [29]. Substantial evidence showed that the major bioactive components of Cs4 are polysaccharides; and Cs4 polysaccharides exhibited versatile biological functions, including antioxidant, anti-aging, anti-tumor, immune-modulation, anti-fatigue, and anti-fibrosis properties [30].

Our hypothesis was that SeNPs prepared using Cs4 polysaccharides could offer better prevention against PD with reduced toxicity. To test this hypothesis, we developed a novel extraction technology specifically designed for the Cs4 polysaccharides-protein complex (PSP) to improve its extraction efficiency and for the purpose of preparing the most stable SeNPs. Using our patented technology, we then prepared a novel SeNPs (Cs4-SeNPs) by using Cs4 PSP as the capping agent. We conducted systematic acute and sub-chronic toxicity evaluations of Cs4-SeNPs in both mice and rats to ensure their biological safety. Subsequently, we investigated the preventative effects and underlying mechanisms of Cs4-SeNPs in comparison with inorganic and organic Se in PD cell model and two PD animal models.

2. Materials and methods

2.1 Chemicals and reagents

Cs4 powder was purchased from Hong Kong Institute of Biotechnology (Hong Kong). Sodium selenite (SeNa, inorganic Se), Seleno-L-methionine (Se-Met, organic Se), Seleno-L-cystine (Se-Cys, organic Se),

iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), iron (III) chloride hexahydrate ($FeCl3. 6H_2O$), 2,4,6-Tris(2-pyridyl)-s-triazine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), apomorphine and MPP^+ and rotenone were purchased from Sigma (USA). Trolox was purchased from Calbiochem (USA). ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was obtained from Roche (Germany). Hydrogen chloride (37%) was obtained from LAB-SCAN Asia Co. Ltd (Thailand). The primary antibody of mouse anti-tyrosine hydroxylase (TH) was purchased from Millipore (USA); primary antibody of mouse anti β-actin and secondary antibody of goat anti-mouse IgG were purchased from Santa Cruz (USA). SH-SY5Y cell line was purchased from ATCC (USA). Medium and culture supplements for cell culture were purchased from Gibco (USA). Fetal bovine serum was obtained from Gibco (Brazil). Antibodies used in cells against α-synuclein (α-syn) (#2642), caspase-3 (#9661), Bcl-2 (#2872), Bax (#2772), p-Akt (#4060), Akt (#4691), p-p38 (#9211), p38 (#9212), LC3B (#2775), P62 (#5114), C/EBP homology protein (CHOP) (#2895) were purchased from Cell Signaling Technology (USA); antibodies against glucose regulated proteins 78 (GPR78) (ab21685) were purchased from Abcam (USA); antibodies against p-mTOR and mTOR were purchased from Abways Technology (China); antibody against TH (#AB152) was purchased from Millipore (USA) and the antibody against β-actin was purchased from BOSTER (China); the secondary antibodies were purchased from Santa Cruz Biotechnology (USA). Maxima SYBR Green qPCR Master mix was purchased from Roche (USA).

2.2 Development and optimization of a novel extraction technology tailor-made for Cordyceps mycelia (Cs4) polysaccharides-protein complex (PSP)

Cs4 powder was pulverized with an ultrafine grinder, after which the particle size was determined using the SALD-2201 Laser Particle Size Analyzer (Shimadzu-GL Sciences Laboratory Supplies Co. Ltd.) and the Scanning Electron Microscope (JSM-6380LV, Japan Electron Optics Laboratory Co. Ltd.). Then, Cs4 ultrafine powders (< 50μM) underwent ultrasonic circulating extraction (ultrasonic field: 20 kHz frequency) in a tailor-made bench-top equipment (Hong Xiang Long Biotechnology Developing Co. Ltd., Beijing, China) prior to dialysis (Molecular Wight (MW) cutoff: 6000-8000) and lyophilization. A total of five major extraction factors including ultrasonic power (W), extraction temperature (℃), intermit–running ratio (s/s), solid–liquid ratio (w/v) and extraction time (min) were firstly investigated by way of single factor analysis using % yield of Cs4 PSP as the evaluation index. Subsequently, the whole extraction process was optimized by Uniform Design with those correlated extraction factors using the particle size and/or stability of resulting Cs4-SeNPs as the evaluation index. All experimental data were analyzed using multiple quadratic stepwise regression analysis; afterwards, the best model with optimal extraction conditions was predicted. To verify the results, a further extraction using the predicted model was undertaken. Apart from protein and carbohydrate contents, the amino acid and monosaccharide profiles of Cs4 PSP were determined for the purposes of quality control as previously described [31].

2.3 Preparation and characterization of SeNPs functionalized by Cs4 PSP

Sodium selenite was added into aqueous Cs4 PSP, after which freshly prepared ascorbic acid was added with stirring. The solution was allowed to react for 24 hours at 4℃. Extensive dialysis (MW cutoff: 8,000) was performed to get the resulting dialysate, Cs4-SeNPs. Concentrated nitric acid (trace metal grade) was added to the Cs4-SeNPs solution, and ETHOS One Advanced Microwave Digestion System (Milestone S.r.l., Italy) was used for microwave digestion with temperature rising from 180℃ to 250℃ before the determination of Se concentration. Digestate was then diluted and filtered, Se concentration in Cs4-SeNPs was determined by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) (Agilent 710, Agilent Technology Inc., USA). Cs4-SeNPs were diluted and injected into Nanosight NS3000 (Malvern Instrument Ltd., UK) for nanoparticle tracking analysis (NTA) to determine its particle size. The particle size was determined every 2 weeks for a total of 14 weeks to track its stability.

2.4 Structural identification of Cs4-SeNPs

The dried Cs4-SeNPs specimen (air dried on a hollow carbon grid) was visualized and captured by JEM-2100F Field Emission Electron Microscope (JEOL Ltd., Japan) using 200kV electron beam. One single nanoparticle was selected for further analysis in order to determine its elemental composition and crystallography under 150,000× magnification using high-resolution transmission electron microscopy and energy-dispersive X-ray analyzer (HR-TEM-EDX) (JEOL Ltd., Japan), which was then analyzed using the software Image J. The pellet discs of the mixture containing Cs4-SeNP or Cs4 PSP with KBr (potassium bromide) were then prepared, and their infrared spectra were obtained and compared using the NicoletTM iSTM 50 Fourier Transform Infrared Spectrometer (FT-IR) (Bruker, USA). The major functional groups that link Cs4 PSP and SeNPs were determined.

2.5 Acute toxicity study in mice

40 Specific-Pathogen-Free (SPF)-grade ICR male and female mice (18-22g) were purchased from the Guangdong Medical Experiment Animal Center (Animal production license No.: SCXK (Yue) 2013-0002; Quality certificate No.: 44007200018880). They were randomly and equally assigned to 4 dose groups, each group consisting of 5 males and 5 females. The dose for the acute toxicity test was determined in accordance with the standard GB 15193.3, using the Horn's method. As stated in this method, an initial screening was directly conducted with a dose of 215 mg/kg, and the observed toxicity was relatively mild. Therefore, subsequent dose groups with higher concentrations were formulated. The mice were orally administered with Cs4-SeNPs once in four doses (215, 464, 1000 and 2150mg Se/kg BW) and then observed for 14 days.

2.6 Sub-chronic toxicity study in rats

40 Sprague Dawley (SD) male rats (58.3 ± 3.7g) and 40 SD female rats (59.3 ± 4.1g) (SPF grade) were purchased from the Southern Medical University Laboratory Animal Center (Production license No.: SCXK (Yue) 2016-0041, Quality certificate No.: 44002100012629). The animal diets were purchased from the Guangdong Medical Experimental Animal Center. The animal laboratory used for the toxicity study is a barrier environment with the license number: SCXK (Yue) 2013-0011. The humidity was 40%–70%, and

the room temperature was 20–26℃. In the year of 2022, an upper tolerable level (UL) of 255 μg Se/day in adults was established by EFSA (European Food Safety Authority) [32]. Our experiment included three dose (Low: 0.3 mg Se/kg BW; Mid: 0.9 mg Se/kg BW; High: 3 mg Se/kg BW in rats) groups of Cs4-SeNPs, which are about 12, 36 and 120 times the updated ULs of Se in human body. After 3 days of habituation to the environment, male or female rats were randomly assigned to 4 treatment groups (including Control, Low, Mid and High dose group, respectively). Different doses of Cs4-SeNPs were administered to male and female rats via gavage once each day for a total of 90 days, and their control groups were treated with an equal volume of distilled water accordingly. The body weight and food intakes were determined once per week, and the physiological conditions were closely and continuously monitored throughout the whole study period. In the end, animals were housed individually in metabolic cages for 24 h for the collection of urine and feces. Then, all animals were sacrificed, blood was withdrawn from the abdominal aorta, different organs were collected for further investigations.

2.7 Animal experimental design for efficacy evaluation

MPTP, 6-OHDA and rotenone are commonly used neurotoxins to induce PD in animal models [33]. The common pathogenic mechanism of all three neurotoxins is oxidative stress, which ultimately leads to the apoptosis of DA neurons. However, each neurotoxin also has its own unique pathogenic mechanism that mimics different features of PD. Using only one model in the experiment might not fully validate potential effects of the candidates. In the present study, MPTP-induced mouse models and 6-OHDA-induced rat models were used to comprehensively evaluate the potential preventative effects of our Cs4-SeNPs against PD *in vivo*.

48 eight-week-old male C57BL/6J mice (20 ± 2g) and 24 three-month-old male Wistar rats (330 ± 20g) were purchased from the Laboratory Animal Services Centre of the Chinese University of Hong Kong (CUHK, Hong Kong). The animals were housed in an air-conditioned room at (22 ± 2)℃ with 55% ± 5% relative humidity and a 12h light/dark cycle in the Centralized Animal Facilities (CAF) of the Hong Kong Polytechnic University. The experiment was conducted according to the guidelines approved by the Animal Subjects Ethics Sub-committee at the Hong Kong Polytechnic University (ASESC Case No.: 18-19/28-ABCT-R-HMRF) with all efforts being made to minimize animal suffering or discomfort. The mice and rats were given a normal diet (TD.94048, Purified Rodent Diet AIN-93M, Harlan Laboratories Inc. USA) and distilled water.

The PD mouse model was made using MPTP (20 mg/kg), i.p. injected four times in one day, at intervals of two hours. All the mice had adapted to the environment for one week before the experiment started. The mice were randomly divided into 6 groups (n = 8 in each group) and orally fed low and high doses of Se-Met or Cs4-SeNPs for a total of 14 days; MPTP were injected 4 times a day on the 13^{th} day; behavior tests were conducted on the 14^{th} day. The low and high doses were 100μg Se/kg body weight/day and 1000μg Se/kg body weight/day, respectively. The quantities of Se from the same doses of Se-Met and Cs4-SeNPs were kept consistent. The experimental timeline is shown in Fig. 4A.

The PD rat model was made by applying 6-hydroxydopamine (6-OHDA) to the left medial forebrain bundle (MFB) on day 8 after the experiment started. Two microinjections of 6-OHDA (3.0 mg／mL dissolved in saline containing 0.2 mg／mL l-ascorbate) were made into the right MFB: (1) TB: -2.3 mm; AP: -4.4 mm; ML: 1.2 mm; V: 7.8 mm; and (2) TB: +3.4 mm; AP: -4.0 mm; ML: 0.8 mm; V: 8.0 mm. Volumes of 2.0 μL of 6-OHDA were microinjected at a rate of 1.0 μL／min into these two sites, respectively; and normal rats were microinjected with the same volume of saline at the same rate. After the injection, the microinjection needle was left in place for a further 5 min before being slowly extracted. Experimental groups included (1) the normal control group (Normal), which was treated with distilled water via daily oral gavage continuously for 4 weeks, with microinjection of saline on day 8; (2) the PD model group (Model), which was treated with distilled water via daily oral gavage continuously for 4 weeks, with microinjection of 6-OHDA on day 8; (3) the Cs4-SeNPs treated group (Cs4-SeNPs), which was treated with Cs4-SeNPs (70 μg Se/kg/d) via daily oral gavage continuously for 4 weeks, with microinjection of 6-OHDA on day 8. The dosage of Cs4-SeNPs was converted from a lower dosage (100 μg Se/kg/d) used in mice. The experimental timeline is shown in Fig. 4D.

2.8 Behavior tests

Pole test and rotarod test for mice. In the pole test, the mice were positioned head up at the top of a vertical rough-surfaced pole, and the time for them to turn around and reach the floor was recorded. In the rotarod test, the mice were put on a rotating rod in an automated accelerating rotarod apparatus (diameter of 1.25 inch) called Omni Rotor (Omnitech Electronics Inc., Columns, OH, USA), and the latency of the mice to fall from the accelerating rod was recorded. The methods used are described in detail in our previously published papers [19, 34]. Before testing, each mouse was trained 3-5 times followed by 3 real tests with each test lasting for a maximum of 3 min.

Rotational behavioral test for rats. Rats were tested for their rotational behavior induced by apomorphine injection (2.5 mg/kg, i.p.) in automated ‘rotometer’ bowls for 30 min. The net rotational asymmetry score was expressed as 360° turns per 30 min. Direction ipsilateral to the side of lesion was given positive value.

2.9 Sample collection

All mice or rats underwent an overnight fasting before sacrifice. On the day of sacrifice, mice or rats were euthanized by pentobarbitone via i.p. injection (10mg/100g Body weight). Blood from 8 mice in each group was withdrawn from their hearts, serum was prepared and stored at -80℃. Both sides of brain striatum, substantia nigra pars compacta (SNpc) or midbrain tissues were harvested and stored at -80℃.

2.10 Culture of SH-SY5Y cells and establishment of PD cell model

All cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and placed in a cell culture chamber at 37℃ and under a humidified atmosphere with 5% CO_2. After the cells adhered and were grown to 80% confluence,

the culture medium was removed and washed with an appropriate amount of PBS and digested with 0.25% trypsin. Cells in the logarithmic phase of growth were selected for the following experiments.

Cell models have limitations and cannot systematically exhibit the pathological characteristics of the disease as animal models do, but detailed mechanistic studies can only be conducted in cell models. The common pathogenic mechanism of neurotoxins of MPTP, 6-OHDA and rotenone is oxidative stress. After testing, rotenone (300nmol/L) induced SH-SY5Y cells were ultimately chosen to study the oxidative stress-related mechanisms of Cs4-SeNPs after Cs4-SeNPs were confirmed to provide preventative neuroprotection in both MPTP and 6-OHDA induced animal models. In signaling pathway experiments, SH-SY5Y cells were pre-treated with different doses of Cs4-SeNPs for 1 hour, followed by co-treatment with rotenone for an additional 6 or 24 hours to examine the effects of Cs4-SeNPs on the expression of relevant genes or proteins.

2.11 Western-blot analysis

The animal midbrain tissues or cells were collected and homogenized in ice-cold lysis buffer. Lysates were centrifuged and total proteins were acquired. Equal amounts of proteins from brain tissues or cells were separated by SDS-PAGE in gels followed by transference to PVDF membrane. Membranes were blocked with 5% non-fat milk followed by incubation with correspondent primary and secondary antibodies. Bands were visualized using a chemiluminescence kit under the ECL system. Densitometry was performed using the Image J software.

2.12 Real-time RT-PCR

As described in our previous paper [34, 35], brain tissues (about 20mg) were mixed with Trizol for homogenization and total RNA extraction. The complementary DNA (cDNA) was synthesized from 2μg of total RNA using transcriptase with random hexamers (Thermo Fermentas, USA). Real-time RT-PCR was performed in an iCycler iQ5 system (Bio-Rad Laboratories). The thermal profile included an initial denaturation at 95℃ for 5min followed by forty cycles of denaturation at 95℃ for 10s, annealing at 59℃ for 30s, and extension at 72℃ for 30s. 18S ribosomal RNA (18S rRNA), GAPDH or β-actin gene was used as the endogenous reference for normalization. Each sample was conducted in duplicate. The expression levels of genes were analyzed by the methods above including tyrosine hydroxylase (TH) and some selenoproteins, which included Iodothyronine deiodinase type1, 2, 3, (Dio1, Dio2, Dio3); Glutathione peroxidase 1, 2, 3, 4 (GPX1, GPX2, GPX3, GPX4); Selenoprotein H (Selh); Selenoprotein I (Seli); Selenoprotein K (Selk); Selenoprotein M (Selm); Selenoprotein N (Sepn1); Selenoprotein O (Selo); Selenoprotein P (Sepp1); Selenoprotein S (Sels); Selenoprotein T (Selt); Selenoprotein V (Selv); Selenoprotein W (Sepw1); 15 kDa selenoprotein (Sep15); Methionine-R-sulfoxide reductase (MsrB1 or Sepx1); Selenophosphate synthetase 2 (Sephs2); and Thioredoxin reductase 1, 2, 3 (Txnrd1, Txnrd2, Txnrd3). The data were normalized with 18S rRNA, GAPDH or β-actin expression levels in the samples, and each mean gene expression was calculated by assigning a relative value of 1.0 to the group mean of the

Normal group, whereas all other values were relative to the group mean of the Normal group. The sequences of the primers used in this study were summarized in the supplementary Table S1.

2.13 In vitro antioxidant assay

The antioxidant activities of different Se forms were examined by Trolox equivalent antioxidant capacity (TEAC) assay and Ferric reducing ability of plasma (FRAP) assay. Different concentrations of samples were added to the ABTS solution and FRAP reagent, respectively, and stored at room temperature for 20 min in the dark. Trolox was selected as the external standard. The absorbance was measured at 734 nm by Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, China). The antioxidant activity of TEAC was shown in μmol Trolox/g Se. As for the FRAP assay, the FRAP reagent was freshly prepared by mixing 0.3M acetate buffer (pH 3.6), 0.1M TPTZ (0.4M HCl), and 0.2M $FeCl_3 \cdot 6H_2O$ in a volume ratio of 10:1:1. The mixed solutions of 100μl samples with different concentrations and 953μl of FRAP reagent were further stored in the dark for 15 min. $FeSO_4 \cdot 7H_2O$ was chosen as an external standard. The absorbance was measured at 593 nm using a microplate reader, and the activity of FRAP was expressed in μmol Fe^{2+}/g Se.

2.14 Intracellular reactive oxygen species (ROS)

To investigate the anti-oxidative effect of different Se forms, these were added directly to SHSY-5Y cells at a final concentration of 10μM together with 1μM rotenone. After 24h exposure, the cell medium was replaced with a new medium supplemented with 2μM DCFH-DA. After a 30 min incubation, ROS production was detected by the DCFH-DA assay kit. Fluorescent signal values shown as O.D. readings were normalized to total protein content and relative to control values. Cells were stained with DCFH-DA for 45 min at 37℃ in dark, and fluorescent signal was visualized and captured under fluorescence microscopy.

2.15 Statistical analysis

Animals in the present experiment were randomly assigned to each group, and experimenters were blinded for all analysis purposes. Data from these experiments are reported as mean ± SEM or SD. Subsequent statistical analyses were performed using PRISM version 9.4.0 (GraphPad, USA). Analyses of treatments on different parameters were performed using one-way ANOVA. When significant variations were found by one-way ANOVA, an unpaired two-tailed t-test with Welch's correction was performed as a post-test between any two groups. Differences with p values of less than 0.05 were considered statistically significant. For those significant results, Welch-corrected t values, degrees of freedom (df) with p values, differences between means (dbm), and 95% confidence intervals (95% CI) are provided in the results for statistical rigor.

3. Results

3.1 Novel extraction technology for preparation of Cs4 polysaccharides

Due to the relatively low yield of traditional polysaccharide extraction methods and to improve stability of the prepared SeNPs, we specifically developed a novel extraction technology (micronization +

ultrasonic-circulating extraction) tailor-made for Cs4 PSP (polysaccharides-protein complex) prior to the preparation of Cs4-SeNPs.

In this study, the particle size of Cs4 powder was significantly reduced after micronization with D50 < 50μM (Fig. 1). The Cs4 powder underwent extraction that involves the optimization of ultrasonic-circulating extraction conditions. As shown in Table S2-1 and Figure S1 & S2, all five major extraction factors including ultrasonic power (W), extraction time (min), extraction temperature (℃), solid–liquid ratio (w/v) and intermit–running ratio (s/s) were found to correlate with the yield (%) of Cs4 polysaccharides-protein complex (PSP) to different extents showing a correlation coefficient (R) of 0.792, 0.937, 0.959, 0.804 and 0.997, respectively. Optimization by Uniform Design using extraction time (min), extraction temperature (℃), ultrasonic power (W) and solid–liquid ratio (w/v) at 6, 6, 6 and 3 levels [i.e. $U6^4(6\times6\times6\times3)$] indicated that the optimal extraction conditions for the maximum yield of Cs4 PSP (11.31%) were extraction time = 78.4 min, extraction temperature = 45℃, ultrasonic power = 540W and solid–liquid ratio = 1/30 (w/v) calculated by predicted extraction equations (Table S2-2). The validity of this predicted extraction model was verified through repeated experiments (n = 5; R2 = 0.9867). Considering that the Cs4 polysaccharide, with a purity of 65%-75%, is more suitable for the preparation of our selenium nanoparticles (Cs4-SeNPs) in terms of particle size, it is essential to consider both the yield and purity of the crude polysaccharide during the extraction process. This approach is necessary to pursue process improvements and further optimization. (see Figure S3 & S4).

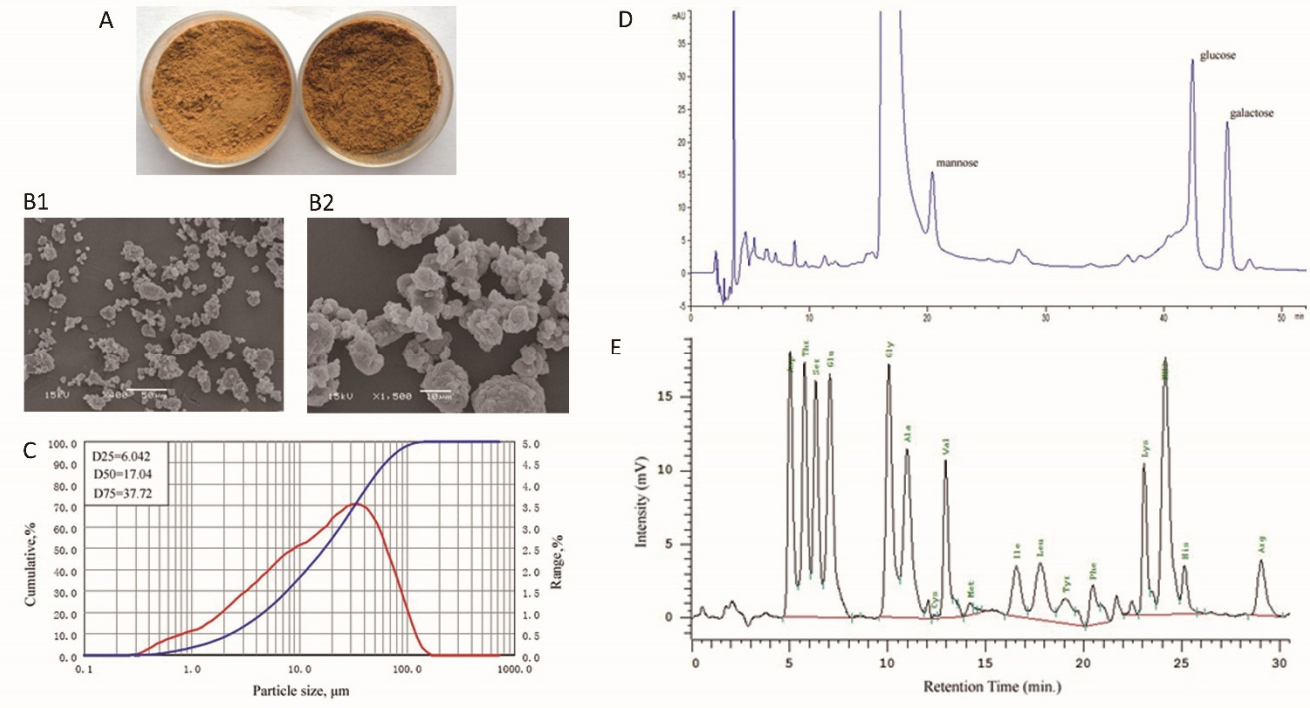

Figure 1 (A) Pictures of Cs4 powder appearance before (Left) and after (Right) micronization; (B) Microscopic morphology of micronized Cs4 powder under 400× (B1) and 1500× (B2) magnifications; (C) Laser particle size distribution graph shows the micronized Cs4 powder has D50 = 17.04μm, D75 = 37.72μm. (D) High performance liquid chromatography (HPLC) profiles of sugars in extracted Cs4 polysaccharides-protein complex (PSP); (E) High performance cation exchange chromatography profiles (HPCEC) of amino acids in extracted Cs4 PSP.

Finally, the resulting yield of Cs4 PSP (11.03%; purity: 76.48%) was significantly higher than that obtained by the traditional hot water extraction method (7.85%). A further composition analysis (Table 1) showed that our prepared Cs4 PSP contained 5.86% of polysaccharides, and its major sugar components included mannose, glucose, and galactose (molar ratio: 2:2:1). Meanwhile, our Cs4 PSP contained 17 amino acids (Total: 12.71%). High performance liquid chromatography (HPLC) profiles of sugars and high-performance cation exchange chromatography (HPCEC) profiles of amino acids in our Cs4 PSP were shown in Fig.1D, 1E respectively. Strikingly, Cs4-SeNPs prepared by using the Cs4 PSP originated from Uniform Design were found to exhibit very high stability without precipitation for 14 weeks (Fig. 2F), longer than Cs4-SeNPs prepared by using Cs4 PSP derived from traditional methods (6-8 weeks).

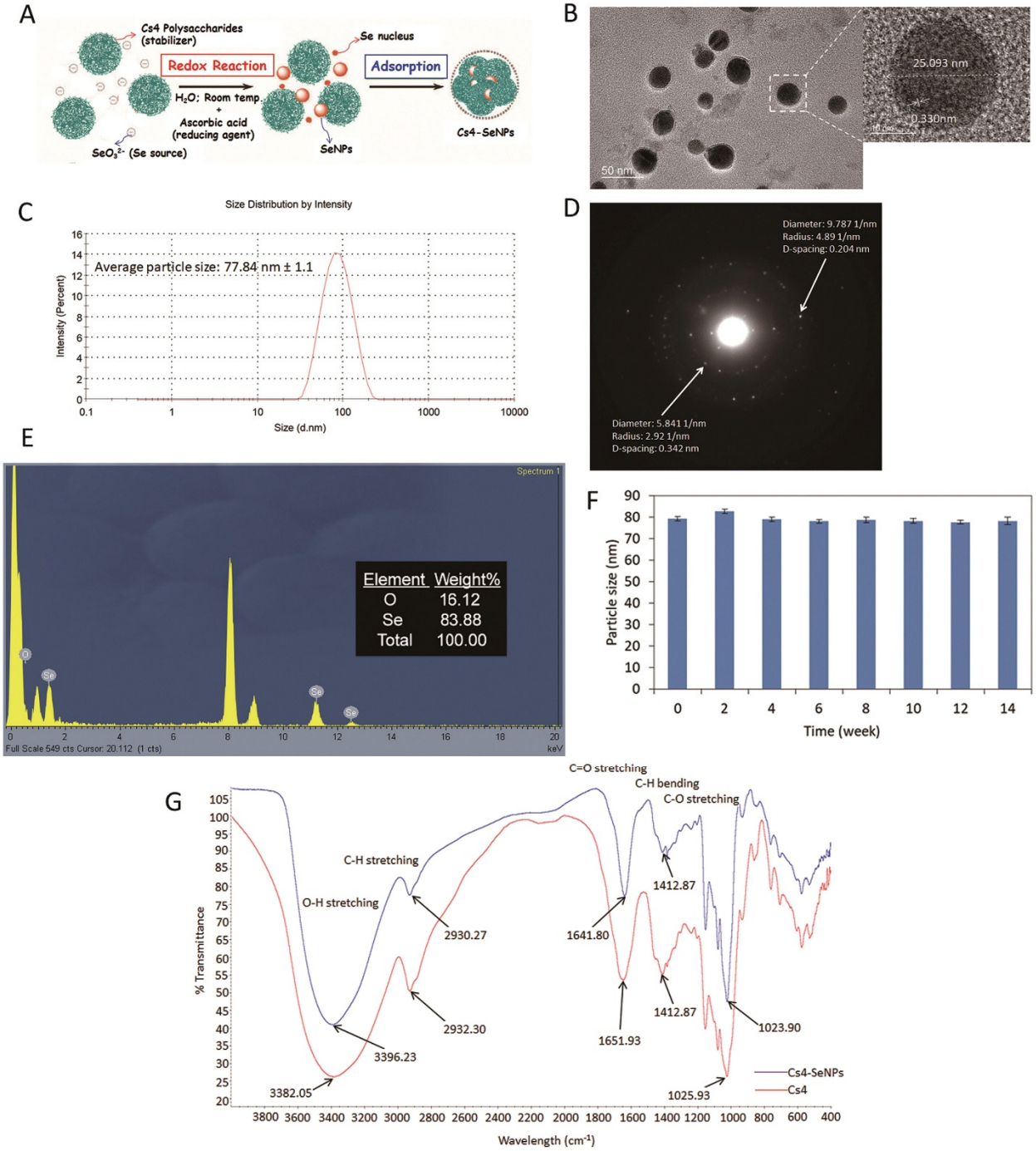

Figure 2 Characterization of Cs4-SeNPs. (A) Schematic illustration of Cs4-SeNPs preparation. (B) Representative TEM image of Cs4-SeNPs. (C) Particles size distribution of Cs4-SeNPs measured by Nanosight NS300. (D) HR-TEM image of

SAED pattern of Cs4-SeNPs. (E) Representative EDX analysis of Cs4-SeNPs. (F) Particles size stability. (G) FT-IR analysis of Cs4-SeNPs and Cs4 polysaccharides-protein complex (PSP). Values are expressed as mean ± SD, n = 4. Cs4-SeNPs, selenium nanoparticles functionalized by Cs4 polysaccharides; NTA, nanoparticle tracking analysis; TEM, transmission electron microscopy; HR-TEM, high-resolution transmission electron microscopy. SAED, selected area electron diffraction; EDX, energy-dispersive X-ray analyzer; FT-IR, fourier transform infrared spectrometer; KBr, potassium bromide.

Table 1 Composition analysis of our prepared Cs4 PSP

	Polysaccharides	**Proteins**
Content	5.86g/100g	12.71g/100g
Monosaccharide or amino acid type	Mannose, Glucose, Galactose (2:2:1)	Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, Lys, His, Arg

3.2 Preparation and characterization of Cs4-SeNPs

Plant polysaccharides are often selected to be the capping agent for the preparation of SeNPs [36], but different polysaccharides might enhance different functions of SeNPs as revealed in our previous findings [28]. Novel SeNPs functionalized with Cs4 PSP based on our patented nanotechnology (US patent no.: 9,072,669) were successfully prepared in this study. Decoration of SeNPs with Cs4 PSP was done by dropping ascorbic acid solution to the mixture of corresponding PSP and sodium selenite solution (Fig. 2A). Cs4 PSP accounts for approximately 82% of the total mass of Cs4-SeNPs, while Se constitutes about 18%. Thus, the mass ratio of Cs4 PSP to Se in Cs4-SeNPs is roughly 4.5:1. From the results of TEM imaging (Fig. 2B), SeNPs themselves appeared as well-dispensed spherical particles in water with an average diameter of around 25nm, while Cs4-SeNPs had an average particle size of (77.8 ± 8.1)nm (Fig 2C), which were smaller than other mushroom PSPs decorated SeNPs (91-102nm) based on our previous findings [28].

The fringe spacing measured in an HR-TEM image was about 3.30Å (Fig. 2B), and the corresponding selected area electron diffraction (SAED) pattern of Cs4-SeNPs exhibited a series of concentric rings composed of white dots (Fig. 2D). The results from HR-TEM-EDX (Fig. 2E) showed that Cs4-SeNPs contained a very high level of Se (83.9%), indicating a successful fabrication of SeNPs using Cs4 PSP. The chemical binding of Cs4 PSP to the surface of SeNPs was further investigated by FT-IR spectrometry. As shown in Fig. 2G, two major absorption peaks at 1651.93 cm^{-1} and 3382.05 cm^{-1} in the IR spectrum of Cs4 PSP corresponded to the stretching vibrations of the carbonyl groups (–C = O–) from its proteins and hydroxyl groups (–OH–) from its polysaccharides, respectively. Interestingly, Cs4 PSP shared a FT-IR spectrum similar to that of Cs4-SeNPs, indicating that Cs4 PSP formed part of the Cs4-SeNP nanocomposites. Nevertheless, both absorption peaks of the carbonyl and hydroxyl groups in Cs4 PSP shifted, namely from 1651.93 cm^{-1} to 1641.80 cm^{-1} and from 3382.05 cm^{-1} to 3396.23 cm^{-1}, respectively, after forming Cs4-SeNPs, indicating the occurrence of interaction between the carbonyl and hydroxyl groups of Cs4 PSP as well as the Se atoms. These results suggested that the SeNPs were capped by Cs4 PSP mainly through the formation of Se-O bonds, giving rise to a stable spherical structure of SeNPs, which were also similar to other SeNPs prepared using mushroom PSPs [28]. More importantly, Cs4-SeNPs were highly stable, and could remain stable in aqueous solution for up to 14 weeks (Fig. 2F).

3.3 Acute toxicity of Cs4-SeNPs in vivo

The acute oral toxicity of Cs4-SeNPs was evaluated in ICR mice (Table 2). After a 13h oral administration, the mice began to die in the high dose groups. Subsequently, the dead mice were dissected, and flatulence was observed. The median lethal dose (LD50) for male mice was 511 mg Se per kg body weight (BW) (with 95% confidence limits of 255-1020 mg/kg BW), and the LD50 for female mice was 619 mg Se per kg BW (with 95% confidence limits of 342-1120 mg/kg BW). The LD50 of Cs4-SeNPs (511-619 mg Se/kg) was much less toxic than the reported LD50 of inorganic selenite (8-12 mg Se/kg) [37] and organic Se-Met (23.9-30.1 mg Se/kg) [38] in mice.

Table 2 The oral acute toxicity of Cs4-SeNPs in mice

Dosage (mg/kg BW)	Animal (n=5)		Mortaility (no.)		LD50 (mg/kg BW)	
	Male	Female	Male	Female	Male	Female
2150	5	5	5	5	511	619
1000	5	5	4	4		
464	5	5	2	1		
215	5	5	1	1		

Note : BW, body weight; LD50, median lethal dose

3.4 Sub-chronic toxicity of Cs4-SeNPs in vivo

To ensure safety, we further investigated the sub-chronic toxicity of Cs4-SeNPs in both male and female SD rats. All rats under investigation were found to be in good condition showing normal physiological characteristics, appearance, behavior, urine, and fur during the 90-day study period. As shown in Fig. 3A, weekly BW of female rats in the high dose group decreased significantly compared with the control group during the week 2 to 11 period (Fig. 3A2). Accordingly, it was found that the weekly food intake of female rats in the high dose group notably went down during the period from week 2 to 4, and from week 8 to 13 (vs. control; Fig. 3B2). But the weekly BW and food intake of male rats were not very much affected throughout the whole study period (Fig. 3A1 and 3B1). Organ weights and relative organ weights (relative to BW) were used to evaluate the toxicity index in animals. Our results indicated that the relative organ weights of the liver, kidney, spleen, and testis in the high dose group of male rats had increased significantly, whereas the relative weights of the brain, heart, and liver in the high dose group of female rats increased greatly in comparison with their respective controls (Table S3). However, the lesions of major organs were idiopathic and the Cs4-SeNPs caused no visible histological damage to the major organs within 90 days (Table S7).

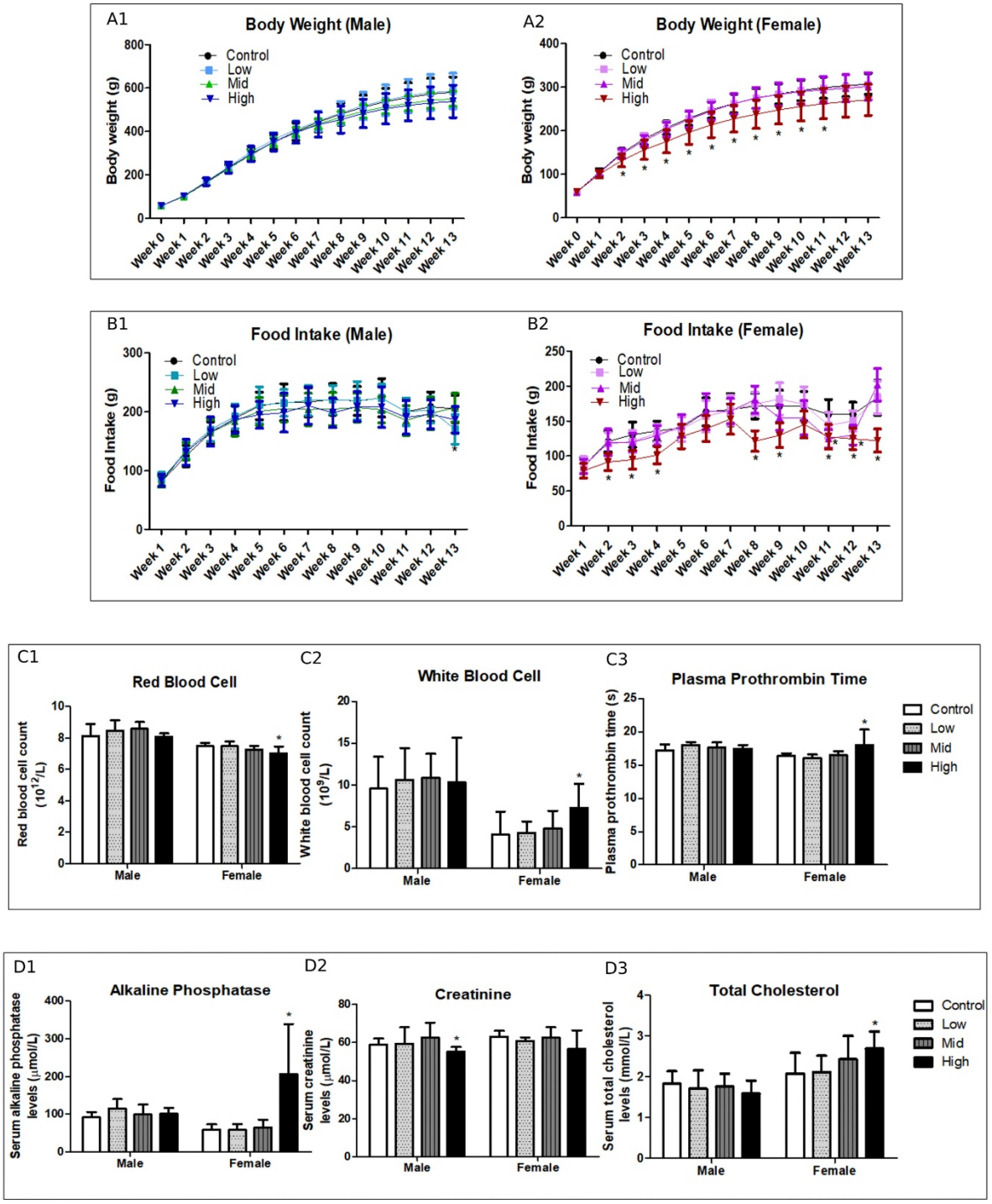

Figure 3 Body weight, food intake and the hematology indicators or biochemical indices being markedly influenced in rats after treatment with low (0.3 mg Se/kg/day), mid (0.9 mg Se/kg/day) or high (3 mg Se/kg/day) dose of Cs4-SeNPs for 90 days. (A) Body weight in male (A1) and female rats (A2); (B) Food intake in male and female rats; (C1) Red blood cell count, (C2) White blood cell count, (C3) Plasma prothrombin time in male and female rats; (D) Serum levels of alkaline phosphatase (D1), creatinine (D2), and total cholesterol (D3). Values are expressed as mean ± SD, n = 10. *P < 0.05 vs. Control in male or female rats. Cs4-SeNPs, selenium nanoparticles functionalized by Cs4 polysaccharides.

In addition, the hematology indicators, biochemical indices, and urinalysis were also measured in rats after treatment with three doses of Cs4-SeNPs for 90 days. As shown in Table S4 and Fig. 3C, the red blood cell (RBC) count was lower (t = 2.655, df [degree of freedom] = 11.05, P = 0.0223; dbm [differences between means] = 0.470 and 95%CI [95% confidence interval]: 0.081 to 0.859), the white blood cell (WBC) count was higher (t = 2.534, df = 17.97, P = 0.0208; dbm = 3.230 and 95%CI: 0.551 to 5.909), and

the plasma prothrombin time (PPT) was longer (t = 2.246, df = 9.522, P = 0.0498; dbm = 1.650 and 95%CI: 0.002 to 3.298) in the high dose group of female rats (vs. control). But in male rats, no hematology indicators were found to have been influenced by either dose of Cs4-SeNPs. Among all the measured biochemical indices (Table S5), serum ALP (t = 3.099, df = 9.171, P = 0.0125; dbm = 147.7 and 95%CI: 40.19 to 255.2) (Fig. 3D1) and total cholesterol (TC) levels (t = 2.450, df = 17.54, P = 0.025; dbm = 0.610 and 95%CI: 0.086 to 1.134) (Fig. 3D3) rose significantly in the high dose group of female rats, but serum Cr (t = 2.450, df = 17.98, P = 0.0122; dbm = 3.800 and 95%CI: 0.934 to 6.666) (Fig. 3D2) was reduced greatly in the high dose group of male rats (vs. control). The results of the urinalysis indicated no obvious difference between either dose group and the control group in both male (Table S6-1) and female (Table S6-2) rats. Taken together, the no-observed-adverse-effect-level (NOAEL) of Cs4-SeNPs in male and female rats was 0.9 mg Se/kg (1.8 mg/kg, approximate equivalent dose in mice).

3.5 Effects of Cs4-SeNPs on motor functions and TH expressions in the midbrain and levels of neurotransmitters in the striatum of PD mice in comparison with organic Se

The pole test and the rotarod test were employed to evaluate the bradykinesia of mice by measuring the total descent time down the pole or the latency to fall (second) from the rod [19]. Our results showed that PD model mice (vs. Normal) exhibited locomotor deficits with longer total descent time (t = 8.285, df = 10.91, P < 0.0001; dbm = 6.041 and 95%CI: 4.434 to 7.647) down the pole (Fig. 4B1) and shorter riding time (t = 6.279, df = 11.92, P < 0.0001; dbm = 66.63 and 95%CI: 43.49 to 89.76) on the rod (Fig. 4B2). Low or high dose Se-Met treatment failed to change much of the descending time and riding time of PD mice in the pole test and rotarod test (Fig. 4B1, B2), respectively. The total time to descend for PD mice treated with low (t = 3.420, df = 10.48, P = 0.0061; dbm = 2.509 and 95%CI: 0.8845 to 4.133) or high (t = 4.927, df = 9.008, P = 0.0008; dbm = 3.246 and 95%CI: 1.756 to 4.736) dose Cs4-SeNPs in the pole test decreased significantly; and the riding time in the rotarod test increased significantly for PD mice treated with high dose Cs4-SeNPs (t = 3.889, df = 11.51, P = 0.0023; dbm = 49.69 and 95%CI: 21.72 to 77.66). The results suggested that the oral administration of Cs4-SeNPs (at high dose in particular) could improve motor functions of PD mice, and this was what Se-Met treatment (with the same quantities of Se as Cs4-SeNPs) could not do.

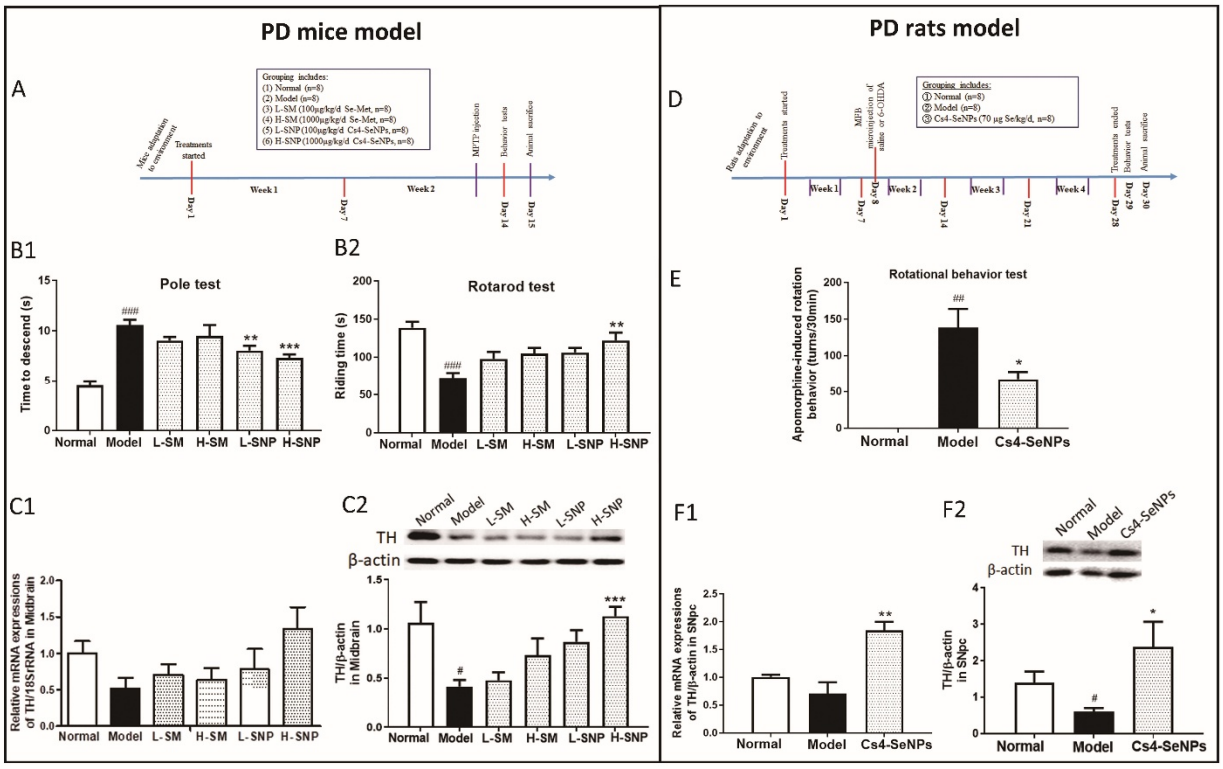

Figure 4 Neuroprotective effects of Cs4-SeNPs in either PD mice or PD rats model. (A) Experimental timeline for animal experiments in PD mice model; (B1) Pole test; (B2) Rotarod test. (C1, C2) mRNA and protein expressions of TH in midbrain of mice. (D) Experimental timeline for animal experiments in PD rats model; (E) Rotational behaviour test; (F1, F2) mRNA and protein expressions of TH in SNpc of rats. TH mRNA expressions were shown as quantitative ratio of TH to 18S rRNA or β-actin gene and relative to group mean of Normal group. For protein expressions, one representative band for TH and endogenous β-actin from the damaged side of SNpc in each group; and the following bar chart shows the ratio of TH/β-actin in each group. Values are expressed as mean ± SEM, n = 6-8. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ Model vs. Normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Model group. Cs4-SeNPs, selenium nanoparticles functionalized by Cs4 polysaccharides; PD, Parkinson's disease; TH, tyrosine hydroxylase; SNpc, substantia nigra pars compacta.

TH is a marker of dopaminergic neurons and TH expressions in the midbrain could represent the status of dopaminergic neuronal loss in PD mice [19, 39]. The results showed that TH protein expressions in PD mice declined greatly (t = 2.898, df = 6.018, P = 0.0273; dbm = 0.6473 and 95%CI: 0.1012 to 1.193) (Model vs. Normal, Fig. 4C2 and Fig S7), indicating that the PD mouse model was successfully established in our present experiment. Moreover, only pre-treatment of high dose Cs4-SeNPs (t = 6.070, df = 9.015, P = 0.0002; dbm = 0.7155 and 95%CI: 0.4489 to 0.9821) could (while high dose Se-Met could not) significantly improve TH protein expressions in the midbrain of PD mice (vs. Model, Fig.4C2).

As shown in Table 3-1, PD model mice had significantly lower levels of DA (t = 5.024, df = 7.841, P = 0.0011; dbm = 4.991 and 95%CI: 2.692 to 7.289) together with its metabolites of DOPAC (t = 6.867, df = 6.558, P = 0.0003; dbm = 293.9 and 95%CI: 191.3 to 396.5), HVA (t = 3.191, df = 10.19, P = 0.0094; dbm = 18.18 and 95%CI: 5.520 to 30.85) (Model vs. Normal). The decline of striatal DA, DOPAC and HVA in the MPTP induced PD mouse model due to dopaminergic neuronal loss had been reported in previously published papers [40, 41], implying that our PD mouse model had been successful. In comparison with organic Se of Se-Met, only the pre-treatment of high dose Cs4-SeNPs was found to significantly increase the striatal DOPAC levels (t = 3.301, df = 5.552, P = 0.0184; dbm = 130.5 and 95%CI: 31.85 to 229.2) in PD mice (vs. Model).

Table 3-1 Striatal neurotransmitters levels in mice from different groups

Neurotransmitters (ng/mg tissue)	Groups					
	Normal	**Model**	**L-SM**	**H-SM**	**L-SNP**	**H-SNP**
DA	6.68 ± 0.92	**1.69 ± 0.37**$^{\#\#}$	1.61 ± 0.28	1.93 ± 0.46	1.75 ± 0.34	2.59 ± 0.97
DOPAC	359.9 ± 41.8	**66.0 ± 9.1**$^{\#\#\#}$	84.3 ± 18.3	107.1 ± 39.8	164.9 ± 44.6	196.5 ± 38.5*
HVA	35.8 ± 3.6	**17.6 ± 4.4**$^{\#\#}$	15.4 ± 1.8	22.0 ± 5.3	36.6 ± 8.1	41.2 ± 9.8

Note: Values are expressed as mean ± SEM, n = 6-8. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ Model vs. Normal group; $^{*}P < 0.05$, vs. Model group.

3.6 Effects of Cs4-SeNPs on motor functions and expressions of TH in the lesioned side of substantia nigra pars compacta (SNpc) and levels of neurotransmitters in the striatum of PD rats

To further validate the preventative neuroprotection of Cs4-SeNPs against PD, another PD rat model created by microinjecting 6-OHDA into the left MFB was used in this study. A low dose (100μg Se/kg/day) of Cs4-SeNPs in PD mice was converted into the testing dose (70μg Se/kg/day) in PD rats. After treatments, rats were tested for their rotational behavior induced by apomorphine injection. It was observed that normal rats showed zero rotation; in comparison, the PD rat model had about 138 positive rotational turns in the direction of the lesioned side (Model vs. Normal, Fig. 4E). Also, the oral administration of Cs4-SeNPs at a comparatively lower dose for 4 weeks could significantly reduce the rotational behaviour of PD rats (t = 2.548, df = 5.195, P = 0.0496; dbm = 70.33 and 95%CI: 0.1634 to 140.5) (vs. Model, Fig. 4E). Moreover, TH protein expressions (t = 2.602, df = 5.500, P = 0.0439; dbm = 0.834 and 95%CI: 0.032 to 1.635) (Model vs. Normal, Fig. 4F2) on the damaged side of SNpc significantly decreased in response to microinjection of 6-OHDA in PD model rats. And Cs4-SeNPs treatment could significantly improve both TH gene (t = 4.382, df = 9.777, P = 0.0014; dbm = 1.134 and 95%CI: 0.555 to 1.712) (Fig. 4F1) and protein (t = 2.595, df = 6.121, P = 0.040; dbm = 1.817 and 95%CI: 0.112 to 3.522) (Fig. 4F2 and Fig S8) expressions on the damaged side of SNpc (vs. Model), indicative of positive neuroprotection provided by Cs4-SeNPs against PD in 6-OHDA induced PD rat model.

As shown in Table 3-2, the PD rat model had comparatively lower levels of DA and its metabolites (DOPAC and HVA), 5-HT and its precursor (5-HTP), although the statistical analysis was not significant. Treatment of Cs4-SeNPs was found to significantly improve the levels of DA (t = 2.933, df = 9.998, P = 0.015; dbm = 0.011 and 95%CI: 0.003 to 0.020), DOPAC (t = 2.677, df = 5.208, P = 0.0422; dbm = 204.8 and 95%CI: 10.47 to 399), 5-HTP (t = 3.346, df = 8.168, P = 0.0098; dbm = 0.202 and 95%CI: 0.063 to 0.341), 5-HIAA (t = 2.247, df = 10.69, P = 0.0468; dbm = 0.237 and 95%CI: 0.004 to 0.470) on the damaged side of striatum of PD rats (vs. Model). Except for DA and its metabolites, a decline in 5-HT and its metabolites have previously been reported in PD patients and animal models [42]. The upregulation of DA, 5-HT, and their metabolites by Cs4-SeNPs further confirmed their neuroprotective effects against PD.

Table 3-2 Neurotransmitters levels in the lesioned side of striatum of rats from different groups

Categories	Neurotransmitters (ng/mg tissue)	Groups		
		Normal	Model	Cs4-SeNPs
DA and its metabolites	DA	5.64 ± 1.99	2.28 ± 0.27	3.41 ± 0.27*
	DOPAC	712.9 ± 250.5	545.0 ± 75.7	749.8 ± 10.9*

	HVA	104.9 ± 44.0	49.5 ± 7.0	65.2 ± 7.1
5-HT precursors and its metabolites	5-HTP	0.86 ± 0.38	0.28 ± 0.05	0.48 ± 0.04**
	5-HT	2.43 ± 1.35	**0.51 ± 0.13**	0.26 ± 0.03
	5-HIAA	0.48 ± 0.07	**0.56 ± 0.08**	0.80 ± 0.07*

Note: Values are expressed as mean ± SEM, n = 6. *P < 0.05, **P < 0.01 vs. Model group. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HTP, 5-hydroxytrptophan; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid.

3.7 Effects of Cs4-SeNPs on selenoproteins expressions in the SNpc of PD rats and on the total GPX activities in PD mice and rats

Se manifests its functions mainly in the form of synthesized selenoproteins in the organisms [43]. In this study, we detected the mRNA levels of 24 selenoproteins in the SNpc of rats by real-time PCR (Fig. S6). As shown in Fig. 5 and Fig. S6, most of the selenoprotein gene expressions did not change very much in the PD rat model compared with normal rats, except for the Selv mRNA levels which were found to be significantly lower in the SNpc of PD rats (Normal vs. Model). The treatment with Cs4-SeNPs for 4 weeks specifically enhanced the mRNA expressions of GPX2 (t = 2.387, df = 10.99, P = 0.036; dbm = 1.470 and 95%CI: 0.115 to 2.826), Sepx1 (t = 2.618, df = 10.83, P = 0.0242; dbm = 0.2618 and 95%CI: 0.041 to 0.482), Selo (t = 2.777, df = 10.88, P = 0.0182; dbm = 0.244 and 95%CI: 0.050 to 0.437), Sep15 (t = 2.242, df = 10.65, P = 0.047; dbm = 0.478 and 95%CI: 0.007 to 0.951), Sephs2 (t = 3.723, df = 9.794, P = 0.004; dbm = 0.788 and 95%CI: 0.315 to 1.260), Txnrd1 (t = 3.551, df = 10.15, P = 0.005; dbm = 0.327 and 95%CI: 0.122 to 0.532).

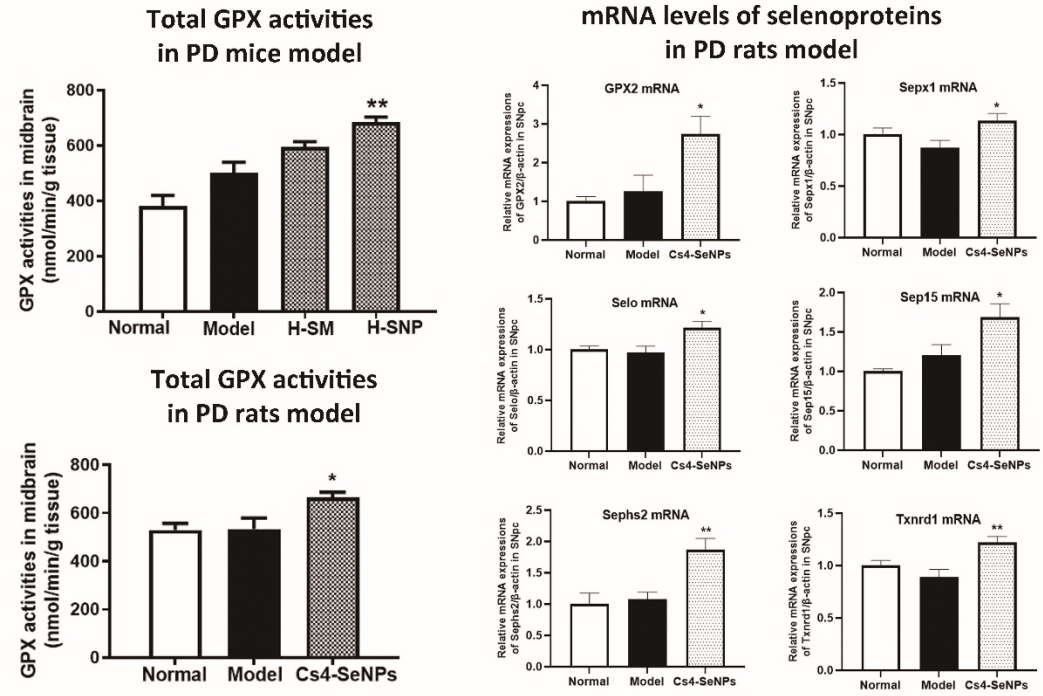

Figure 5 Effects of Cs4-SeNPs on the total GPX activities in the midbrain of mice or rats and on the mRNA expressions of representative selenoproteins in the SNpc of rats. GPX activities were measured by corresponding ELISA kit and expressed as nmol/min/g tissue. mRNA expressions were shown as quantitative ratio of specific selenoprotein (GPX2, Sepx1, Selo, Sep15, Sephs2, Txnrd1) to β-actin gene and relative to group mean of Normal group. All values are expressed as mean ± SEM, n = 6-8. *P < 0.05, **P < 0.01 vs. Model group. Cs4-SeNPs, selenium nanoparticles functionalized by Cs4 polysaccharides; GPX, glutathione peroxidase; SNpc, substantia nigra pars compacta. GPX2, glutathione peroxidase 2; Sepx1, methionine-R-sulfoxide reductase; Selo, selenoprotein O; Sep15, 15 kDa selenoprotein; Sephs2, selenophosphate synthetase 2; Txnrd1, thioredoxin reductase 1.

Although we also measured 6 selenoprotein expressions in the PD mice (Fig. S5), neither of these treatments could significantly influence these selenoprotein gene expressions. Also, the total GPX activities in the midbrain of mice and rats were measured. The results showed that the GPX activities did not change substantially in PD mice compared with normal mice, although high dose Cs4-SeNPs (for 2 weeks) did significantly improve the total GPX activities in the midbrain of PD mice (t = 4.389, df = 7.154, P = 0.003; dbm = 183.5 and 95%CI: 85.09 to 282.0) (vs. Model), which was what Se-Met at the same Se dose could not do (Fig. 5). Similarly, the total GPX activities did not change much in the PD rats compared with normal rats; but the treatment with Cs4-SeNPs (lower lose for 4 weeks) did significantly improve the total GPX activities in the midbrain of PD rats (t = 2.579, df = 7.063, P = 0.036; dbm = 132.2 and 95%CI: 11.23 to 253.2) (Fig. 5). The significant preventative neuroprotection against PD performed by Cs4-SeNPs in both PD mice and PD rats were closely associated with their ability to improve the GPX activities.

3.8 Antioxidant properties of different Se forms

Se exerts important antioxidant effects in organisms. Our present experiments compared the antioxidant properties of different Se forms including inorganic selenite, organic Se-Met, and Cs4-SeNPs using *in vitro* TEAC and FRAP assays. Our results indicated that Cs4-SeNPs showed the strongest antioxidant activity, while selenite showed the lowest antioxidant activity at the same Se dose in both TEAC (Fig. 6A) and FRAP (Fig. 6B) assays.

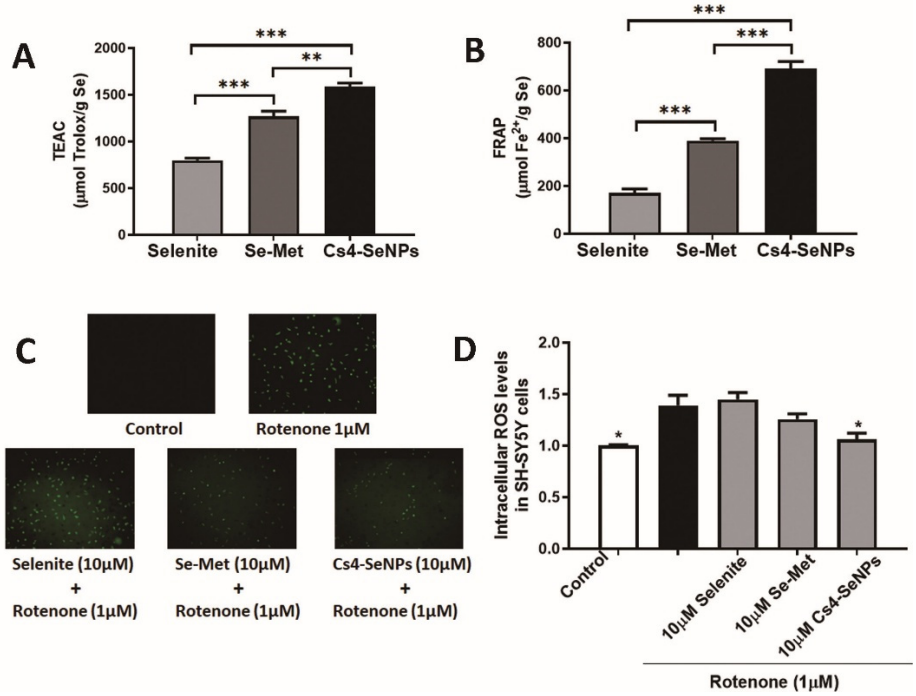

Figure 6 Antioxidant activity of different Se forms measured by TEAC assay (A) and FRAP assay (B). Data were presented as means ± SEM from three independent experiments with n = 3. **P < 0.01, ***P < 0.001, selenite vs. Se-Met or Se-Met vs. Cs4-SeNPs or selenite vs. Cs4-SeNPs. (C, D) ROS generation in response to different Se forms in rotenone induced SH-SY5Y cells. (C) Cells with/without treatment were stained with DCFH-DA under fluorescence microscopy. (D) Intracellular ROS generation was detected by DCFH-DA assay kit and expressed as O.D. readings normalized to total protein content and relative to control. Data were presented as means ± SEM from three independent experiments with n = 3. *P < 0.05 vs. negative control with rotenone induction. Se, selenium; TEAC, trolox equivalent antioxidant capacity; FRAP, ferric reducing ability of plasma; ROS, intracellular reactive oxygen species.

SH-SY5Y cells are human neuroblastoma cells with moderate levels of dopamine-β hydroxylase activity and are commonly used for the preparation of *in vitro* PD cell models. Rotenone is a natural organic insecticide with high lipophilicity, which can cross the blood-brain barrier and have a role in the production of mitochondrial respiratory chain complex I in brain tissue, thereby reducing ATP production and increasing ROS synthesis, leading to endoplasmic reticulum stress (ERs) and apoptosis [44]. Rotenone can induce PD-like pathological changes and symptoms in vertebrates and invertebrates, so it is often used to prepare PD animal or cell models of PD [45]. We also detected intracellular ROS generation in response to different Se forms in rotenone induced PD cell model. The results showed that rotenone (1μmol/L) significantly increased ROS generation in SH-SY5Y cells (t = 5.387, df = 2.040, P = 0.031; dbm = 0.462 and 95%CI: 0.100 to 0.823) (vs. Control), mimicking high oxidative stress levels in PD. In comparison with the same Se dose (10μmol/L) of inorganic selenite and organic Se-Met, only Cs4-SeNPs could significantly downregulate the increased intracellular ROS levels induced by rotenone in SH-SY5Y cells (t = 3.860, df = 3.564, P = 0.023; dbm = 0.401 and 95%CI: 0.098 to 0.703) (vs. negative control with rotenone induction, Fig. 6C, D). These results indicated that Cs4-SeNPs had the highest antioxidant properties compared with inorganic selenite and organic Se-Met; and only Cs4-SeNPs significantly prevented the increase of cellular ROS generation in PD cell model.

3.9 Effects of Cs4-SeNPs on protein expression of TH and α-synuclein and apoptosis proteins in PD cell model

The pathological hallmarks of PD are accumulation and aggregation of α-Synuclein in the SNpc of the midbrain and other neural structures, leading to apoptosis of dopaminergic neurons [34]. TH expression is a marker of dopaminergic neurons. Our results demonstrated that rotenone (300nm) significantly decreased TH (Figure 7A, B and Fig. S9) (t = 4.375, df = 3.192, P = 0.0194, dbm = 0.443 and 95%CI: 1.754 to 0.131) but increased α-Synuclein (Fig. 7A, C and Fig. S9) (t = 3.424, df = 4.075, P = 0.0259, dbm = 0.807 and 95%CI: 0.157 to 1.457) protein expressions in SH-SY5Y cells. These results indicated that rotenone induced PD cell model could mimic major PD pathological features. After incubation together with rotenone, it was found that Cs4-SeNPs could significantly improve the decreased TH (at concentration of 0.1-1μM, Fig. 7A, B) (0.1μM: t = 3.194, df = 3.312, P = 0.0432, dbm = 0.256 and 95%CI: 0.014 to 0.498; 1μM: t = 4.502, df = 3.396, P = 0.0156, dbm = 0.322 and 95%CI: 0.109 to 0.535) and suppress the increased α-Synuclein (at concentrations of 0.1-10μM, Fig. 7A, C) (0.1μM: t = 4.07, df = 3.572, P = 0.0191, dbm = 0.923 and 95%CI: 0.262 to 1.583; 1μM: t = 3.223, df = 5.619, P = 0.0198, dbm = 0.879 and 95%CI: 0.201 to 1.557; 10μM: t = 2.577, df = 5.581, P = 0.0448, dbm = 0.699 and 95%CI: 0.023 to 1.735) protein expressions in PD cell model, which confirmed its neuroprotective effects *in vitro*.

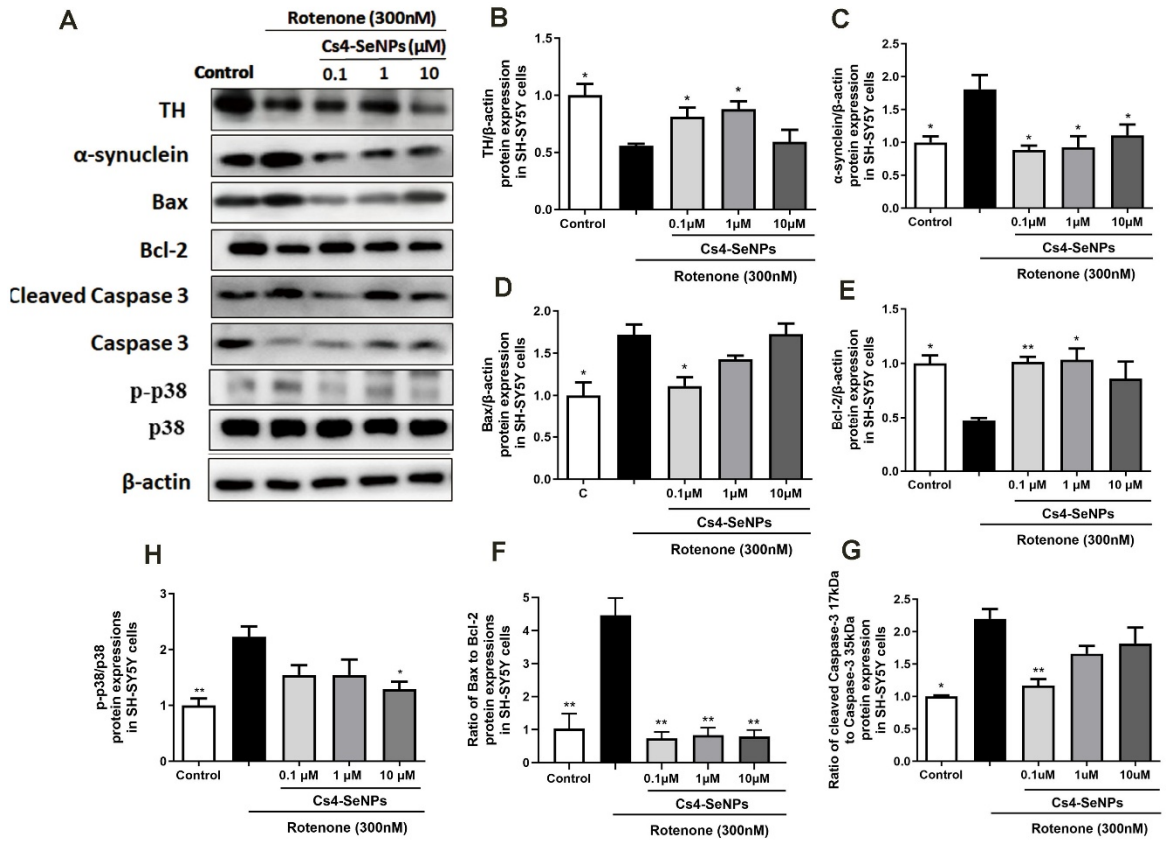

Figure 7 Neuroprotective effects of Cs4-SeNPs against apoptosis in PD cell model. Protein expressions of TH, α-synuclein, and apoptosis proteins (Bax, Bcl-2, cleaved caspase 3, caspase 3, p-p38, p38) were measured by western-blot in rotenone induced SH-SY5Y cells. One representative band for the specific protein and endogenous β-actin from each group was shown in the left panel (A); and the following bar chart shows the ratio of the specific protein to β-actin in each group (B-H). Data were presented as means ± SEM from three independent experiments with n = 3. **P* < 0.05, ***P* < 0.01 vs. negative control with rotenone induction. Cs4-SeNPs, selenium nanoparticles functionalized by Cs4 polysaccharides; PD, Parkinson's disease; TH, tyrosine hydroxylase.

Our results demonstrated that rotenone (300nm) significantly increased Bax (t = 3.597, df = 3.82, *P* = 0.0247, dbm = 0.717 and 95%CI: 0.153 to 1.281) and caspase-3 (t = 7.946, df = 2.055, *P* = 0.0143, dbm = 1.197 and 95%CI: 0.565 to 1.829) (Fig. 7A, D, G and Fig. S9), but decreased Bcl-2 (Fig.7A, E) (t = 6.759, df = 2.526, *P* = 0.0112, dbm = 0.528 and 95%CI: 0.251 to 0.806) protein expressions, leading to a higher ratio of Bax to Bcl-2 (Fig. 7F and Fig. S9) (t = 8.584, df = 3.894, *P* = 0.001, dbm = 3.423 and 95%CI: 2.304 to 4.543) in SH-SY5Y cells; and at the same time, it promoted the phosphorylation level of p38 (Fig. 7A, H and Fig. S9) (t = 5.506, df = 3.48, *P* = 0.0079, dbm = 1.228 and 95%CI: 0.571 to 1.885) in SH-SY5Y cells. These results demonstrated rotenone induced apparent neuron apoptosis. Treatment with 0.1μmol/L Cs4-SeNPs directly inhibited the increased Bax (Fig. 7A, D) (t = 3.731, df = 3.863, *P* = 0.0216, dbm = 0.607 and 95%CI: 0.148 to 1.059), while 0.1-1μmol/L Cs4-SeNPs upregulated the decreased Bcl-2 protein expressions (Fig. 7A, E) (0.1μM: t = 10.12, df = 3.202, *P* = 0.0015, dbm = 0.543 and 95%CI: 0.378 to 0.708; 1μM: t = 5.213, df = 2.264, *P* = 0.0267, dbm = 0.561 and 95%CI: 0.146 to 0.976), leading to a significantly lower ratio of Bax to Bcl-2 in response to 0.1-10μmol/L Cs4-SeNPs (Fig. 7A, F) (0.1μM: t = 9.627, df = 2.235, *P* = 0.0073, dbm = 1.338 and 95%CI: 0.796 to 1.879; 1μM: t = 14.14, df = 2.63, *P* = 0.0015, dbm = 1.245 and 95%CI: 0.941 to 1.548; 10μM: t = 11.27, df = 2.564, *P* = 0.003, dbm = 3.67 and

95%CI: 2.527 to 4.813) in the rotenone induced cell model of PD. Meanwhile, Cs4-SeNPs greatly decreased the rotenone induced cleaved caspase-3 expressions (at concentration of 0.1μmol/L, Fig. 7A, G) (t = 5.753, df = 3.442, *P* = 0.0071, dbm = 1.028 and 95%CI: 0.499 to 1.557) and phosphorylation of p38 (at concentration of 10μmol/L, Fig. 7A, H) (t = 4.106, df = 3.611, *P* = 0.0182, dbm = 0.936 and 95%CI: 0.275 to 1.596) in SH-SY5Y cells. These results clearly indicated Cs4-SeNPs protected dopaminergic neurons by inhibiting neuron apoptosis.

3.10 Signalling pathways mediating the anti-apoptotic actions of Cs4-SeNPs in PD cell model

Endoplasmic reticulum stress (ERs) and autophagy imbalance induce apoptosis and participate in the pathogenesis of PD [46]. Selenium protein K (Selk) is present in the endoplasmic reticulum and has been reported to be closely related to ERs. Our results showed that SelK gene expression (t = 7.969, df = 5.922, *P* = 0.0002, dbm = 0.615 and 95%CI: 0.426 to 0.804) significantly decreased in response to rotenone induction, and Cs4-SeNPs (at concentration of 0.1-1μM) treatment could specifically upregulate SelK gene expression (Fig. 8A) (0.1μM: t = 5.152, df = 5.184, *P* = 0.0032, dbm = 0.482 and 95%CI: 0.244 to 0.719; 1μM: t = 5.173, df = 3.335, *P* = 0.0107, dbm = 1.153 and 95%CI: 0.482 to 1.823). We also found that rotenone treatment up-regulated the expression of GPR78 (t = 4.843, df = 3.979, *P* = 0.0085, dbm = 1.731 and 95%CI: 0.583 to 2.159) and CHOP (t = 12.96, df = 3.286, *P* = 0.0006, dbm = 1.758 and 95%CI: 1.347 to 2.170) (Fig. 8B, C, D and Fig. S10), and Cs4-SeNPs (at concentration of 0.1-10μM) treatment could inhibit GPR78 (0.1μM: t = 4.843, df = 3.979, *P* = 0.0085, dbm = 1.371 and 95%CI: 0.583 to 2.159; 1μM: t = 9.806, df = 3.15, *P* = 0.0018, dbm = 2.17 and 95%CI: 1.484 to 2.855; 10μM: t = 11.2, df = 2.629, *P* = 0.0028, dbm = 2.29 and 95%CI: 1.573 to 3.008) and CHOP (0.1μM: t = 15.89, df = 3.898, *P* = 0.0001, dbm = 1.721 and 95%CI: 1.417 to 2.025; 1μM: t = 5.418, df = 2.512, *P* = 0.0192, dbm = 1.119 and 95%CI: 0.383 to 1.855) protein expression (Fig. 8B, C, D). These results suggest that Cs4-SeNPs may alleviate ERs via increasing SelK synthesis specifically, decreasing GPR78 and CHOP protein expressions, thereby protecting against ERs mediated neuron apoptosis.

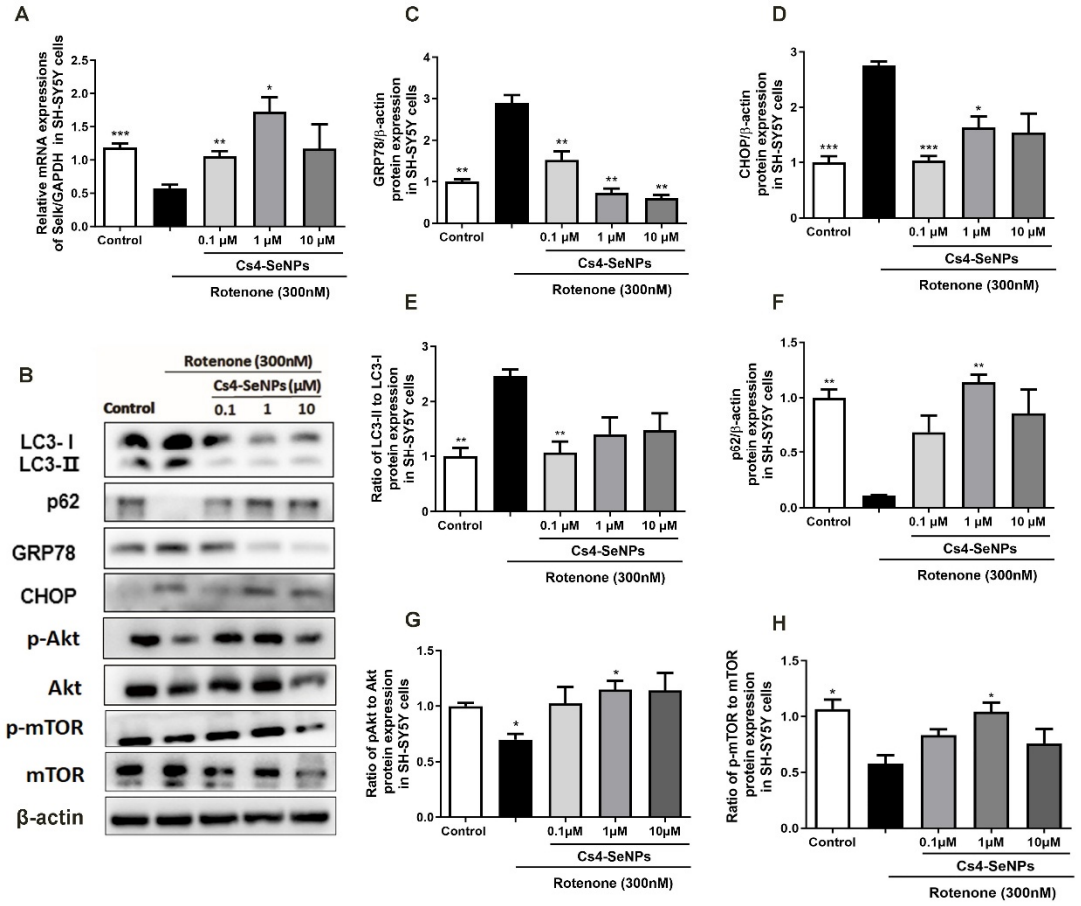

Figure 8 Signalling pathways mediating the anti-apoptotic actions of Cs4-SeNPs in PD cell model. SelK mRNA expression was measured by qPCR (A). ERs proteins (GPR78, CHOP), autophagy proteins (LC3-I, LC3-II, p62) and critical proteins (p-mTOR, mTOR, p-Akt, Akt) in related signalling pathways were measured by western-blot in rotenone induced SH-SY5Y cells. One representative band for the specific protein and endogenous β-actin from each group was shown in the left panel (B); and the following bar chart shows the ratio of the specific protein to β-actin in each group (C-H). Data were presented as means ± SEM from three independent experiments with n = 3. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. negative control with rotenone induction. Cs4-SeNPs, selenium nanoparticles functionalized by Cs4 polysaccharides; PD, Parkinson's disease; ERs, Endoplasmic reticulum stress; SelK, Selenoprotein K; GPR78, glucose regulated proteins 78; CHOP, C/EBP homology protein; LC3, microtubule-associated protein 1A/1B-light chain 3; mTOR, mammalian target of rapamycin.

ERs can induce autophagy, and over-activated autophagy leads to apoptosis [47]. Up-regulation of LC3-II/I ratio and down-regulation of ubiquitinated p62 is a marker of autophagy activation [48]. Studies have found that LC3-II expression is significantly up-regulated in rotenone-induced PD animals [49]. Our results showed that rotenone could significantly enhance the ratio of LC3-II to LC3-I protein expression (t = 7.103, df = 3.661, $P = 0.0029$, dbm = 1.463 and 95%CI: 0.869 to 2.056) and decrease the expression of p62 protein (t = 11.48, df = 2.042, $P = 0.0069$, dbm = 0.893 and 95%CI: 0.565 to 1.222) in SH-SY5Y cells, indicating again that rotenone could induce autophagy. Cs4-SeNPs treatment significantly inhibited rotenone-induced autophagy by suppressing LC3-II/I ratio (0.1μmol/L Cs4-SeNPs, Fig. 8B, E and Fig. S10) (t = 5.666, df = 3.165, $P = 0.0094$, dbm = 1.396 and 95%CI: 0.634 to 2.157) and increasing p62 expressions (1μmol/L Cs4-SeNPs, Fig. 8B, F and Fig. S10) (t = 14.56, df = 2.051, $P = 0.0042$, dbm = 1.034 and 95%CI: 0.735 to 1.332). Our further studies found that rotenone induces autophagy via inactivating PI3K/Akt/mTOR pathway as shown by reduced phosphorylation of Akt (t = 5.108, df = 3.097, $P = 0.0134$, dbm = 0.301 and 95%CI: 0.117 to 0.485) and mTOR (t = 4.263, df = 3.963, $P = 0.133$, dbm = 0.489 and 95%CI: 0.169 to

0.809) proteins in SH-SY5Y cells (Fig. 8B, G, H and Fig. S10). This result is consistent with previous report [49]. Cs4-SeNps (1μmol/L) significantly improved the decreased phosphorylation of Akt (p-Akt/Akt; Fig. 8B, G) (t = 4.79, df = 3.459, P = 0.0124, dbm = 0.450 and 95%CI: 0.172 to 0.728), and Cs4-SeNps (1μmol/L) significantly inhibited the phosphorylation of mTOR (p-mTOR/mTOR; Fig. 8B, H) (t = 4.14, df = 3.985, P = 0.0145, dbm = 0.466 and 95%CI: 0.153 to 0.779) in the rotenone induced PD cell model. These results demonstrated that Cs4-SeNPs play anti-apoptotic effects by inhibiting the activation of ERs and PI3K/Akt/mTOR signaling pathway mediated autophagy. And the effects of Cs4-SeNPs against ERs are highly related to the increased selenoprotein synthesis of SelK in ER.

4. Discussion

Se, as an essential antioxidant trace element, plays an important role in various neurodegenerative disorders, including AD and PD [50]. However, it is inorganic Se with the highest toxicity, but not ordinary used organic Se, could play the preventative effects against PD [19]. Although many studies have reported that SeNPs by using different preparation methods could protect from neurotoxin induced damage in PD cell or animal models [23-26], they didn't conduct systematic comparisons of the efficacy and toxicity with ordinary Se. In this study, we chose the functional agent of Cs4 polysaccharides to prepare novel SeNPs and conducted systematic studies on the toxicity, preventive effects, and underlying mechanisms of our novel SeNPs in comparison with ordinary inorganic and organic Se.

To improve the extraction efficiency of Cs4 polysaccharide and consider the stability of the prepared SeNPs, we specifically developed a novel extraction technology (micronization + ultrasonic-circulating extraction) tailor-made for Cs4 PSP (polysaccharides-protein complex) prior to the preparation of Cs4-SeNPs. Micronization (ultra-fine grinding or milling) is a mechanical and high shearing operation to downsize the particles of food material to the micron range, which can boost the functional and physiochemical properties and help to delay retrogradation, induce gelatinization, reduce syneresis and fermentation time, and improve the dissolution rate of foods [51]. Micronization provides an important basis for polysaccharide extraction. The ultrasonic-assisted extracting method is also a relatively novel technique being applied to plant polysaccharide extraction, which has been proven to be much more efficient than the traditional hot water extraction method [52]. A novel and efficient extraction technique (ultrasonic-circulating extraction integrated with superfine pulverization) has been successfully developed by our team to extract crude polysaccharides from the fruiting bodies of *Ganoderma lucidum* and this innovative method not only yields a higher extract compared to conventional methods, such as hot-water extraction (HWE), ultrasonic-assisted extraction (UAE), and superfine grinding-HWE extraction [31], but also results in total polysaccharides and their major fractions exhibiting enhanced antioxidant activities *in vitro* and stronger antioxidant activity *in vivo* [52, 53]. Based on the traditional hot water extraction method, the average yield of Cs4 polysaccharides in our previous study was low (about 7.85% only), but this optimized extraction technology (superfine-pulverization + ultrasonic-circulating extraction) specifically tailored to Cs4 polysaccharides greatly improved the extraction yield to be 11.03%. Our prepared Cs4 PSP

contains abundant polysaccharides and amino acids, which potentiate the stability of Cs4-SeNPs for 14 weeks, longer than that of Cs4-SeNPs prepared using Cs4 PSP derived from traditional methods (6 - 8 weeks). SeNPs were capped by Cs4 PSP mainly through the formation of Se-O bonds, giving rise to a stable spherical structure of Cs4-SeNPs with a particle size of about 77nm.

In order to compare the bioactive effects among the SeNPs (selenium nanoparticles stabilized with non-bioactive PEG), SeNPs plus Cs4 (combination treatment with SeNPs and Cs4 PSP; Cs4+SeNPs) and Cs4-SeNPs (our prepared SeNPs stabilized with bioactive Cs4 PSP; Cs4-SeNPs), PD cell model was pre-treated with the same Se (1μM) and Cs4 (0.4μg/ml) dose of SeNPs, Cs4+SeNPs and Cs4-SeNPs for 1 hour, followed by co-treatment with rotenone for an additional 24 hours to examine their effects on cell viability by CCK8 method. As shown in Figure S13, only Cs4-SeNPs exhibited significant protective effects against rotenone-induced toxicity. The results demonstrate that the Cs4-SeNPs we prepared exert a synergistic neuroprotective effect through the organic combination of SeNPs and Cs4 PSP, with efficacy superior to that of conventional SeNPs, and not a simple addition of the effects of the Cs4 PSP and SeNPs.

Before efficacy evaluation, systematic acute and sub-chronic toxicity evaluation of Cs4-SeNPs was performed to ensure its biological safety. The acute toxicity results found that the LD50 of Cs4-SeNPs (511-619 mg Se/kg) was much less toxic than the reported LD50 of inorganic selenite (8-12 mg Se/kg) [37] and organic Se-Met (23.9-30.1 mg Se/kg) [38] in mice. The NOAEL of Cs4-SeNPs in male and female rats was 0.9 mg Se/kg (1.8 mg/kg, approximate equivalent dose in mice), that is much higher than the NOAEL of inorganic and organic Se (0.14-0.2 mg Se/kg) [54]. The doses applied in our study (0.1-1mg Se/kg in mice and 0.07mg Se/kg in rats) are proven to be within the safe range. Our findings verified again the lower toxicity of SeNPs than common food sources of organic and inorganic selenocompounds as demonstrated in other reports [55]. One article utilizing HSA-SeNPs (selenium nanoparticles stabilized with human serum albumin (HSA)) has compared the efficacy and toxicity profiles with organic Se and BSA-SeNPs (selenium nanoparticles stabilized with bovine serum albumin). The study demonstrated that the LD50 of HSA/Se NPs, sodium selenite, and BSA-SeNPs in mice were 95.9, 12.3, and 87.5 mg Se/kg, respectively [26]. Our results demonstrated our prepared Cs4-SeNPs have much lower toxicity than the reported HSA-SeNPs. Our prepared Cs4 PSP—rich in polysaccharides and amino acids—not only caps Cs4-SeNPs primarily via Se-O bond formation to yield stable spherical particles (≈77 nm) but also enhances the stability of these Cs4-SeNPs for 14 weeks, a duration significantly longer than the 6–8 weeks observed for Cs4-SeNPs prepared using Cs4 PSP derived from traditional methods; this superior stability is thus speculated to be a key factor contributing to the lower toxicity of our Cs4-SeNPs compared to those from other preparation methods.

In our study, a mouse PD model induced by MPTP was used to compare the preventative effects of Cs4-SeNPs and Se-Met with the same Se content. The results showed that preventative oral administration of Cs4-SeNPs for 2 weeks at a high dose (1000 μg Se/kg/d) in acute PD mouse model and for 4 weeks at low dose (70 μg Se/kg/d) in PD rat model could significantly improve motor functions and prevent dopaminergic

neuronal loss, but organic Se-Met at the same Se dosage level could not do the same. These results are consistent with our previous findings that showed the inability of organic Se-Met to prevent dopaminergic neuronal loss in MPTP injected mouse model [19]. On the other hand, inorganic Se was able to provide neuroprotection against PD with higher bioavailability in PD mouse than Se-Met, but it is extremely toxic [19]. To further validate the preventative neuroprotection of Cs4-SeNPs against PD, another PD rat model created by microinjecting 6-OHDA into the left MFB was used in this study. The results showed that Cs4-SeNPs could significantly improve the motor ability of PD rats in apomorphine-induced rotation experiment, increase the expression of TH gene and protein in substantia nigra, and increase the content of dopamine and its metabolites in the striatum.

Se exercises its biological functions in the brain via selenoproteins that were mainly expressed in neurons, among which, Selp and GPX4 were regarded as the important contributors to neurological integrity [6]. Although the relationship between many other selenoproteins and PD remain unclear, most of the selenoproteins exhibited antioxidant activities. Our results showed that Cs4-SeNPs could increase the total GPX activity and the mRNA levels of GPX2, Seli, Sepx1, Selo, Sep15, Sephs2 and Txnrd1 selenoproteins, which may play an important role in fighting against the PD pathogenesis. However, the same dose of Se-Met could not significantly increase the total GPX activity in PD mice.

Our previous bio-distribution experiments showed that Se accumulated to a peak in the brain at 6 hours after oral administration of Cs4-SeNPs, followed by a gradual decline (Figure S11). This pharmacokinetic result is consistent with previously published data on SeNPs and HSA-SeNPs [26]. At the peak, the original form of SeNPs in the brain was barely detectable, while GPX and SeGalNAc levels increased significantly (Figure S12), GPX in particular. In the present study, considering the extremely low levels of SeGalNAc in the midbrain of mice, we compared the GPX mRNA levels and total GPX activity in response to Cs4-SeNPs and Se-Met at the same dose in the midbrain of PD mice. Our results showed high dose Cs4-SeNPs (for 2 weeks) did significantly improve the total GPX activities in the midbrain of PD mice, which was what Se-Met at the same Se dose could not do (Fig. 5). From these results, we can clearly conclude that Cs4-SeNPs have a higher Se brain uptake rate than organic Se. However, inorganic Se killed PD mice at very low doses, making it difficult to directly compare Cs4-SeNPs with inorganic Se in terms of anti-PD effects.

These results demonstrate that Cs4-SeNPs have higher Se boosting capacity and the underlying mechanisms behind the preventative effects of Cs4-SeNPs are mainly attributed to their stimulatory effects on some specific antioxidant selenoprotein expressions and activities in the midbrain of PD mice or rats. Our further mechanism studies verified that Cs4-SeNPs had the highest antioxidant properties compared to inorganic and organic Se, and that Cs4-SeNPs could protect against ROS induced dopaminergic neuron apoptosis as indicated by the upregulation of the decreased TH, Bcl-2, the downregulation of the increased α-Synuclein and Bax protein expressions, caspase cleavage and phosphorylation of p38 in the PD cell model.

Endoplasmic reticulum stress (ERs) and autophagy imbalance induce apoptosis and are involved in the pathogenesis of PD [56]. GRP78 and CHOP are the key signalling molecules in ERs-mediated apoptosis. When

ERs occurs, GRP78 dissociates from the three trans-membrane ERs effector proteins in the ER and induces the expression of CHOP through the downstream signalling pathway, which induces apoptosis by regulating the Bcl-2 protein family [57]. The biological effect of Se *in vivo* is mediated mainly by selenoproteins and many selenoproteins are closely related to ERs. Among them, selenoprotein K (SelK) is a single channel transmembrane protein present in ER. It has been reported that CHOP levels significantly increased in the cortex and hippocampus of SelK-/- mice, confirming that loss of SelK gene can induce ERs in neurons and eventually lead to apoptosis [58]. Our results showed that Cs4-SeNPs play anti-apoptotic effects by inhibiting the activation of ERs and PI3K/Akt/mTOR signaling pathway (Figure S15 and S16) mediated autophagy in rotenone induced PD cell model. And the effects of Cs4-SeNPs against ERs are highly related to the increased selenoprotein synthesis of SelK in ER, as Cs4-SeNPs treatment significantly increased SelK mRNA (Figure 8A) and protein (Figure S14) levels in PD cell model. But SelK gene expression was not obviously changed in response to Cs4-SeNPs in PD rat model (Figure S6). Regarding the differential expression of SelK in animals and cells, the discrepancy is primarily attributed to the time points of observation. Our preliminary Se distribution experiments showed that Se accumulation in the brain peaked at 6 hours post-gastric gavage of Cs4-SeNPs, followed by a gradual decline (Figure S11). Our animal experiments for efficacy evaluation only reflected the average Se level after long-term gavage, whereas the cell experiments were conducted at 6 hours post-Cs4-SeNPs treatment. These two scenarios cannot be directly compared.

A schematic diagram of the molecular mechanism for the neuroprotective effects of Cs4-SeNPs is summarized in Figure 9. In brief, neurotoxins such as MPTP, 6-OHDA, and rotenone induce oxidative stress in the brain, leading to the death of dopaminergic neurons. Our prepared Cs4-SeNPs exhibit stronger antioxidant activity and lower toxicity compared to commonly used inorganic and organic Se compounds. Preventive administration of our Cs4-SeNPs can enhance the synthesis and activity of selenoproteins in neuronal cells, thereby increasing the neurons' resistance to oxidative stress. Additionally, during this process, the increased expression of specific selenoprotein helps to counteract ERs and inhibit excessive autophagy.

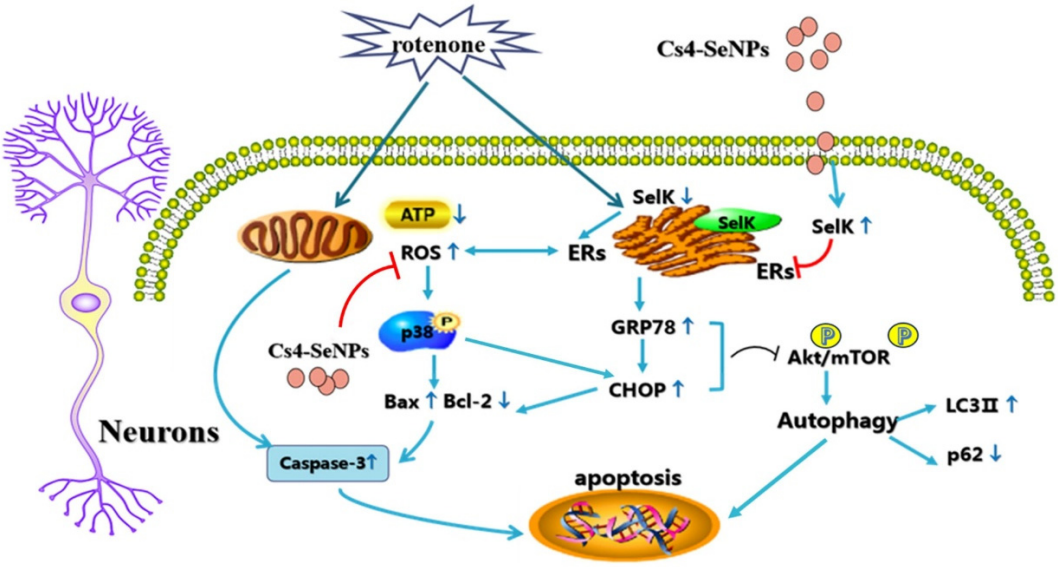

Figure 9 Schematic diagram of the molecular mechanism for the neuroprotective effects of Cs4-SeNPs against rotenone-induced dopaminergic neuronal injury.

Since many clinical observations have revealed higher levels of Se in patients with PD [10-14], whether Se acts as a cause or effect of PD sparked controversy. But many clinical [15, 16] and pre-clinical [17-19] studies have demonstrated the preventative effects of Se against PD. And the elevated levels of Se in PD patients may have multiple causes, which do not necessarily contradict the preventive role of Se against PD. Our study demonstrated that Se intake at an appropriate dose and chemical form could help prevent the occurrence of PD. However, whether Se, including our Cs4-SeNPs, could serve as a therapeutic agent requires further investigation. Cs4 PSP also has antioxidant capacity with the potential of neuroprotection in PD animal model. In our current animal experiment, the effective dose of Se administered to PD mice was 1 mg/kg, which corresponds to a Cs4 PSP dose of 4.5 mg/kg. Based on our previous research experience and the other similar studies with polysaccharides in PD mouse models, polysaccharides typically require an oral dose of 30-200mg/kg administered over a period of up to one month [34, 59], or at least an oral dose of 60-100mg/kg [60] before their effects can be observed in PD mice. A 4.5 mg/kg dose of polysaccharides administered to PD mice for only two weeks would not be capable of producing significant neuroprotective effects. It is speculated that the neuroprotective effect of Cs4-SeNPs results from the synergistic action between Cs4 PSP and Se.

4. Conclusion

Our findings proved that SeNPs that were functionalized by tailor-made Cs4 PSP potentiated the preventative neuroprotection of Se against PD with higher antioxidant activities and lower toxicity. This study provides solid scientific evidence for the development of an economical and safe oral agent in the prevention of PD, which will help to curb the spiralling costs of neurodegenerative disease treatments in the healthcare system.

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Availability of data and materials

All data are available in the main text or Supplementary Materials. The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declared no conflict of interest.

References

1. Armstrong MJ, Okun MS: **Diagnosis and Treatment of Parkinson Disease: A Review.** *Jama* 2020, **323:**548-560.
2. Seppi K, Ray Chaudhuri K, Coelho M, Fox SH, Katzenschlager R, Perez Lloret S, Weintraub D, Sampaio C: **Update on treatments for nonmotor symptoms of Parkinson's disease-an evidence-based medicine review.** *Mov Disord* 2019, **34:**180-198.
3. Kumar S, Goyal L, Singh S: **Tremor and Rigidity in Patients with Parkinson's Disease: Emphasis on Epidemiology, Pathophysiology and Contributing Factors.** *CNS Neurol Disord Drug Targets* 2022, **21:**596-609.
4. Chakrabarti S, Bisaglia M: **Oxidative Stress and Neuroinflammation in Parkinson's Disease: The Role of Dopamine Oxidation Products.** *Antioxidants (Basel)* 2023, **12:**955.
5. Zhang F, Li X, Wei Y: **Selenium and Selenoproteins in Health.** *Biomolecules* 2023, **13:**799.
6. Chen J, Berry MJ: **Selenium and selenoproteins in the brain and brain diseases.** *J Neurochem* 2003, **86:**1-12.
7. Leiter O, Zhuo Z, Rust R, Wasielewska JM, Grönnert L, Kowal S, Overall RW, Adusumilli VS, Blackmore DG, Southon A, et al: **Selenium mediates exercise-induced adult neurogenesis and reverses learning deficits induced by hippocampal injury and aging.** *Cell Metab* 2022, **34:**408-423.e408.
8. Alim I, Caulfield JT, Chen Y, Swarup V, Geschwind DH, Ivanova E, Seravalli J, Ai Y, Sansing LH, Ste Marie EJ, et al: **Selenium Drives a Transcriptional Adaptive Program to Block Ferroptosis and Treat Stroke.** *Cell* 2019, **177:**1262-1279.e1225.
9. Qi Z, Duan A, Ng K: **Selenoproteins in Health.** *Molecules* 2023, **29:**136.
10. Zhao HW, Lin J, Wang XB, Cheng X, Wang JY, Hu BL, Zhang Y, Zhang X, Zhu JH: **Assessing plasma levels of selenium, copper, iron and zinc in patients of Parkinson's disease.** *PLoS One* 2013, **8:**e83060.
11. Zhang YJ, Sun HL, Wang T, Liu XX, Liu C, Li WJ, Li X: **Selenium level does not differ in blood but increased in cerebrospinal fluid in Parkinson's disease: a meta-analysis.** *Int J Neurosci* 2021, **131:**95-101.
12. Adani G, Filippini T, Michalke B, Vinceti M: **Selenium and Other Trace Elements in the Etiology of Parkinson's Disease: A Systematic Review and Meta-Analysis of Case-Control Studies.** *Neuroepidemiology* 2020, **54:**1-23.
13. Chen Q, Hu X, Zhang T, Ruan Q, Wu H: **Association between Parkinson disease and selenium levels in the body: A systematic review and meta-analysis.** *Medicine (Baltimore)* 2024, **103:**e37919.
14. Qureshi GA, Qureshi AA, Memon SA, Parvez SH: **Impact of selenium, iron, copper and zinc in on/off Parkinson's patients on L-dopa therapy.** *J Neural Transm Suppl* 2006**:**229-236.
15. Zeng Z, Cen Y, Luo X: **Association between blood selenium with parkinson's disease in the US (NHANES 2011-2020).** *Environ Sci Pollut Res Int* 2023, **30:**117349-117359.
16. Shahar A, Patel KV, Semba RD, Bandinelli S, Shahar DR, Ferrucci L, Guralnik JM: **Plasma selenium is positively related to performance in neurological tasks assessing coordination and motor speed.** *Mov Disord* 2010, **25:**1909-1915.
17. Ellwanger JH, Molz P, Dallemole DR, Pereira dos Santos A, Muller TE, Cappelletti L, Goncalves da Silva M, Franke SI, Pra D, Pegas Henriques JA: **Selenium reduces bradykinesia and DNA damage in a rat model of Parkinson's disease.** *Nutrition* 2015, **31:**359-365.
18. Zafar KS, Siddiqui A, Sayeed I, Ahmad M, Salim S, Islam F: **Dose-dependent protective effect of selenium in rat model of Parkinson's disease: neurobehavioral and neurochemical evidences.** *J Neurochem* 2003, **84:**438-446.
19. Sun C, Du Z, Liu X, Yang Y, Zhou S, Li C, Cao X, Zhao Q, Wong K, Chen W, Dong X: **Selenium Forms and Dosages Determined Their Biological Actions in Mouse Models of Parkinson's Disease.** *Nutrients* 2022, **15**.
20. Liu M, Jiao Q, Du X, Bi M, Chen X, Jiang H: **Potential Crosstalk Between Parkinson's Disease and Energy Metabolism.** *Aging Dis* 2021, **12:**2003-2015.
21. Genchi G, Lauria G, Catalano A, Sinicropi MS, Carocci A: **Biological Activity of Selenium and Its Impact on Human Health.** *Int J Mol Sci* 2023, **24:**2633.
22. Zhang T, Qi M, Wu Q, Xiang P, Tang D, Li Q: **Recent research progress on the synthesis and biological effects of selenium nanoparticles.** *Front Nutr* 2023, **10:**1183487.

23. Zhao H, Song J, Wang T, Fan X: **Selenium nanoparticles decorated with polysaccharides from Sargassum fusiforme protects against 6-OHDA-induced neurotoxicity in PC12 cells and rat model of Parkinson's disease.** *Nanomedicine* 2024, **59:**102755.
24. Salaramoli S, Amiri H, Joshaghani HR, Hosseini M, Hashemy SI: **Bio-synthesized selenium nanoparticles ameliorate Brain oxidative stress in Parkinson disease rat models.** *Metab Brain Dis* 2023, **38:**2055-2064.
25. Yue D, Zeng C, Okyere SK, Chen Z, Hu Y: **Glycine nano-selenium prevents brain oxidative stress and neurobehavioral abnormalities caused by MPTP in rats.** *J Trace Elem Med Biol* 2021, **64:**126680.
26. Xu K, Huang P, Wu Y, Liu T, Shao N, Zhao L, Hu X, Chang J, Peng Y, Qu S: **Engineered Selenium/Human Serum Albumin Nanoparticles for Efficient Targeted Treatment of Parkinson's Disease via Oral Gavage.** *ACS Nano* 2023, **17:**19961-19980.
27. Huang G, Liu Z, He L, Luk KH, Cheung ST, Wong KH, Chen T: **Autophagy is an important action mode for functionalized selenium nanoparticles to exhibit anti-colorectal cancer activity.** *Biomater Sci* 2018, **6:**2508-2517.
28. Zeng D, Zhao J, Luk KH, Cheung ST, Wong KH, Chen T: **Potentiation of in Vivo Anticancer Efficacy of Selenium Nanoparticles by Mushroom Polysaccharides Surface Decoration.** *J Agric Food Chem* 2019, **67:**2865-2876.
29. Sang Q, Pan Y, Jiang Z, Wang Y, Zhang H, Hu P: **HPLC determination of massoia lactone in fermented Cordyceps sinensis mycelium Cs-4 and its anticancer activity in vitro.** *J Food Biochem* 2020, **44:**e13336.
30. Yan JK, Wang WQ, Wu JY: **Recent advances in Cordyceps sinensis polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: A review.** *J Funct Foods* 2014, **6:**33-47.
31. Kang Q, Chen S, Li S, Wang B, Liu X, Hao L, Lu J: **Comparison on characterization and antioxidant activity of polysaccharides from Ganoderma lucidum by ultrasound and conventional extraction.** *Int J Biol Macromol* 2019, **124:**1137-1144.
32. Alexander J, Olsen AK: **Selenium - a scoping review for Nordic Nutrition Recommendations 2023.** *Food Nutr Res* 2023, **67:**67.
33. He S, Ru Q, Chen L, Xu G, Wu Y: **Advances in animal models of Parkinson's disease.** *Brain Res Bull* 2024, **215:**111024.
34. Dong XL, Wang X, Liu F, Liu X, Du ZR, Li RW, Xue CH, Wong KH, Wong WT, Zhao Q, Tang QJ: **Polymannuronic acid prevents dopaminergic neuronal loss via brain-gut-microbiota axis in Parkinson's disease model.** *Int J Biol Macromol* 2020, **164:**994-1005.
35. Liu X, Du ZR, Wang X, Sun XR, Zhao Q, Zhao F, Wong WT, Wong KH, Dong XL: **Polymannuronic acid prebiotic plus Lacticaseibacillus rhamnosus GG probiotic as a novel synbiotic promoted their separate neuroprotection against Parkinson's disease.** *Food Res Int* 2022, **155:**111067.
36. Ferro C, Florindo HF, Santos HA: **Selenium Nanoparticles for Biomedical Applications: From Development and Characterization to Therapeutics.** *Adv Healthc Mater* 2021, **10:**e2100598.
37. Jacevic V, Jokić G, Dragojevic Simic V, Bokonjić D, vučinić S, Marina V: **Acute toxicity of sodium selenite in rodents: pathomorphological study.** *Mil Med Sci Lett* 2011, **80:**90-96.
38. Wang H, Zhang J, Yu H: **Elemental selenium at nano size possesses lower toxicity without compromising the fundamental effect on selenoenzymes: comparison with selenomethionine in mice.** *Free Radic Biol Med* 2007, **42:**1524-1533.
39. Cao X, Du ZR, Liu X, Wang X, Li C, Zhou SN, Liu JR, Xu PY, Ye JL, Zhao Q, et al: **Low and high doses of oral maslinic acid protect against Parkinson's disease via distinct gut microbiota-related mechanisms.** *Biomed Pharmacother* 2023, **165:**115100.
40. Lin CY, Huang CY, Chen CM, Liu HL: **Focused Ultrasound-Induced Blood-Brain Barrier Opening Enhanced α-Synuclein Expression in Mice for Modeling Parkinson's Disease.** *Pharmaceutics* 2022, **14:**444.
41. Qiao CM, Quan W, Zhou Y, Niu GY, Hong H, Wu J, Zhao LP, Li T, Cui C, Zhao WJ, Shen YQ: **Orally Induced High Serum Level of Trimethylamine N-oxide Worsened Glial Reaction and Neuroinflammation on MPTP-Induced Acute Parkinson's Disease Model Mice.** *Mol Neurobiol* 2023, **60:**5137-5154.
42. Miquel-Rio L, Sarriés-Serrano U, Pavia-Collado R, Meana JJ, Bortolozzi A: **The Role of α-Synuclein in the Regulation of Serotonin System: Physiological and Pathological Features.** *Biomedicines* 2023, **11:**541.

43. Kayrouz CM, Seyedsayamdost MR: **Enzymatic strategies for selenium incorporation into biological molecules.** *Curr Opin Chem Biol* 2024, **81:**102495.
44. Innos J, Hickey MA: **Using Rotenone to Model Parkinson's Disease in Mice: A Review of the Role of Pharmacokinetics.** *Chem Res Toxicol* 2021, **34:**1223-1239.
45. Raza C, Anjum R, Shakeel NUA: **Parkinson's disease: Mechanisms, translational models and management strategies.** *Life Sci* 2019, **226:**77-90.
46. Wang D, Qu S, Zhang Z, Tan L, Chen X, Zhong HJ, Chong CM: **Strategies targeting endoplasmic reticulum stress to improve Parkinson's disease.** *Front Pharmacol* 2023, **14:**1288894.
47. Wang H, Dou S, Zhu J, Shao Z, Wang C, Cheng B: **Regulatory effects of ghrelin on endoplasmic reticulum stress, oxidative stress, and autophagy: Therapeutic potential.** *Neuropeptides* 2021, **85:**102112.
48. Runwal G, Stamatakou E, Siddiqi FH, Puri C, Zhu Y, Rubinsztein DC: **LC3-positive structures are prominent in autophagy-deficient cells.** *Sci Rep* 2019, **9:**10147.
49. Zhang Y, Guo H, Guo X, Ge D, Shi Y, Lu X, Lu J, Chen J, Ding F, Zhang Q: **Involvement of Akt/mTOR in the Neurotoxicity of Rotenone-Induced Parkinson's Disease Models.** *Int J Environ Res Public Health* 2019, **16:**3811.
50. Cardoso BR, Roberts BR, Bush AI, Hare DJ: **Selenium, selenoproteins and neurodegenerative diseases.** *Metallomics* 2015, **7:**1213-1228.
51. Chen T, Zhang M, Bhandari B, Yang Z: **Micronization and nanosizing of particles for an enhanced quality of food: A review.** *Crit Rev Food Sci Nutr* 2018, **58:**993-1001.
52. Chen TQ, Kan YJ, Yang C, Wu JG, Wu JZ: **Bioactivities and Molecular Characterizations of the Polysaccharide(s) from Ultrasonic-Circulating Extracts of Lingzhi or Reishi Medicinal Mushroom, Ganoderma lucidum (Agaricomycetes).** *Int J Med Mushrooms* 2020, **22:**455-466.
53. Chen TQ, Wu JG, Kan YJ, Yang C, Wu YB, Wu JZ: **Antioxidant and Hepatoprotective Activities of Crude Polysaccharide Extracts from Lingzhi or Reishi Medicinal Mushroom, Ganoderma lucidum (Agaricomycetes), by Ultrasonic-Circulating Extraction.** *Int J Med Mushrooms* 2018, **20:**581-593.
54. Jia X, Li N, Chen J: **A subchronic toxicity study of elemental Nano-Se in Sprague-Dawley rats.** *Life Sci* 2005, **76:**1989-2003.
55. Au A, Mojadadi A, Shao JY, Ahmad G, Witting PK: **Physiological Benefits of Novel Selenium Delivery via Nanoparticles.** *Int J Mol Sci* 2023, **24:**6068.
56. Nakka VP, Prakash-Babu P, Vemuganti R: **Crosstalk Between Endoplasmic Reticulum Stress, Oxidative Stress, and Autophagy: Potential Therapeutic Targets for Acute CNS Injuries.** *Mol Neurobiol* 2016, **53:**532-544.
57. Zhao J, Yang P, Lu LI, Yi T, Li Y, Mao W, Zhou Q, Lin KE: **A study on expression of GRP78 and CHOP in neutrophil endoplasmic reticulum and their relationship with neutrophil apoptosis in the development of sepsis.** *J Biosci* 2024, **49**.
58. Jia SZ, Xu XW, Zhang ZH, Chen C, Chen YB, Huang SL, Liu Q, Hoffmann PR, Song GL: **Selenoprotein K deficiency-induced apoptosis: A role for calpain and the ERS pathway.** *Redox Biol* 2021, **47:**102154.
59. Wang SY, Li MM, Wang L, Pan J, Sun Y, Wu JT, Naseem A, Jiang YK, Kuang HX, Yang BY, Liu Y: **Schisandra chinensis (Turcz.) Baill neutral polysaccharides alleviate Parkinson's disease via effectively activating MCL-1 expression regulation of autophagy signaling.** *Int J Biol Macromol* 2024, **279:**134952.
60. Li QM, Xu H, Zha XQ, Zhang FY, Luo JP: **Polygonatum cyrtonema polysaccharide alleviates dopaminergic neuron apoptosis in Parkinson's disease mouse model via inhibiting oxidative stress and endoplasmic reticulum stress.** *Int J Biol Macromol* 2025, **311:**143986.